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Author(s)	Adam, Ahmed Adbel Gadir; Takahashi, Yoshiyuki; Katagiri, Seiji et al.
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Effects of oxygen tension in the gas atmosphere during *in vitro* maturation, *in vitro* fertilization and *in vitro* culture on the efficiency of *in vitro* production of mouse embryos

Ahmed Abdel Gadir Adam, Yoshiyuki Takahashi*, Seiji Katagiri and Masashi Nagano

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Abstract

Effects of oxygen (O₂) tension in the gas atmosphere during *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) on the efficiency of *in vitro* production of mouse embryos were examined. Mouse oocytes recovered from large antral follicles were subjected to IVM in Waymouth medium for 15, 16 and 17 hr under 5 or 20% O₂ and then subjected to IVF and IVC under 5 or 20% O₂ tension. Lowering the O₂ tension in the gas atmosphere for IVM from 20 to 5% improved the cleavage rate after IVF when the oocytes were subjected to IVM for 15 hr; however, no improvement in the cleavage rate was observed when the culture period for IVM was extended to 16 and 17 hr. Lowering the O₂ tension to 5% for IVM and IVC improved the development of the cleaved oocytes to the blastocyst stage, regardless of the culture period for IVM. However, the O₂ tension for IVF had no remarkable effect on the subsequent embryonic development. These results demonstrate that 5% O₂ is superior to 20% O₂ for IVM and IVC, and suggest that 20% O₂ for IVM may delay oocyte maturation and/or the acquisition of fertilizability and impair the developmental competence of oocytes.

Key words : development; fertilization; maturation; mouse oocyte; oxygen tension

Introduction

In vitro production (IVP) of embryos pro-

vides an excellent source of low cost embryos for basic research and for the application of embryo biotechnologies such as nuclear trans-

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

*Corresponding author : Yoshiyuki Takahashi. Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818.

Fax : +81-11-706-5231.

E-mail : ytaka@vetmed.hokudai.ac.jp

fer and production of transgenic animals. The successful development and application of IVP of embryos and related technologies is critically dependent on a whole range of basic protocols including *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). One issue that has to be resolved in relation to IVP of embryos is the problem of oxidative stress. The oxygen (O_2) tension in the female reproductive tract is very low compared with that in the atmosphere⁸ and O_2 at the atmospheric level was found to be toxic to mammalian embryos, probably due to the formation of intracellular reactive oxygen species (ROS)¹². The O_2 tension at the atmospheric level increases the production of ROS, such as hydrogen peroxide and superoxide, which are converted into more toxic hydroxyl radicals in the presence of trace amounts of transitional metals such as iron¹². These highly toxic ROS can cause severe cellular damage through membrane lipid peroxidation, enzyme inactivation and DNA damage in many cell types including the oocytes²⁶ and spermatozoa².

It is well known that in farm animals, IVC of embryos is more successful at a lower (4-8%) O_2 tension especially in the absence of somatic cell support⁴. Lowering the O_2 tension in the gas atmosphere for IVC has also been shown to be beneficial for mouse embryonic development^{18,26}, although some workers have failed to confirm this advantage on the development of mouse embryos^{1,17}.

In routine IVP, IVM is the most critical step for generating oocytes with high developmental competence. The O_2 tension in the follicular fluid decreases with follicular growth suggesting that a lower O_2 tension is required for the maturation of oocytes¹⁶. Nevertheless, a few studies have examined the effect of O_2 tension for IVM on the developmental competence of oocytes and the results are inconsis-

tent among studies. In mice, 5% O_2 for IVM was reported to be beneficial⁹ or detrimental¹¹ to the nuclear maturation and to have no effect on the subsequent development of oocytes⁶. In cattle, IVM under 20% O_2 was superior to that under 5% O_2 for nuclear maturation in medium containing 5.6 mM glucose¹⁹, whereas IVM under 5% O_2 gave higher rates of cleavage and development into blastocysts than that under 20% O_2 in medium containing 20 mM glucose¹⁰.

