Development of a simple, portable carbon dioxide incubator for in vitro production of bovine embryos

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Abstract

The objective of this study was to develop a simple and portable CO_2 incubator using effervescent granules (EG) and to examine the effect of negative and positive air pressure for in vitro maturation (IVM), fertilization (IVF) and culture (IVC) of bovine oocytes. In experiment 1, cumulus-oocyte complexes (COCs) were matured (22 h), fertilized (5 h) and cultured (7 days) using 0.25, 0.5 or 1.0 g of EG per 0.6 l added to maintain an optimum level of CO_2 (approximately 3, 6 or 12%, respectively) for in vitro production of embryos. Control oocytes, zygotes and embryos were cultured in a standard CO_2 incubator. The blastocyst production rates observed on Days 7 to 9 after insemination were 20.5±4.2%, 18.5±3.9% and 28.7±5.1% for the 0.25 g EG, 0.5 g EG treatments and control, respectively. These rates were significantly higher (P<0.05) than that of the 1.0 g EG treatment (8.7±2.6%). The number of cells in the inner cell mass (ICM) and trophectoderm (TE) produced from blastocysts using the control procedure were 40.8±2.9 and 81.2±5.3, respectively, and were higher (P<0.05) compared to the 0.50 g EG (34.6±2.9 and 66.8±5.7) and 1.0 g EG treatments (33.4±3.4 and 67.2±7.3). In experiment 2, COCs were placed in a small box with 0.25 g of EG so that the effects on IVM, IVF and IVC of positive or negative air pressure could be compared. The blastocyst production rate observed in the negative air pressure treatment (29.6±4.6%) was higher (P<0.01) than that of the positive air pressure treatment (6.2±1.5%) or the normal treatment pressure (P<0.05; 18.7±4.2%) but did not differ from that of the control (30.7±4.4%). These results indicate that this simple type of incubator with negative air pressure can be successfully used for in vitro production of bovine embryos and could be used at the field level.

Author Keywords: Incubator; Cattle-reproductive technology; In vitro culture; Embryo; Oocyte

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1. Introduction

There has been rapid progress in recent years in the development of systems for in vitro production of bovine embryos for experimental and commercial purposes. Only a moderate proportion of bovine zygotes derived from in vitro maturation (IVM) and in vitro fertilization (IVF) techniques develop into blastocysts in vitro. Extensive studies have been undertaken to improve embryo culture conditions to enhance the developmental competence of the majority of those embryos. However, these efforts have not achieved a dramatic improvement in the developmental competence of IVM, IVF and in vitro culture (IVC) derived bovine embryos. We have recently developed a simple, portable and economical CO_2 incubator, using effervescent granules (EG). We have also tested the effect of negative or positive air pressure (±0.03 cm Hg) in the incubation box, by adding or aspirating air from the box, as there may be negative pressure in the genital tract during the maturation and fertilization of oocytes in the oviduct and during the transfer of embryos from the oviduct to the uterus (Fisher and Bavister, 1993).

The composition of the atmosphere in which bovine embryos develop is one of the most important factors in their development; 2.5–5% CO_2 is regard as the optimum range for IVMFC (Thompson et al., 1990; Voelkel and Hu, 1992; Pinyopummintr and Bavister, 1995). Whitten (1957) reported on the minimum oxygen requirement for in vitro survival of mouse embryos and also on oxygen toxicity which occurred if the concentration was high. More recently, researchers have evaluated the effect of altering the atmosphere during the development of embryos from mice (Eppig et al., 1990; Quinn and Harlow, 1978; Umaoka et al., 1992), hamster (Mckiernan and Bavister, 1990), swine (Wright, 1977), sheep (Wright et al., 1976; Betterbed and Write, 1985) and cattle (Tervit et al., 1972; Thompson et al., 1990; Nagao and Nakasuji, 1990; Fukui et al., 1991; Yang et al., 1994). While there is general agreement that an oxygen concentration of 5% to 10% is optimal for culturing embryos, there are a few reports (Betterbed and Write, 1985; Wright et al., 1992) that do not support this view. There are no reports comparing the effect of negative or positive air pressure on IVMFC of bovine oocytes.

The purpose of these experiments were to develop a simple portable carbon dioxide incubator using EG, and to determine the effect of negative or positive pressure in a portable incubator, so as to make IVM, IVF and IVC economically feasible in the field.

