Pregnancies after co-culture of cumulus cells with bovine embryos derived from in-vitro fertilization of in-vitro matured follicular oocytes

K. Goto, Y. Kajihara, S. Kosaka, M. Koba, Y. Nakanishi and K. Ogawa

Laboratory of Animal Reproduction, Department of Animal Science, Faculty of Agriculture, Kagoshima University, Kagoshima 890 Japan

Summary. Bovine follicular oocytes surrounded by cumulus cells for more than one-third of their surface were matured, fertilized and developed *in vitro* utilizing a co-culture system with bovine cumulus cells. Embryos developed into blastocysts were non-surgically transferred to the uteri of cows at Day 6, 7 or 8 (Day 0 = oestrus). Out of 6 recipient cows (19 blastocysts transferred), 3 became pregnant. One of the 3 pregnant cows carried twins.

The results of this study demonstrated the viability of embryos obtained from in-vitro maturation of bovine oocytes followed by in-vitro fertilization and culture to the blastocyst stage *in vitro*.

Keywords: in-vitro; maturation; fertilization; cattle

Introduction

In-vitro fertilization of oocytes would have a major impact on bovine embryo transfer (Brackett *et al.*, 1982; Brackett, 1983). A wide variety of culture media has been used but only limited in-vitro development of preimplantation cow embryos has been reported (Brock & Rowson, 1952; Thibault, 1966; Brinster, 1968; Sreenan, 1968; Sreenan *et al.*, 1968; Onuma & Foote, 1969; Seidel *et al.*, 1971; McKenzie & Kenney, 1973). Tervit *et al.* (1972) reported the development of 8-cell bovine embryos into blastocysts.

Wright *et al.* (1976b) obtained development of 4–8-cell bovine embryos cultured *in vitro* into blastocysts. Several investigators have reported that bovine embryos are capable of hatching *in vitro* (Renard *et al.*, 1976; Wright *et al.*, 1976a, b; Boone *et al.*, 1978; Flechon & Renard, 1978; Massip & Mulnard, 1980). However, development of early cow embryos *in vitro* has generally been arrested at the 8- to 16-cell stage (Eystone *et al.*, 1987a, b). One of the breakthroughs to overcome this problem was the finding of a co-culture system for the culture of early bovine embryos fertilized *in vitro*. Co-culture of bovine embryos with bovine fibroblasts promoted embryo hatching and attachment *in vitro* (Kuzan & Wright, 1982). Camous *et al.* (1984) reported that successful culture through the 8- to 16-cell block required the presence of an active biological component such as Day-14 trophoblastic vesicles. Voelkel *et al.* (1985) found that the bovine endometrial fibroblast monolayer system was excellent for both short- and long-term culture of halved bovine embryos. Eystone *et al.* (1987b) obtained the development of bovine embryos (5- to 8-cell) into blastocysts in a co-culture system utilizing oviducal epithelium.

In contrast, Kajihara *et al.* (1987) and Y. Fukuda (personal communication) have reported that the co-culture of bovine embryos with bovine cumulus cells is an excellent system for the in-vitro development of embryos fertilized *in vitro*: one-cell embryos developed into blastocysts and

hatched blastocysts, but the viability of embryos (blastocysts) obtained from this system after their transfer to the cow uterus remains to be examined. Therefore, we conducted an experiment to verify their viability.

Materials and Methods

Collection of oocytes. The ovaries were collected from cows at a local slaughter house and brought to the laboratory in physiological saline (0.85% (w/v) NaCl) at 32–38°C within 2 h. Follicular oocytes (5–10/ovary) were collected by puncturing follicles of diameter 1–7 mm with a needle.

Culture of oocytes. After washing twice with Whittingham's modified Dulbecco's phosphate-buffered saline (mPBS) (Whittingham, 1971) and once with the maturation medium (25 mM-Hepes TCM 199 with Earle's salts; Gibco, Cat. 380-2340, NY, U.S.A.) supplemented with 5% neonatal calf serum (Gibco, Lot. 22P-4457), the oocytes (60–90 oocytes) surrounded by cumulus cells for more than one-third of their surface (Fig. 1) were introduced into the maturation medium (2.5 ml), covered with paraffin oil (Nakarai Chemical, No. 26117, Kyoto, Japan), in a polystyrene culture dish (35 M/M, Termo, Tokyo, Japan) and cultured for 19–22 h in a CO₂ incubator (5% CO₂ in air) at 39°C.

Fig. 1. Oocytes before maturation.

Fig. 2. Oocytes after maturation. Expansion of cumulus cells is visible.

