Effect of Oxygen Concentration in the Gas Atmosphere during *In Vitro* Insemination of Bovine Oocytes on the Subsequent Embryonic Development *In Vitro*

Yoshiyuki TAKAHASHI and Hiroshi KANAGAWA

Laboratory of Theriogenology, Department of Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

(Received 14 July 1997/Accepted 1 October 1997)

ABSTRACT. *In vitro* matured bovine oocytes were co-incubated with sperm for 18 hr in a droplet of fertilization medium under a gas atmosphere of 5% CO₂ with 5 or 20% O₂. After removing the cumulus cells, they were fixed to examine their fertilization rate, or cultured for another 154 hr in a chemically defined medium under 5% O₂ to determine their development to the blastocyst stage. There was no difference between the 5 and 20% O₂ groups in the fertilization rate. However, the percentage of inseminated oocytes which developed to the blastocyst stage was higher when *in vitro* insemination was conducted under 5% O₂ compared with that under 20% O₂ (34.4 vs. 24.7%, $P < 0.05$). — **KEY WORDS:** fertilization, oocyte, oxygen.

J. Vet. Med. Sci. 60(3): 365–367, 1998

Oxygen tension in the female reproductive tract is lower than that in the atmospheric gas phase [5]. It is well known that oxygen at excessive tension gives an oxidative stress to mammalian cells, including embryos [1]. An oxygen concentration of 5 to 10% in the gas atmosphere has been reported to be optimal for the development of bovine embryos when they are cultured *in vitro* without feeder cells [4, 6, 8, 14–16, 20]. When bovine embryos were co-cultured with feeder cells such as oviduct cells, cumulus/granulosa cells, or other somatic cells, the optimal oxygen tension varied according to the cell type, medium and culture system itself [6, 8, 19].

The *in vitro* insemination of bovine oocytes is usually performed by co-incubating spermatozoa with oocytes surrounded by cumulus cells. It is therefore expected that the optimal oxygen concentration in the gas atmosphere during *in vitro* insemination may also vary according to the *in vitro* insemination conditions. However, empirical protocols for the *in vitro* insemination of bovine oocytes commonly utilize atmospheric gas conditions of 5% CO₂ in air (approximately 20% O₂) with different details (i.e., media, medium volume, oocytes density, oil covering etc.) [10]. Few attempts have been made to determine the effects of the oxygen concentration during *in vitro* insemination on the fertilization of bovine oocytes [10, 18] or the subsequent embryo development *in vitro* [18]. In the present study, the hypothesis that the *in vitro* insemination of bovine oocytes under a low oxygen concentration (5% O₂), which is similar to the physiological level [5], would improve the fertilization rate and the subsequent embryonic development was tested by co-incubating *in vitro* matured oocytes with frozen-thawed spermatozoa using our *in vitro* insemination procedure [12–14].

The *in vitro* maturation of follicular oocytes was performed as described previously [14]. Briefly, bovine cumulus-oocyte complexes (COCs) aspirated from small antral follicles of slaughterhouse-obtained bovine ovaries, were washed twice with HEPES-buffered Tyrode's medium (TALP-HEPES) [2]. The oocytes with morphologically

normal ooplasm and with an intact cumulus were selected for culture. The *in vitro* maturation 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 follicle stimulating hormone (from porcine pituitary, Sigma), 1 µg/ml of estradiol-17β (Sigma), 0.2 mM sodium pyruvate, and 50 µg/ml of gentamicin sulfate. After being washed twice with the maturation medium, selected COCs were transferred to 50-µl drops of maturation medium (10–13 COCs per drop) under paraffin oil in a 60-mm plastic dish (Falcon 1007, Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.). They were then 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.).

