

Development of *In Vitro* Matured/Fertilized Bovine Embryos in a Chemically Defined Medium: Influence of Oxygen Concentration in the Gas Atmosphere

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ABSTRACT. The influence of oxygen concentrations in the gas atmosphere on the development of IVM/IVF bovine embryos was determined by culturing them in a microdrop of modified synthetic oviduct fluid medium supplemented with amino acids, insulin and PVA (mSOFai). After removing the cumulus cells at 18 hr post-insemination, presumptive zygotes were cultured in mSOFai for 104–106 hr under 5% CO₂ with various O₂ concentrations (2.5 to 20%). Reduced O₂ (5–10%) improved the development to the morula stage, and 5% O₂ gave the highest development. In the next experiment, morulae obtained after 102–104 hr of culture, were further cultured for 50 hr in mSOFai with 2 mM glucose under 5 and 20% O₂. An increase in the mean cell number in blastocysts, but not in the frequency of blastocysts, was observed under 5% O₂. In the third experiment, zygotes were cultured for 152–154 hr in mSOFai under 5 and 20% O₂, or cocultured with bovine oviduct epithelial cells in TCM199 + 10% FCS under 5% CO₂ in air. Percentage of blastocysts for mSOFai in 5% O₂ doubled to that for 20% O₂, and was similar to that for coculture. Moreover, mean cell number in the blastocysts for mSOFai in 5% O₂ was significantly higher than that for coculture. Results demonstrate that oxygen concentration critically affects embryonic development through zygotes to blastocysts, and suggest that around 5% O₂ is optimal. It also indicates that bovine zygotes can be cultured up to the blastocyst stage using a chemically defined medium with rates similar to those of a conventional coculture system. — **KEY WORDS:** bovine, culture, defined medium, embryo, oxygen.

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Oxygen is essential for the energy producing metabolism of mammalian cells, however living cells will receive oxidative stress under excessive concentrations due to the auto-production of reactive oxygen species [12]. Common gaseous environment for embryo culture has been 5% CO₂ in humidified air (i.e., approx. 20% O₂), whereas it is indicated that preimplantation embryos of rats, hamsters, rabbits and monkeys are exposed to quite lower oxygen tension *in vivo* [9]. Therefore, it is easily expected that lower oxygen levels in the gaseous atmosphere might favor embryonic development *in vitro*. Indeed, the use of 5 to 10% O₂, or lower, with 5% CO₂ has been reported to be advantageous for preimplantation embryos of mouse [1, 25, 26, 32, 35], hamster [22], rabbit [8, 17, 18], and sheep [30, 31, 34]. However, lower O₂ concentration was reported to have no advantage for sheep [4, 36], mouse [24], and cat embryos [13].

It is difficult to culture bovine embryos through the 8- to 16-cell stage. Development of coculture systems with somatic cells such as oviduct epithelial cells [7], cumulus cells [14], and buffalo rat liver cells [33], made it possible to overcome the cleavage block. They are now applied to IVF programs worldwide in the culture of zygotes to the blastocyst stage. However, favorable O₂ varies depending on the type of somatic cells, suggesting that the effects of O₂ concentration on the embryos might be mediated indirectly through the coculture system used [33]. Using somatic cell-free media, several investigators have reported that reducing O₂ tension from 20 to 5% improved the development of IVM/IVF bovine zygotes to the blastocysts stage [6, 10, 23, 37]. The effect of various O₂ concentrations

(0 to 20%) on the development of 2- to 8-cell stage bovine embryos was also determined by Thompson *et al.* [31] using a semi-chemically defined medium containing BSA with a stoppered test-tube system. The standard method for culturing preimplantation embryos is the “microdrop method”. However, none of these studies examined the development of bovine zygotes in a microdrop of chemically defined medium under various O₂ tensions. The critical effect of oxygen concentration on embryo development *in vitro* can be influenced by the type of system employed such as microdrop vs. stoppered test-tube [4, 36], and the presence vs. the absence of oil covering [22, 33]. Suboptimal O₂ conditions could cause more detrimental effects on embryonic development in chemically defined, protein-free media.

In the present experiments, IVM/IVF bovine zygotes/embryos were cultured in a microdrop of chemically defined medium covered with oil. Since embryonic susceptibility against higher O₂ tension is expected to be higher at the developmental stage earlier than the morula to blastocyst stages, the optimal O₂ concentration required for the development of zygotes to the morula stage was initially determined by culturing zygotes under 2.5 to 20% O₂. Information about the effects of O₂ tension during culture of the advanced stage bovine embryos is not available. Therefore, the effects of lowering O₂ tension on the development of morulae to blastocysts were then examined separately. Development of zygotes to the blastocyst stage in a defined medium with a reduced O₂ tension was also compared with that for conventional coculture system.