Sperm preparation. Frozen semen prepared from epididymal spermatozoa in 0.5 ml plastic straws was thawed in hot water (32–35°C) and then washed 3 times with BO medium (Brackett & Oliphant, 1975) without bovine serum albumin but supplemented with 5 mM-caffeine by centrifugation at 700 g for 5 min. Then the spermatozoa were pre-incubated for 2–3 h in a CO₂ incubator (5% CO₂ in air) at 39°C as a 110 µl microdrop (15–18 × 10⁶ cells/ml) in BO medium containing bovine serum albumin (5 mg/ml; Sigma, A-4387, St Louis, MO, U.S.A.) and caffeine (2.5 mM) before insemination. The microdrops were covered with liquid paraffin.

Insemination and subsequent culture. The method used was similar to that of Kajihara *et al.* (1987). Briefly the following procedure was used. After incubation for 19–22 h the cumulus–oocyte complexes (Fig. 2) were transferred to the sperm microdrop (about 15 oocytes/microdrop). After 6 h of insemination, the ova with cumulus cells were transferred into the development medium (25 mM-Hepes TCM 199 supplemented with 2.5–10% calf serum) and cultured for further development in a CO₂ incubator at 39°C. The culture medium (2.5 ml) in a polystyrene dish (35 M/M) was covered with liquid paraffin. The cumulus cells surrounding embryos were removed by pipetting after insemination for 72–96 h but the cumulus cell layer (forming a monolayer at this time) attached to the bottom of culture dish was not removed and the embryos were cultured on this layer. The incubation medium was replaced with new medium every 36–48 h. All of the culture media used were supplemented with antibiotics (100 i.u. penicillin/ml + 100 µg streptomycin/ml). The embryos were examined under the microscope every 24 h after the removal of cumulus cells from the embryos.

Fig. 3. Matured oocyte (metaphase II), showing first polar body (P) and metaphase chromosome (C).

Fig. 4. Fertilized ovum, showing a male pronucleus (M), female pronucleus (F) and a sperm tail (arrow).

Maturation and fertilization rates. Some of the ova used were randomly picked up for the calculation of maturation and fertilization rates. Maturation and fertilization rates of ova were examined 23–24 h after maturation and 20 h after insemination, respectively, after fixing ova in methanol:acetic acid (3:1, v/v) and then stained with 1% aceto-orcin for microscopic evaluation. The ova exhibiting a metaphase II chromosome figure with the first polar body (Fig. 3) were regarded as matured. Ova which had one set of male and female pronuclei and one sperm tail (Fig. 4) were regarded as normally fertilized.

Embryo transfer and pregnancy diagnosis. Embryos that developed into blastocysts (Figs 5 & 6) were non-surgically transferred to the uteri of cows at Day 6, 7 or 8 (Day 0 = oestrus) of the cycle. Pregnancy diagnosis was done by ultrasound and rectal palpation after 55 days.

Fig. 5. Overall view of the cultured ova at 10 days after in-vitro fertilization. Hatching and hatched blastocysts are visible.

Fig. 6. One of the two expanded blastocysts transferred to Cow 4 on 11 May 1987. The cumulus cell layer at the bottom of the culture dish is visible.

Results

The maturation rate of oocytes examined after 23–24 h of incubation was 82.9% (63/76). The fertilization rate examined after 20 h of insemination was 63.0% (160/254).

After in-vitro maturation and fertilization (6 trials), 25% (171/684) of oocytes used developed to at least the 8-cell stage by 3–4 days after in-vitro fertilization. At 5–6 and 7–8 days after in-vitro fertilization development to morulae and blastocysts was 21.1% (144/684) and 15.1% (103/684), respectively.

The results of the embryo transfer are shown in Table 1. A female calf (41 kg) born to Cow 4 appears to be completely normal.

Discussion

In-vitro fertilization of cow ova began in the 1970s (Edwards, 1973; Bregulla *et al.*, 1974; Iritani & Niwa, 1977; Brackett *et al.*, 1978) and, in 1981, the first calf was born after in-vitro fertilization (Brackett *et al.*, 1982). Although other calves have since been born (Brackett *et al.*, 1984), the technology used has been considered unsuitable for field application. This is mainly due to the surgical approach that was required for the recovery of ova and for the transfer of embryos to the oviduct of recipients.