The *in vitro* insemination was conducted according to the procedure described elsewhere [14]. In brief, frozen sperm from a Holstein bull were thawed at 37°C for 30 sec, and subsequently layered onto a Percoll gradient (45 and 90%). The Percoll gradient was centrifuged at 700 × g for 20 min. After the top layers were removed, the sperm pellet was resuspended in 6 ml of modified Brackett and Oliphant isotonic medium [3] without bovine serum albumin (BSA), penicillin and streptomycin but with 1.7 µg/ml phenol red (Sigma) and 25 µg/ml gentamicin sulfate, which was designated as mBO medium [12]. They were then washed again by centrifugation at 500 × g for 5 min. After the centrifugation and aspiration of the supernatant, the sperm pellet was resuspended to 10 × 10⁶ cells/ml in mBO medium. Initial *in vitro* insemination droplets were prepared with 50-µl of mBO medium containing 6 mg/ml of BSA (Fatty acid-free, Sigma) and 5 mM theophylline (Sigma). The droplets were covered with paraffin oil in a 35-mm plastic dish (Falcon 1008), and were kept at 39°C under 5 or 20% O₂ for more than 3 hr before use. Fifty µl of sperm suspension was introduced into the initial insemination droplet to give a final insemination droplet of 5 × 10⁶ cells/ml sperm concentration, 3 mg/ml BSA, and 2.5 mM theophylline. COCs were then immediately put into the

Table 1. The effect of oxygen concentration in the gas atmosphere on the *in vitro* fertilization rate

Item	Concentration of oxygen (%)	
	5	20
No. of replicates (oocytes)	5 (111)	5 (129)
% of total fertilization	92.4 ± 5.2	89.5 ± 4.6
% of normal fertilization	78.0 ± 7.5	78.5 ± 5.3
% of polyspermy	6.7 ± 4.3	4.7 ± 3.5

Values are means ± SD determined at 18 hr after insemination.

above final insemination droplets (10 to 13 COCs per 100- μ l droplet), and co-incubated with sperm for 18 hr at 39°C under 5 or 20% O₂. For the incubation under 5 and 20% O₂, an oxygen-adjustable N₂-O₂-CO₂ incubator (5% O₂ and 5% CO₂; BPN 110, Tabai Espec Co., Osaka, Japan) and a CO₂ incubator (5% CO₂ in air) were used, respectively. After the co-incubation with sperm, the COCs were transferred to a 10 ml conical tube (Falcon 2059) and vortexed for 3 to 4 min in 1 ml of TALP-HEPES to remove their cumulus cells.

The first experiment was carried out to determine the effect of oxygen concentration in the gas atmosphere during *in vitro* insemination on the fertilization rate. The cumulus-free oocytes were fixed with a mixture of ethanol and acetic acids (3:1), and stained with 1% aceto-orcein. The evaluation of the fertilization was made under a phase-contrast microscope. The oocytes were considered as fertilized when they had an enlarged sperm head(s) or male pronucleus(ei) with corresponding sperm tail(s). The percentage of normal fertilization was determined as the ratio of the oocytes with a pair of pronuclei and corresponding sperm tail to the total number of oocytes evaluated. The results are shown in Table 1. There were no significant differences between the 5 and 20% O₂ concentration preparations in any of the criteria examined ($P > 0.1$; Student's *t*-test).

The effect of oxygen concentration in the gas atmosphere during *in vitro* insemination on the subsequent embryonic development was examined in the next experiment. After *in vitro* insemination under 5 or 20% O₂, the cumulus-free oocytes (presumptive zygotes) were washed five times with a protein-free chemically defined medium. They were then cultured in a 40- μ l droplet of the defined medium (25 to 30