MATERIALS AND METHODS

Recovery and culture of ovarian oocytes: Bovine cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (2 to 6 mm in diameter) on the ovaries obtained from a slaughterhouse as described previously [28]. They were washed twice with HEPES-buffered Tyrode's medium (TALP-HEPES) [3] supplemented with 3 mg/ml BSA (Fraction V, Sigma Co., St. Louis, MO, U.S.A.), 0.2 mM sodium pyruvate (Sigma), and 50 µg/ml of gentamicin sulfate (Sigma). Only oocytes with intact and unexpanded cumulus cells were selected for culture according to the criteria defined by Leibfried and First [16]. Maturation *in vitro* was accomplished in HEPES-buffered TCM199 (Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 10% heat-treated fetal calf serum (Gibco), 0.02 units/ml of FSH (from porcine pituitary, Sigma), 1 µg/ml of estradiol-17β (Sigma), 0.2 mM sodium pyruvate, and 50 µg/ml of gentamicin sulfate. After washing once with the maturation medium, selected COCs were transferred to 50 µl-drops of maturation medium (10 to 12 COCs per drop) under paraffin oil in 60-mm plastic dish (Falcon 1007, Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.). They were cultured for 22 hr at 39°C in humidified air with 5% CO₂ (in a CO₂ incubator, Napco 5100, Napco Scientific Co., Tualatin, OR, U.S.A.).

***In vitro* fertilization of oocytes:** *In vitro* fertilization was performed using frozen semen from a single ejaculate of a Holstein bull (semen donated by the Hokkaido Livestock Improvement Association, Hokkaido, Japan). Frozen sperm were thawed at 37°C for 30 sec and subsequently layered onto the Percoll gradient (45 and 90%). A 90% Percoll stock solution was prepared with a 9:1 mixture of Percoll (Pharmacia, Uppsala, Sweden) and 10-strength modified Brackett and Oliphant isotonic solution [5] without BSA but with 1.7 µg/ml phenol red and 50 µg/ml gentamicin sulfate (mBO) [29]. The Percoll gradient was prepared in a 15-ml plastic conical tube (Falcon 2095) with 2 ml of 90% Percoll added to the tube, which was layered with 2 ml of 45% Percoll (1:1 mixture of 90% Percoll and single strength of mBO). The gradient was centrifuged at 700 × g for 20 min. After removing the top layers, the sperm pellet was resuspended in mBO (about 6 ml), and washed again by centrifugation at 500 × g for 5 min. *In vitro* insemination was carried out using the procedure as described previously [29]. Briefly, a 100 µl of fertilization droplet was prepared by adding a 50 µl aliquot of sperm suspension to 50 µl of the mBO containing 6 mg/ml fatty acid-free BSA (Sigma) and 5 mM theophylline (Sigma) to give a final concentration of 5 × 10⁶ cells/ml. After washing once with mBO, the COCs were put into the fertilization droplets (10 to 12 COCs per drop), and coincubated for 18 hr with sperm at 39°C in humidified air with 5% CO₂. In the preliminary experiments, *in vitro* insemination resulted in more than 95% sperm penetration of oocytes and around 85% of normal fertilization (two pronuclei).

***In vitro* culture of presumptive zygotes and embryos:** Presumptive zygotes were stripped of cumulus cells after coincubation with sperm by vortexing in TALP-HEPES [27]. The zygotes were then washed five times with culture medium, and incubated at 39°C in a 50 µl-microdrop (20 to 25 zygotes per drop) covered with paraffin oil under the gas atmosphere of 5% CO₂ and with various O₂ tensions. The culture droplets were equilibrated overnight with the appropriate gas atmosphere before use. For 2.5 to 15% O₂, oxygen-adjustable N₂-O₂-CO₂ incubators (BPN 110, Tabai Espec Co., Osaka, Japan) were used, whereas a CO₂ incubator (Napco) was used for 20% O₂ (5% CO₂ in air). Modified synthetic oviduct fluid medium [28] supplemented with 20 amino acids, 10 µg/ml insulin (from bovine pancreas, Sigma) and 1 mg/ml PVA (Sigma) instead of BSA (designated as mSOFai), was used as a basal medium (Table 1). This basal medium was prepared using a reverse osmosis-filtered water with a resistance of more than 18 megΩ. A premixture solution of 12 essential amino acids for basal medium Eagle (BME, Flow Laboratories, Irvine, Scotland) with 1 mM glutamine (Sigma) and 7 nonessential amino acids for minimum essential medium (MEM, Flow) were added. Two days (44 to 46 hr) after *in vitro* insemination, the initial cleavage rate was checked under a dissecting microscope. Embryonic development to the morula and blastocyst stages were examined under a stereomicroscope 120 to 124 and 170 to 172 hr after insemination, respectively. The cell numbers of all embryos that developed to the 8-cell (Experiment 1) and blastocyst stages (Experiments 2 and 3) were determined by an air-dry procedure [28, 29].