Concerning the effect of O_2 tension during IVF, there are a few reports and the results are inconsistent. Takahashi and Kanagawa²¹ observed that bovine oocytes are fertilized at the same rate under 5 and 20% O_2 tension, while Tanghe *et al.*²² reported that lowering the O_2 tension decreased the fertilization rates of oocytes. In mice, the effect of O_2 tension for IVF has not been reported. Therefore, the optimal O_2 tension for the various steps of IVP, especially for IVM and IVF, is still ambiguous. We hereby examined the effects of O_2 tension for IVM, IVF and IVC on the efficiency of IVP of embryos using the mouse as a model.

Materials and Methods

Experimental animals : Male and female ICR mice (Japan SLC Inc., Shizuoka, Japan) were housed and bred to produce offspring in the animal housing facility in the Graduate School of Veterinary Medicine, Hokkaido University. The animals were handled according to the guidelines laid down by the university. They were kept in light- and temperature-controlled conditions (12 hr light : 12 hr dark, 23°C), and given chow pellets and water *ad libitum*.

Preparation of oocytes : Immature (21-23 days old) female mice were treated with 5 IU of equine chorionic gonadotropin (Teikoku

Hormone Mfg. Co. Ltd., Tokyo, Japan) and sacrificed 48 hr later by cervical dislocation. The ovaries were collected into a 35-mm plastic dish (Falcon 1008, Becton Dickinson, Franklin Lakes, NY, USA) containing 2.5 ml of Leibovitz's L-15 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 4 mg/ml of bovine serum albumin (Sigma Chemical Co., St Louis, MO, USA) and 50 µg/ml of gentamicin sulfate (Sigma). Cumulus-oocyte-complexes (COCs) were released from the large antral follicles using a 25-G needle. Only the oocytes completely enclosed by cumulus cells were selected. COCs with dark, shrunken or irregular-shaped oocytes were discarded.

In vitro maturation of oocytes : About 25 COCs were transferred to a 50-µl droplet of maturation medium and incubated for 15 to 17 hr at 37°C in a humidified atmosphere of 5% CO₂ in air or 5% CO₂, 5% O₂ and 90% N₂. The maturation medium was Waymouth medium (Gibco) supplemented with 1 IU/ml of porcine follicle stimulating hormone (Antrin R, Denka Pharmaceutical Co. Ltd., Kawasaki, Japan), 10 ng/ml of human recombinant epidermal growth factor (Sigma), 5% fetal calf serum (Gibco), 0.23 mM sodium pyruvate and 50 µg/ml of gentamicin sulfate.

In vitro fertilization of oocytes : After IVM, oocytes surrounded by the cumulus cells were subjected to IVF by adapting the procedure of Toyoda *et al.*²⁵⁾. Briefly, spermatozoa collected from the caudal epididymis of matured (3 - 6 months old) male mice were incubated at 37°C for 1 hr in a 0.4-ml droplet of modified Krebs-Ringer bicarbonate solution (TYH medium)²⁵⁾ under paraffin oil (Nacal tesque, Kyoto, Japan) in a humidified atmosphere of 5% CO₂ in air or 5% CO₂, 5% O₂ and 90% N₂. An aliquot of sperm suspension was added to a droplet of 0.4 ml of TYH medium

containing about 25 COCs to give a final sperm concentration of 1.5×10^5 cells/ml. The droplets were incubated at 37°C for 5 hr under 5% CO₂ in air or 5% CO₂, 5% O₂ and 90% N₂.

In vitro culture and evaluation of embryonic development : After IVF, presumptive zygotes freed from cumulus cells were transferred to a 25-µl droplet of potassium simplex optimized medium⁷⁾ and cultured *in vitro* at 37°C in a humidified atmosphere of 5% CO₂ in air or 5% CO₂, 5% O₂ and 90% N₂. Cleavage and development to the blastocyst stage were determined at 24 and 120 hr after IVF, respectively. Live cell numbers of the blastocysts obtained after 120 hr of IVC were determined using an air-dry procedure described elsewhere²⁰⁾.

Experimental design : To examine the effects of O₂ tension for IVM, IVF and IVC, the COCs were divided into one of four experimental groups according to the O₂ tension at each step as shown in Table 1. IVM was conducted for 15, 16 and 17 hr in each experimental group.

Table 1. Oxygen (O₂) tension in the gas atmosphere during *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) in each experimental group

Experimental groups	Concentration (%) of O ₂ ^a during		
	IVM	IVF	IVC
20-20-20	20	20	20
5-20-20	5	20	20
5-5-20	5	5	20
5-5-5	5	5	5

^a20% O₂ denotes 5% CO₂ in air, and 5% O₂ denotes 5% CO₂, 5% O₂ and 90% N₂.