2. Materials and methods

In this experiment, a simple portable incubator made of metal (length×width×height=29×26×21 cm, volume of 15.8 l; Fig. 1) was electrically heated to maintain a chamber temperature of 38.5°C. Incubations were performed in small plastic boxes (L×W×H=15×10×4 cm=volume of 0.6 l; Fig. 2A) placed in the incubator. The CO_2 requirement

was achieved by adding 5 ml of distilled water to the EG (420 mg tartaric acid, 460 mg carbonated hydrogen natrium, and 10 mg silicone fiber per gram of granules, Fig. 2B) in a small plastic screw cap (diameter×height=35×20 mm; Fig. 2A) to release CO_2 gas.


Full-size image (23K)

Fig. 1. A simple, portable incubator with plastic container gassed, into CO_2 using effervescent granules. (Incubator, $L \times W \times H$=29×26×21 cm. Volume=15.8 l).


Full-size image (18K)

Fig. 2. (A) Plastic container ($L \times W \times H$=15×10×4 cm. Volume=0.6 l) with small plastic screw cap (diameter×height=35×20 mm) inside to release CO_2 gas. (B) EG in a tube. (C). Vacuum compressor for negative pressure.

Ovaries were obtained from the local abattoir and transported in Ringers solution supplemented with 100 μg/ml penicillin-g and 0.1 μg/ml streptomycin sulfate at 30 to 32°C. Oocytes within follicles of 5 to 6 mm diameter were aspirated using an 18 G needle and then washed with modified-PBS solution. The aspirated oocytes were washed three times in maturation medium TCM 199 (Earl's salt, Gibco, NY, USA) supplemented with superovulated cow serum (SCS) (Matsuoka et al., 1992), 0.01 mg/ml follicle stimulating hormone (FSH, Denka Pharmaceuticals, Kawasaki, Japan), and 50 μg/ml gentamycin (Sigma, St. Louis, USA). Around 80 to 100 oocytes surrounded by cumulus cells were incubated in 2.5 ml of maturation medium in a 35×10 mm petri dish (Falcon 1008, Becton Dickinson, USA) for 22 h at 38.5°C in a plastic container with the designated quantity of EG appropriate to the treatment.

Frozen–thawed spermatozoa were centrifuged twice in Brackett and Oliphant medium (Brackett and Oliphant, 1975) containing 2.5 mM caffeine and 20 μg/ml heparin. A micro drop (100 μl) formed from an aliquot of the sperm suspension (2×10^6 sperm) was covered with mineral oil and incubated with mature oocytes (20 to 25 oocytes per micro drop) for 5 h at 38.5°C in the plastic container plus EG. At 5 h after insemination, the oocytes covered by

cumulus cells were washed and then 20 to 25 oocytes cultured in 500 μl TCM 199 culture medium supplemented with 5% SCS and 5 μg/ml insulin (Wako, Japan) in a four-well dish (Nunclon, Nunc™, Nalge Nunc International, Denmark) (Goto et al., 1988; Boediono et al., 1994). At 48 h after insemination, the cumulus cells surrounding the embryos were removed by repeated pipetting and then co-cultured on the cumulus cell layer which had formed at the bottom of the culture well. The culture medium and EG were replaced every 3 days.

2.1. Experiment 1

This experiment was conducted to measure the effects of EG as the CO_2 source. The quantity of EG granules appropriate to each treatment (0.25 g EG, 0.50 g EG and 1.00 g EG) were placed in small a plastic screw cap (35-mm diameter) with 5 ml of distilled water for IVM, IVF and IVC. The pH and CO_2 concentrations were confirmed using a pH indicator (Iuchi, Tokyo, Japan) and a CO_2 indicator (FHK, Tokyo, Japan) by connecting the equipment to the inlet at the plastic container. Oocyte complexes for the control were incubated in an atmosphere of 5% CO_2 in air at 38.5°C in a standard CO_2 incubator (Sanyo, Tokyo, Japan).

Blastocysts harvested on Day 7 after insemination were used to determine cells numbers in the inner cell mass (ICM) and trophectoderm (TE). Immunosurgery and differential staining techniques were performed with procedures previously described (Iwasaki et al., 1990; Takagi et al., 1994; Suzuki et al., 1995a and Suzuki et al., 1995b). Briefly, the blastocysts were incubated in TCM 199 supplemented with 20% rabbit anti-bovine lymphocyte antiserum for 30–35 min at 38.5°C. Then the embryos were washed three times with TCM 199 supplemented with guinea pig complement and 5% SCS. Propidium iodide (Sigma) and bisbenzimide (Hoechst 33342, Sigma) were added to the complement solution to give final concentrations of 10 μg/ml. Finally, the embryos were washed in PBS with 3 mg/ml BSA and mounted on glass slides. ICM cells were separated from the TE by placing a coverslip over the embryo and applying gentle pressure. The slides were examined under a fluorescence microscope filter 330–380 mm (Nikon Industries, Tokyo, Japan) for ICM cells. This resulted in vital nuclei fluorescing blue (bisbenzimide positive) and nonvital nuclei fluorescing pink (propidium iodide). Color photographs of all ICM cells were taken and the number of viable ICM cells counted. Total numbers of ICM and TE cells were also noted following hypotonic treatment and Giemsa staining.