zygotes per droplet) covered with paraffin oil for 6 days (about 154 hr) at 39°C under a gas atmosphere with 5% CO₂ and 5% O₂ using the oxygen-adjustable N₂-O₂-CO₂ incubator. A modified synthetic oviduct fluid medium supplemented with 1 mM glutamine (Sigma), 12 essential amino acids for basal medium Eagle (Flow Laboratories, Irvine, Scotland), 7 non-essential amino acids for minimum essential medium (Flow), 10 μ l/ml of insulin (Sigma), and 1 mg/ml of polyvinyl alcohol (Sigma), designated as mSOFai [14], was used as a chemically defined medium. Seven days (about 172 hr) after insemination, the cleavage rate and the percentage of presumptive zygotes developed to blastocysts were determined. The total cell numbers of all blastocysts were examined using an air-dry procedure [12]. As shown in Table 2, the *in vitro* insemination under 5% O₂ yielded a higher percentage of blastocysts than that under 20% O₂ ($P < 0.05$; Student's *t*-test). However, there was no difference between the 5 and 20% O₂ preparations in the cleavage rate or total cell number per blastocyst ($P > 0.05$).

No remarkable effect of oxygen concentration in the gas phase was detected on the fertilization rate under the present experimental conditions. This result is in agreement with the finding reported by Van der Westerlaken *et al.* [18]. In contrast, Pinyopummintr and Bavister [10] demonstrated a lower fertilization rate after culturing bovine oocytes through *in vitro* maturation and insemination in 5% O₂ compared with that in 20% O₂. They did not determine the effect of oxygen concentration during the *in vitro* insemination separately from *in vitro* maturation. It is thus not possible to compare the present data with their results.

In the present experiment, 5% O₂ during the *in vitro* insemination improved the subsequent embryonic development to blastocysts. In contrast, Van der Westerlaken *et al.* [18] reported that 5% O₂ during *in vitro* insemination significantly increased the cleavage rate, but had no obvious beneficial effect on the percentage of blastocysts compared with 20% O₂ (24.6 vs. 15.7%, respectively). The discrepancy is obscure, since they did not describe the details of the *in vitro* insemination procedure in their abstract paper [18].

The reason(s) for the improved developmental potential to the blastocyst stage under the present *in vitro* insemination in 5% O₂ is not clear. However, it has been

Table 2. The effect of oxygen concentration in the gas atmosphere during *in vitro* insemination on the subsequent *in vitro* development of presumptive zygotes

Item	Concentration of oxygen (%)	
	5	20
No. of replicates (zygotes)	5 (150)	5 (143)
% of cleaved zygotes	79.9 ± 3.7	77.3 ± 5.7
% of blastocysts	34.4 ± 6.7 ^{a)}	24.7 ± 6.2 ^{b)}
Total cell numbers per blastocyst (N)	193.4 ± 67.1 (52)	196.5 ± 63.1 (36)

Values are means ± SD determined at around 172 hr after insemination. a), b) Values with different superscripts denote a significant difference ($P < 0.05$).

shown that the blastocyst formation rate in mice embryos was significantly decreased after a 1-hr exposure of pronuclear embryos to 20% O₂ [9, 17]. It is likely that the present *in vitro* insemination procedure in 5% O₂ might reduce the oxidative stress and/or production of reactive oxygen species (ROS) [1, 7] resulting in an improvement of the subsequent embryonic development as a latent effect. Alternatively, the time course of the *in vitro* fertilization in 5% O₂ might differ from that in 20% O₂. The delay in the sperm penetration causes the aging of oocytes and reduces the viability of fertilized oocytes [11]. Further experiments on the oxidative stress/production of ROS during *in vitro* insemination and the time course of fertilization are needed to clarify the beneficial effect of *in vitro* insemination under 5% O₂ on fertilization and subsequent embryonic development.

In conclusion, the present results demonstrated that lowering the oxygen concentration from 20 to 5% during *in vitro* insemination does not affect the fertilization rate, but improves the subsequent embryonic development to the blastocyst stage under the present experimental conditions.

ACKNOWLEDGMENTS. This study was supported by a Grant-in-Aid for Scientific Research (No. 09660296) from the Ministry of Education, Science, Sports and Culture, Japan to Y. Takahashi. The authors thank the staff of the Ebetsu Meat Inspection Center for the collection of bovine ovaries, and the Hokkaido Livestock Improvement Association for the donation of frozen bull sperm.

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