Experiment 1: Presumptive zygotes were cultured for 104 to 106 hr in mSOFai under the gas atmospheres with 5% CO₂ and various O₂ concentrations (2.5, 5, 7.5, 10, 15 and 20%), and the influence of O₂ concentration on embryonic development to the morula stage was examined. Two to three treatments were conducted on each day (i.e. replicates)

Table 1. Composition of modified synthetic oviduct fluid medium supplemented with amino acids and insulin (mSOFai)

Component	Concentration
NaCl	107.70 mM
KCl	7.16
KH ₂ PO ₄	1.19
CaCl ₂	1.71
MgCl ₂	0.49
NaHCO ₃	25.07
Sodium lactate	3.30
Sodium pyruvate	0.33
L-glutamine	1.00
BEM essential amino acids	12 ^{a)}
MEM non-essential amino acids	7 ^{b)}
Insulin	10.0 µg/ml
Phenol red	1.3 µg/ml
Gentamicin sulfate	50.0 µg/ml
Polyvinyl alcohol	1.0 mg/ml

^{a)} 12 essential amino acids for basal medium Eagle (BME).

^{b)} 7 nonessential amino acids for minimum essential medium (MEM).

Table 2. Effects of oxygen concentration in the gas atmosphere on the development of bovine presumptive zygotes to the morula stage

Oxygen concentration (%)	No. of replicates (zygotes)	% of cleaved zygotes	% of morulae at Day 5 ^{a)}	Total cell No. in morulae (N)
2.5	10 (247)	77.7 ± 1.2	32.7 ± 3.3 ^{b,c,d)}	33.1 ± 1.4 ^{b)} (81)
5	8 (181)	78.3 ± 1.9	40.0 ± 2.7 ^{b)}	32.7 ± 1.7 ^{b)} (72)
7.5	7 (159)	81.6 ± 1.3	36.9 ± 3.3 ^{b,c)}	33.0 ± 1.8 ^{b)} (58)
10	8 (194)	80.1 ± 1.9	34.8 ± 3.6 ^{b,c)}	33.1 ± 1.5 ^{b)} (68)
15	7 (149)	78.3 ± 1.3	27.2 ± 2.4 ^{c,d)}	31.2 ± 2.0 ^{b)} (40)
20	7 (149)	77.2 ± 2.1	22.3 ± 3.9 ^{d)}	24.0 ± 1.4 ^{c)} (33)

Values are mean ± SEM. ^{a)} 122–124 hr post-insemination. ^{b,c,d)} Values with different superscripts differ (at least $P < 0.05$).

of experimentation using two sets of N₂-O₂-CO₂ incubators and one set of CO₂ incubator. All embryos that developed beyond the 8-cell stage were assigned for cell counting 122 to 124 hr after *in vitro* insemination. The embryos possessing 16 cells or more were judged as morulae as described previously [28]. In this experiment, O₂ and CO₂ pressures, and pH of mSOFai droplets which were incubated at the various O₂ concentrations for 104 to 106 hr without embryos, were also measured using a blood gas analyzer (Auto Blood Gas Analyzer IL1304, Instrumentation Laboratory, Milano, Italy). Calibration of these values was done under 39°C.

Experiment 2: Presumptive zygotes were cultured for 102 to 104 hr in mSOFai under a gas atmosphere of 5% O₂ and 5% CO₂. To examine the effect of O₂ on the development of morulae to the blastocyst stage, embryos which developed to the morula stage were divided into two groups, and were further cultured for 50 hr in mSOFai supplemented with 2 mM glucose under two different gas atmospheres of 5 and 20% O₂. All of the embryos that reached the blastocyst stage 170 to 172 hr after *in vitro* insemination were fixed and their total cell numbers were counted.