2.2. Experiment 2

This experiment was conducted to determine the effects of negative and positive pressure on IVM, IVF and IVC. After placing 0.25 EG with 5 ml of distilled water in the plastic container, 20 ml of air was removed from the container using a vacuum compressor (Fujihira, Tokyo, Japan; Fig. 2C) to create a negative pressure (−0.03 cm Hg); pressure was not changed for the normal treatment (0.00 cm Hg), and 20 ml of air was added to the container using an air pump (Fujihira) to create a positive pressure treatment (+0.03 cm Hg). Control oocytes/embryos were incubated as in experiment 1. The pH and CO_2 concentration were measured using the same methods as in experiment 1.

2.3. Statistical analysis

All experiments were replicated five times. Mean proportions were subjected to least-square analysis variance following arcin transformation, except for pH, the mean total number of ICM cells and the percentage of live ICM cells of blastocysts. Treatments were compared using Student's *t*-test. Differences at a probability value (*P*) of 0.05 or less were considered statistically significant.

3. Results and discussion

In these experiments, we have tested the effect on bovine oocyte IVM, IVF and IVC of atmospheres containing 2.5% to 12.2% CO_2 in air using EG in small incubation boxes placed in a simple portable incubator and the pH of the media were examined. Our aim was to aid the transfer of in vitro embryo production technology to the field and also to make its use economically feasible, particularly in developing countries.

The carbon dioxide concentration (Table 1) was higher ($P<0.01$) than control (5.0±0.08%) for 0.5 g EG (6.2±0.75) and 1.0 g EG (12.2±1.48%) treatments, but lower than control for 0.25 g EG (2.5±0.42%). The pH of the culture medium was higher ($P<0.01$) for control (7.5±0.08) and 0.25 g EG (7.4±0.19) than 0.50 g EG (6.9±0.08) or 1.00 g EG (6.5±0.08) treatments. The cleavage rates (Table 2) were lower than control for 0.25 g EG and 0.50 g EG ($P<0.05$) and lower still for 1.00 g EG ($P<0.01$). Blastocyst production rate was higher for control than 0.25 g EG and 0.50 g EG ($P<0.05$) or 1.00 g EG ($P<0.01$).

Table 1. pH and CO_2 concentration of culture medium in different gas atmospheres produced by effervescent granule in a portable incubator

EG (g)	pH range	CO_2 concentration (percentage)
0.25 EG	7.4 ± 0.19^{a}	2.5 ± 0.42^{d}
0.50 EG	6.9 ± 0.08^{b}	6.2 ± 0.75^{b}
1.00 EG	6.5 ± 0.08^{c}	12.2 ± 1.48^{a}
Control	7.5 ± 0.08^{a}	5.0 ± 0.08^{c}

Columns with different superscripts are significantly different ($P< 0.01$).

Table 2. The effect of different quantities (g) of EG as the carbon dioxide source on development of IVMFC bovine embryos, in a portable incubator compared to control (standard CO_2 incubators)

EG (g)	No. of oocytes inseminated per replicate (n=5) (range)	Percentage of oocytes which we	
		Cleavage	Blastocyst*
0.25 EG	60 (55–65)	58.5±7.5[b]	20.5±4.2[b]
0.50 EG	63 (57–66)	56.5±5.7[b]	18.5±3.9[b]
1.00 EG	83 (80–86)	40.7±7.1[c]	8.7±2.6[c]
Control	61 (56–66)	77.1±6.2[a]	28.7±5.1[a]

*Blastocyt percentage was counted from number of inseminated oocytes.
Columns with superscripts are significantly different (a–b, $P< 0.05$; a–c, $P< 0.01$).