Statistical analysis : The effects of O₂ tension and IVM culture period were analyzed using two-way analysis of variance followed by Fisher's protected least significant

difference as a post *hoc* test. Data analysis was performed using StatView software (Abacus Concepts Inc., Berkeley, CA, USA).

Results

As shown in Table 2, significant interaction between the effects of O₂ tension in the gas atmosphere and the culture period for IVM on the cleavage rate was observed ($P < 0.001$). When IVM was conducted for 15 hr, oocytes in the 20-20-20 group showed a lower cleavage rate than those in other groups ($P < 0.05$). After 16 hr of IVM, the cleavage rate in the 20-20-20 group was lower than that in the 5-5-5 group ($P < 0.05$), but similar to that in the other two groups. When the oocytes were cultured for 17 hr, there were no differences among the four groups. The cleavage rates of oocytes in the 20-20-20 and 5-5-20 groups improved as the culture period for IVM increased ($P < 0.05$).

Table 2. Effect of O₂ tension during IVM, IVF and IVC on the cleavage of oocytes

Experimental groups *	% of cleaved oocytes subjected to IVM for different culture periods (n)		
	15 hr	16 hr	17 hr
20-20-20	49.0±6.6 ^{aA} (90)	74.4±3.1 ^{aB} (98)	86.4±5.0 ^c (103)
5-20-20	77.8±7.0 ^b (95)	79.0±4.5 ^{ab} (94)	80.0±3.2 (95)
5-5-20	75.0±2.1 ^{bA} (96)	76.3±4.8 ^{abA} (79)	84.2±3.3 ^B (96)
5-5-5	83.0±8.3 ^b (97)	82.7±3.1 ^b (91)	83.3±2.2 (95)

* See footnote in Table 1.

^{A-C} and ^{a,b} Values (means±SD of 4 replicates) with different superscripts in the same row and same column differ significantly ($P < 0.05$).

As shown in Table 3, there was no interaction between the effects of O₂ tension in the gas atmosphere and the culture period for IVM on the development of cleaved eggs to the blastocyst stage. Development to the blas-

tocyst stage was affected by the O₂ tension in the gas atmosphere, regardless of the culture period for IVM. The proportion of blastocysts in the 20-20-20 group was lower than that in the other three groups ($P < 0.001$).

Table 3. Effect of O₂ tension during IVM, IVF and IVC on the development of cleaved oocytes to the blastocyst stage

Experimental groups *	% of blastocysts based on numbers of cleaved oocytes that were subjected to IVM for different culture periods (n)			
	15 hr	16 hr	17 hr	Total
20-20-20	36.4±4.7 (44)	40.7±9.0 (73)	40.7±5.7 (89)	39.3±6.5 ^a (206)
5-20-20	56.7±4.5 (74)	59.7±7.2 (74)	55.8±8.8 (76)	57.4±6.6 ^{b*} (224)
5-5-20	49.0±6.5 (74)	54.4±4.3 (77)	57.1±4.5 (81)	53.5±5.9 ^b (223)
5-5-5	60.8±8.9 (81)	61.5±7.4 (75)	59.5±2.0 (79)	60.6±6.2 ^c (235)

* See footnote in Table 1.

% value in each experimental group was the mean ± SD of 4 replicates.

^{a-c} Values with different superscripts in the same column differ significantly ($P < 0.001$).

As shown in Table 4, both the O₂ tension in the gas atmosphere and the culture period for IVM affected the live cell numbers in blastocysts without interaction ($P < 0.05$). Regardless of the culture period for IVM, the cell number in the blastocysts was lower in the 20-20-20 group than the other three groups ($P < 0.05$). A longer culture period for IVM resulted in higher cell numbers in the blastocysts ($P < 0.05$), regardless of the O₂ tension in the gas atmosphere.