Experiment 3: To confirm the efficacy of embryo culture under a gas atmosphere of 5% O₂, presumptive zygotes were cultured for 152 to 154 hr under gas atmospheres of 5 and 20% O₂. In this experiment, zygotes were cultured for 102 to 104 hr in mSOFai. They were then transferred to fresh mSOFai supplemented with 2 mM glucose, and further cultured for 50 hr. Presumptive zygotes were also cocultured with bovine oviduct epithelial cells in TCM199 supplemented with 10% FCS as described previously [8, 28], and embryonic development to the blastocyst stage were compared to that of somatic cell-free culture with mSOFai.

Statistical analysis: In Experiments 1 and 3, differences in the mean percentages of initial cleavage, morulae and blastocysts among experimental groups were analyzed by one-way analysis of variance (ANOVA). If a significant treatment effect was revealed by ANOVA, comparisons were made by Duncan's multiple range-test. In Experiment 2, mean percentages of embryos developing to the blastocyst stage were analyzed by Student's *t*-test. The total cell number in morulae (Experiment 1) and blastocysts

Table 3. Oxygen concentrations in the incubator gas atmosphere and oxygen pressures in the culture microdrops

Oxygen concentration (%) ^{a)}	No. of replicates	O ₂ pressure (mmHg) ^{b)}
2.5	6	57.0 ± 1.1
5	6	72.2 ± 1.6
10	5	98.8 ± 2.4
20	5	155.6 ± 1.4

Values are mean ± SEM. ^{a)} Oxygen concentrations in the incubator gas atmosphere. ^{b)} Oxygen pressures in the microdrops.

Table 4. Effects of oxygen concentration on the development of morulae^{a)} to the blastocyst stage

Oxygen concentration (%)	No. of replicates (morulae)	% of blastocysts at Day 7 ^{b)}	Total cell No. in blastocysts (N)
5	5 (57)	62.0 ± 1.6	184.9 ± 10.4 ^{c)} (35)
20	5 (53)	58.0 ± 7.7	134.3 ± 8.8 ^{d)} (31)

Values are mean ± SEM. ^{a)} Morulae were obtained after culture of zygotes for 4 days (120 to 122 hr post-insemination). ^{b)} 170–172 hr post-insemination. ^{c,d)} Different superscripts denote significant difference ($P < 0.001$).

(Experiments 2 and 3) were subjected to logarithmic transformation, and were then assigned for ANOVA and Duncan's multiple range-test or for Student's *t*-test.

RESULTS

Experiment 1: As shown in Table 2, the reduction of O₂ from 20 to 5–10% significantly increased the development of zygotes to the morula stage ($P < 0.05$). Although there was no significant difference among the frequencies of morulae for 2.5, 5, 7.5 and 10% O₂, only 5% O₂ gave a significantly higher development to morulae as compared with 15% O₂ ($P < 0.05$). Oxygen pressures in the microdrops of medium for 2.5, 5, 10 and 20% of O₂ concentrations in the incubator are shown in Table 3. There were no significant differences in the values of CO₂ pressures and pH among the 4 groups; mean values of pCO₂ and pH for

Table 5. Development of presumptive zygotes cultured in mSOFai without somatic cell and cocultured with bovine oviduct epithelial cells (BOEC) in TCM 199+FCS

Media	BOEC	Oxygen conc. (%)	No. of replicates (zygotes)	% of cleaved zygotes	% of blastocysts at Day 7 ^{a)}	Total cell No. in blastocysts (N)
mSOFai	without	5	6 (166)	78.8 ± 1.5	24.9 ± 1.4 ^{b)}	190.6 ± 8.4 ^{b)} (42)
mSOFai	without	20	6 (182)	76.4 ± 0.8	11.6 ± 1.9 ^{c)}	110.4 ± 8.6 ^{c)} (21)
TCM199 +FCS	with	20	6 (163)	80.3 ± 2.1	22.4 ± 1.7 ^{b)}	146.9 ± 8.1 ^{d)} (38)

Values are mean ± SEM. ^{a)} 170–172 hr post-insemination. ^{b,c,d)} Different superscripts denote significant difference (P<0.01).

the four groups were 40.9 mmHg (ranged from 40.3 to 41.7 mmHg) and 7.279 (ranged from 7.275 to 7.283), respectively.

Experiment 2: Percentages of morulae which developed to blastocysts were not significantly different between the two O₂ concentrations. However, total cell numbers in the blastocysts for 5% O₂ was higher (P<0.001) than that for 20% O₂ as shown in Table 4.

Experiment 3: Percentage of blastocysts and their cell numbers for mSOFai in 20% O₂ were lower than that for mSOFai in 5% O₂ and for coculture (P<0.01). Although frequency of blastocysts for mSOFai in 5% O₂ was similar to that for coculture, total cell number in the blastocysts for mSOFai in 5% O₂ was higher (P<0.01) than that for coculture as shown in Table 5.