In this study, the optimum atmospheric environments for IVM and IVF were 2.5% CO_2 (0.25 g EG) or 6.2% CO_2 (0.50 g EG) rather than 12.2% CO_2 (1.0 g EG) in air. In a previous report, Pinyopummintr and Bavister (1995) suggested that the optimal gas composition for IVM and IVF of cumulus-intact bovine oocytes was either 2.5% or 5% CO_2 together with 20% O_2. They found that raising the CO_2 concentration to 10% during IVM and IVF did not improve the outcome; in fact, 10% CO_2 reduced the index of normal fertilization. These results suggest: (a) that bovine oocytes are sensitive to changes in pH during fertilization; and, (b) that culture of bovine oocytes does not induce an increase in pH. Factors other than pH which may affect the outcome include the use of an optimum pressure for IVM, IVF and IVC of bovine oocytes. The results from this study also support this finding, with concentrations of 2.5% or 6.6% of carbon dioxide supporting better results for cleavage and blastocyst rates than 12.2% CO_2. However, there was no difference in pH between the 0.25 g EG treatment and the control. This may be have been due to similar atmospheres in the simple incubator and in the standard CO_2 incubator. In addition, the number of ICM and TE cells of blastocysts (Table 3) produced in 0.25 EG treatment (35.0±3.8 and 83.0±6.9) did not differ from those produced in the control (40.8±2.9 and 81.2±5.3), but were higher than for the 0.50 g EG (34.6±2.9 and 66.8±5.7) and 1.0 g EG treatments (33.4±3.4 and 67.2±7.3).

Table 3. The effect of different quantities (g) of EG as the carbon dioxide source on ICM, TE and total cell number of Day 7 blastocysts in portable incubator

EG (g)	No. of blastocyst on Day 7 (range per replicate)	No. of ICM	No. of TE	Total cel
0.25 EG	34 (6–8)	35.0±3.8[ab]	83.0±6.9[a]	118.0±6
0.50 EG	35 (6–8)	34.6±2.9[b]	66.8±5.7[c]	101.4±6
1.00 EG	26 (5–6)	33.4±3.4[b]	67.2±7.3[c]	100.6±4
Control	38 (7–8)	40.8±2.9[a]	81.2±5.3[a]	122.0±5

Columns with superscripts are significantly different (a–b, $P< 0.05$; a–c, $P< 0.01$).
Full-size table (8K)

The cleavage rate (Table 4) was highest for control (79.2±6.0) and not different from that for the negative pressure treatment (72.6±8.4), but they were higher for normal (66.9±5.7%; P<0.05) and positive pressure (34.2±7.2; P<0.01) treatments. Blastocyst production rates reflected the cleavage rates with the highest values observed for control (30.7±4.4%) and

negative pressure (29.6±4.6%) treatments and lowest for normal (18.7±4.2%; $P<0.05$) and positive pressure (6.2±1.5%; $P<0.01$) treatments.

Table 4. The effect of negative or positive pressure on development of IVMFC embryos using 0.25 g EG in a portable incubator

Pressure (cm Hg)	No. of oocytes inseminated per replicate (range)	Percentage of oocytes which	
		Cleavage	Blastocyst*
−0.03	79 (77–84)	72.6 ± 8.4^{ab}	29.6 ± 4.6^{a}
0.00	83 (82–83)	66.9 ± 5.7^{b}	18.7 ± 4.2^{b}
+0.03	76 (73–78)	34.2 ± 7.2^{c}	6.2 ± 1.5^{c}
Control	75 (70–80)	79.2 ± 6.0^{a}	30.7 ± 4.4^{a}

*Blastocyt percentage was counted from number of inseminated oocytes.
Columns with superscripts are significantly different (a–b, $P< 0.05$; a–c, $P< 0.01$).

The results from these experiments have demonstrated that incubation of bovine embryos under 2.5% to 12.2% CO_2 with negative air pressure and possibly a reduced O_2 concentration due to aspiration of air (Thompson et al., 1990) beneficially affected the development of early stage embryos, whereas positive air pressure, with a possible increased concentration of O_2 in air, and lower concentration of CO_2 in air, resulted in less advanced embryo development.

These results suggest that the whole process of IVM, IVF and IVC can be carried out effectively in a portable incubator able to be used in the field (blastocysts produced on Day 7 from 0.25 g EG treatment are shown in Fig. 3); the blastocyst production rate and the ratio of ICM to TE cells in blastocysts were similar to those obtained in a laboratory standard CO_2 incubator.

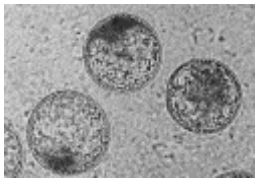
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Fig. 3. Bovine blastocysts produced at Day 7 after insemination after in vitro culture in a simple incubator with CO_2 supplied by effervescent granules.

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