Lambert *et al.* (1986) obtained calves by using in-vivo matured follicular oocytes collected by laparoscopy and by incubation of the in-vitro fertilized eggs in rabbit oviducts before surgical or non-surgical transfer to the uterus of recipient heifers. Hanada (1986) obtained calves from

Table 1. Summary of embryo transfers

Recipient cow	Day of oestrus	Day of transfer	Side of uterine horn (CL)	Stage and no. of embryos transferred (days after insemination)	Diagnosis
1	25 Feb. 1987	5 March 1987	Left (CL) Right	ExBl 1 (7) Bl 1 (7)	Not pregnant
2	27 Feb. 1987	7 March 1987	Left (CL) Right	ExBl 1 (9) ExBl 2 (9)	Not pregnant
3	9 March 1987	16 March 1987	Left (CL) Right	ExBl 1 (10) ExBl 1 (10)	Not pregnant
4	5 May 1987	11 May 1987	Left (CL) Right	Bl 2 (8) ExBl 2 (8)	Pregnant
5	23 July 1987	31 July 1987	Left (CL) Right	ExBl 2 (8) ExBl 1 (8)	Pregnant
6	4 Sept. 1987	10 Sept. 1987	Left Right (CL)	Bl 1 (7) Bl 2 (7)	Pregnant
		11 Sept. 1987*	Left	ExBl 2 (8)	(Twin)

Bl = blastocyst, ExBl = expanded blastocysts; CL = presence of functional corpus luteum.

*Another transfer because blood was observed at the tip of the straw used for transfer the day before.

follicular oocytes matured and fertilized *in vitro* followed by incubation of embryos in rabbit oviducts before non-surgical transfer to the uterus of cows.

In the present study we have obtained pregnancies from cow oocytes which had been matured, fertilized and developed *in vitro* by utilizing a co-culture system with bovine cumulus cells before their non-surgical transfer to the cow uterus. We have obtained a high rate (83%) of nuclear maturation in our culture system. This is partly due to the fact that cumulus cells surrounding the oocyte expanded well during the maturation process. The importance of cumulus expansion during the maturation process has been suggested by Ball *et al.* (1984).

Although the percentage of embryos developed to blastocysts was low (15.1%), the results of this study have verified the viability of embryos (blastocysts) developed in a co-culture system with bovine cumulus cells. Further trials have resulted in 5 pregnancies out of 11 recipient cows which had received frozen-thawed blastocysts (2–3 blastocysts/recipient) obtained from the same method described in this paper. One out of 5 cows was carrying twins. Therefore, the blastocysts obtained from our culture system can be stored in liquid nitrogen.

The method described in this paper seems to be a simple and practical way to obtain calves from in-vitro fertilization. Furthermore, culture systems such as that described here should prove useful for studies of early embryonic development *in vitro* and for the application of embryonic manipulation procedures.

We thank Dr K. Hamana, Department of Veterinary Medicine, Kagoshima University, for help in pregnancy diagnosis; and Mr T. Oku, Mr M. Fujiyama and Mr Y. Yoshida, Nagasaki Prefectural Livestock Experimental Station, for help in embryo transfers.

References

- Ball, G.D., Leibfried, M.L., Ax, R.L. & First, N.L. (1984) Maturation and fertilization of bovine oocyte *in vitro*. *J. Dairy Sci.* **67**, 2775–2785.
- Boone, W.R., Dickey, J.F., Luszcz, L.J., Dantzler, J.R. & Hill, J.R. (1978) Culture of ovine and bovine ova. *J. Anim. Sci.* **47**, 908–913.