DISCUSSION

The present study clearly shows that lowering O₂ concentration from 20 to 5–10 % markedly improves the development of zygotes to the morula stage, and that around 5% of O₂ seems to be suitable for embryonic development under the present culture conditions. These results are similar to the previous findings by Thompson *et al.* [31] in which *in vivo* fertilized bovine embryos at the 2- to 8-cell stages were cultured for 5 days in a 5 ml test-tube with 1 ml of SOF containing 32 mg/ml BSA [30] without oil covering. Thompson *et al.* [31] showed that concentrations of O₂ at 4 to 12% in the atmosphere appeared best for supporting embryonic development to the morula and compacted morula stages. Therefore, it is unlikely that the two culture systems, microdrop under oil and stoppered test-tube, have different O₂ tension effects.

The O₂ tensions in the bovine oviduct and uterus are not known. However, oxygen tensions in several animals measured at 37°C were reported to be much less than half of atmospheric O₂, ranging from high values of about 60 mmHg in the rabbit oviduct, rabbit and hamster uteri, to as low as 11 mmHg in the monkey uterus [9]. Considering some reduction in the O₂ concentration in the region near the embryos in the microdrop under oil covering [2], O₂

pressure in the present microdrop of bulk medium (mSOFai) under the incubator gas atmosphere of 5% O₂ (around 70 mmHg at 39°C) might give the range values of physiological O₂ tension to the embryos.

Oxygen can penetrate cells by simple diffusion depending on the concentration gradient, and is required for their metabolism. However, higher level of O₂ than that of the physiological condition would stimulate the production of reactive oxygen species (ROS) such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical HO· [12]. An increase in the level of H₂O₂ within mouse embryos was observed when they were cultured for 60 min or more under 20% O₂ as compared to 5% O₂ [11]. ROS may cause membrane disruption by lipid peroxidation, enzyme inactivation, structural damage to DNA, and cell death [12]. Reduced O₂ concentration in the gas atmosphere may decrease the O₂ tension within the embryos to physiological levels. Subsequently, the damage by ROS to the embryonic cells might decrease. Incubation under reduced O₂ concentration may also provide adequate energy production to support embryonic development *in vitro*. Khurana and Wales [15] reported that reduced O₂ concentrations (1 to 5%) enhance the utilization of endogenous glycogen pools in cultured mouse morulae.

Results from Experiment 2 clearly demonstrate that the gas atmosphere of 5% CO₂ in air (20% of O₂) which has been widely used for embryo incubation and manipulation, is deleterious for embryonic cell proliferation even at the advanced stages such as morulae to blastocysts. Although, there is no comparable data in bovine embryos, a similar harmful effect of higher O₂ tension was reported in the culture of rabbit morulae [18].

Results of the present coculture, both frequency of blastocysts and mean cell number in blastocysts, were similar to those of the previous studies where *in vitro* maturation, fertilization and coculture were performed under similar conditions [28, 29]. The frequency of blastocysts for mSOFai under 5% O₂ doubled that for 20% O₂, and was comparable to that of coculture. However, culture in mSOFai under 5% O₂ gave a higher cell number in the blastocysts as compared with that in coculture. These data

suggest that blastocysts obtained after culturing zygotes in mSOFai are of better quality than that of coculture as judged by their cell numbers.

Eight days after estrus, bovine embryos develop into the compacted morula to hatched blastocyst stages *in vivo*, and the distribution of compacted morulae, early blastocysts, blastocysts, expanded blastocysts, and hatching/hatched blastocysts were reported to be 14, 21, 24, 36, and 5%, respectively [19]. In the present culture with mSOFai under 5% O₂, the distribution of early/mid, expanded, and hatching/hatched blastocysts at 7 days after insemination (equal to 8 days after estrus) were 50, 32, and 17%, respectively. Their total cell numbers (ranging from 91 to 291 cells) were in the range of *in vivo* developed blastocysts [20, 21]. However, we have not determined the viability of the present blastocysts by transferring them to recipient cattle. Further experiments are required to confirm the *in vivo* viability of the blastocysts obtained after culture in mSOFai.

In conclusion, the present results demonstrate that O₂ concentration in the gas atmosphere critically affects the *in vitro* development of bovine embryos from zygotes to blastocysts, and suggest that around 5% of O₂ is optimal for culturing in the microdrops of mSOFai covered with oil. It also indicates that bovine zygotes can be cultured up to the blastocyst stage using mSOFai without somatic cell support with resulting equivalent embryonic development to that of a conventional coculture system.

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