- Brackett, B.G. (1983) A review of bovine fertilization *in vitro*. *Theriogenology* **19**, 1–15.
- Brackett, B.G., Oh, Y.K., Evans, J.F. & Donawick, W.J. (1978) *In vitro* fertilization of cow ova. *Theriogenology* **9**, Abstr. 89.
- Brackett, B.G. & Oliphant, G. (1975) Capacitation of rabbit spermatozoa *in vitro*. *Biol. Reprod.* **12**, 260–274.
- Brackett, B.G., Bousquet, D., Boice, M.L., Donawick, W.J., Evans, J.F. & Dressel, M.A. (1982) Normal development following *in vitro* fertilization in the cow. *Biol. Reprod.* **27**, 147–158.
- Brackett, B.G., Keefer, L.L., Troop, L.G., Donawick, W.J. & Bennett, K.A. (1984) Bovine twins resulting from *in vitro* fertilization. *Theriogenology* **21**, Abstr. 224.
- Bregulla, K., Gerlach, U. & Hahn, R. (1974) Versuche zur extrakorporalen Reitung, Befruchtung und Embryoenzucht mit Rinderkeimzellen. *Dtsch. Tierarztl. Wochenschr.* **81**, 465–470.
- Brinster, R.L. (1968) *In vitro* culture of mammalian embryos. *J. Anim. Sci.* **27**, 1–15.
- Brock, H. & Rowson, L.E.A. (1952) The production of viable bovine ova. *J. agric. Sci., Camb.* **42**, 479–482.
- Camous, S., Heyman, Y., Méziou, W. & Ménézo, Y. (1984) Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles. *J. Reprod. Fert.* **72**, 479–485.
- Edwards, R.G. (1973) Physiological aspects of human ovulation, fertilization and cleavage. *J. Reprod. Fert., Suppl.* **18**, 87–101.
- Eyestone, W.H., Leibfried-Rutledge, M.L., Northey, D.L., Gilligan, B.G. & First, N.L. (1987a) Culture of one- and two-cell bovine embryos to the blastocyst stage in the ovine oviduct. *Theriogenology* **28**, 1–24.
- Eyestone, W.H., Vignieri, J. & First, N.L. (1987b) Co-culture of early bovine embryos with oviductal epithelium. *Theriogenology* **27**, Abstr. 228.
- Flechon, J.-E. & Renard, J.-P. (1978) A scanning electron microscope study of the hatching of bovine blastocysts *in vitro*. *J. Reprod. Fert.* **53**, 9–12.
- Hanada, A. (1986) *In vitro* fertilization of bovine oocytes. *Consultant for Animal Science* **258**, 10–15 (in Japanese).
- Iritani, A. & Niwa, K. (1977) Capacitation of bull spermatozoa and fertilization *in vitro* of cattle follicular oocytes matured in culture. *J. Reprod. Fert.* **50**, 119–121.
- Kajihara, Y., Goto, K., Kosaka, S., Nakanishi, Y. & Ogawa, K. (1987) *In vitro* fertilization of bovine follicular oocytes and their development up to hatched blastocysts *in vitro*. *Jpn. J. Anim. Reprod.* **33**, 173–180.
- Kuzan, F.B. & Wright, R.W. (1982) Observations on the development of bovine morulae on various cellular and noncellular substrata. *J. Anim. Sci.* **54**, 811–816.
- Lambert, R.D., Sirard, M.A., Bernard, C., Béland, R., Rioux, J.E., Leclerc, P., Ménard, D.P. & Bedoya, M. (1986) *In vitro* fertilization of bovine oocytes matured *in vivo* and collected at laparoscopy. *Theriogenology* **25**, 117–132.
- Massip, A. & Mulnard, J. (1980) Time-lapse cinematographic analysis of hatching of normal and frozen-thawed cow blastocysts. *J. Reprod. Fert.* **58**, 475–478.
- McKenzie, B.E. & Kenney, R.M. (1973) *In vitro* culture of bovine embryos. *Am. J. vet. Res.* **34**, 1271–1276.
- Onuma, H. & Foote, R.H. (1969) *In vitro* development of ova from pre-puberal cattle. *J. Dairy Sci.* **52**, 1085–1087.
- Renard, J.P., Buisson, F.M., Wintenberger-Torres, S. & Menezo, Y. (1976) *In vitro* culture of cow embryos from Day 6 and Day 7. In *Egg Transfer in Cattle*, pp. 159–164. Ed. L. E. A. Rowson. Commission of the European Communities. Luxembourg.
- Seidel, G.E., Jr, Larson, L.L., Spilman, C.H., Hahn, J. & Foote, R.H. (1971) Culture and transfer of calf ova. *J. Dairy Sci.* **54**, 923–926.
- Sreenan, J. (1968) *In vivo* and *in vitro* culture of cattle eggs. *Proc. 6th Int. Congr. Anim. Reprod. & A.I., Paris*, pp. 577–580.
- Sreenan, J., Scanlon, P. & Gordon, I. (1968) Culture of fertilized cattle eggs. *J. agric. Sci., Camb.* **70**, 183–185.
- Tervit, H.R., Whittingham, D.G. & Rowson, L.E.A. (1972) Successful culture *in vitro* of sheep and cattle ova. *J. Reprod. Fert.* **30**, 493–497.
- Thibault, C. (1966) La culture *in vitro* des oeufs de vache. *Annls Biol. anim. Biochim. Biophys.* **6**, 159–164.
- Voelkel, G.F., Amborski, G.F., Hill, K.G. & Godke, R.A. (1985) Use of a uterine-cell monolayer culture system for micromanipulated bovine embryos. *Theriogenology* **24**, 271–281.
- Whittingham, D.G. (1971) Survival of mouse embryos after freezing and thawing. *Nature, Lond.* **233**, 125–126.
- Wright, R.W., Anderson, G.B., Cupps, P.T. & Drost, M. (1976a) Blastocyst expansion and hatching of bovine ova cultured *in vitro*. *J. Anim. Sci.* **43**, 170–174.
- Wright, R.W., Anderson, G.B., Cupps, P.T. & Drost, M. (1976b) Successful culture *in vitro* of bovine embryos to the blastocyst stage. *Biol. Reprod.* **14**, 157–162.

Received 3 November 1987