Discussion

Lowering the O₂ tension in the gas atmosphere for IVM from 20 to 5% improved the developmental competence of oocytes to the blastocyst stage and the quality (the live cell numbers) of blastocysts, regardless of the culture period for IVM. The cleavage rate was

Table 4 . Effect of O₂ tension during IVM, IVF and IVC on live cell numbers in blastocysts

Experimental groups *	Live cell numbers of blastocysts derived from oocytes subjected to IVM for different culture periods (n)			
	15 hr	16 hr	17 hr	Total
20-20-20	35.8±2.4 (11)	38.3±3.7 (26)	41.9±5.4 (33)	39.6±5.0 ^a (70)
5-20-20	42.9±6.4 (32)	47.3±7.8 (26)	50.9±8.3 (28)	46.9±8.1 ^b (86)
5-5-20	42.1±3.8 (21)	47.6±5.7 (23)	49.8±4.6 (25)	46.7±5.7 ^b (69)
5-5-5	43.4±9.2 (25)	49.3±10.7 (25)	53.1±10.2 (28)	48.7±10.7 ^b (78)
Total	42.0±6.9 ^a (89)	45.5±8.5 ^b (100)	48.6±8.6 ^c (114)	-

* See footnote in Table 1.

% value in each experimental group was the mean ± SD of 4 replicates.

^{A-C and a, b} Values with different superscripts within the same row and same column differ significantly ($P < 0.05$).

also improved by lowering the O₂ tension when the oocytes were subjected to IVM for 15 hr; however, no improvement in the cleavage rate was observed when the IVM culture period was extended to 16 and 17 hr. These findings indicate that 20% O₂ for IVM impairs the developmental competence of oocytes and may delay the oocyte maturation and/or acquisition of fertilizability.

In previous studies on mouse¹¹ and bovine oocytes¹⁹, IVM under 20% O₂ gave a higher proportion of oocytes at the MII stage than that under 5% O₂ when the oocytes were cultured using media containing 5.6 mM glucose. However, IVM under 5% O₂ gave a higher blastocyst rate than that under 20% O₂ when bovine oocytes were cultured in medium containing 20 mM glucose¹⁰. The present study supports the latter finding since we cultured mouse oocytes using Waymouth medium, which contains a high concentration (27.7 mM) of glucose. When using a medium containing 20 mM glucose, IVM under 20% O₂

increased the intracellular hydrogen peroxide level in bovine oocytes and reduced the blastocyst formation rate compared with that under 5% O₂¹⁰. Accordingly, in the present study, the developmental competence of mouse oocytes matured in Waymouth medium under 20% O₂ might be impaired by oxidative damage due to the increase in the intracellular ROS level. In contrast to the present results, a previous study reported no differences in the cleavage and development to the blastocyst stage between mouse oocytes matured in Waymouth medium under 5 and 20% O₂⁶. The discrepancy may be attributed in part to the difference in the strain of mice used. The present study used ICR mice (blocking strain) in which embryos are sensitive to the culture conditions and show cleavage arrest at the two-cell stage (two-cell block) when cultured in classical chemically defined media. Whereas the previous study⁶ used oocytes and sperm collected from F1 mice (C57BL/6L × SJL), in which two-cell block seldom occurs, and obtained very high rates of cleavage (about 85%) and development to the blastocysts (90%), regardless of the O₂ tension for IVM. Therefore, the beneficial effect of low O₂ tension for IVM would have been difficult to detect in the previous study.

In the present study, lowering the O₂ tension for IVF from 20 to 5% had no remarkable effect on the subsequent development of mouse oocytes. This result is comparable to those reported earlier which showed that bovine oocytes were fertilized at the same rate under both 5 and 20% O₂ tension, although the subsequent development to blastocysts was improved when IVF was conducted under 5% O₂²¹. In humans, Duomoulin *et al.*⁵ failed to demonstrate any advantage for the use of 5% O₂ over 20% O₂ during IVF. In contrast to the previously mentioned studies, Tangle *et al.*²² reported that lowering the O₂ tension to

5% decreased the fertilization rate of bovine oocytes. The reason for this discrepancy is not clear but the requirements for O₂ may vary with different media and supplements. Further studies are needed to determine the optimum O₂ tension for IVF.

Lowering the O₂ tension for IVC from 20 to 5% improved the proportion of cleaved oocytes that developed into blastocysts. This finding is in agreement with previous reports in which reducing the O₂ tension from 20 to 4 - 8% improved the embryonic development in sheep²³⁾, cattle²⁴⁾, hamsters¹⁵⁾, rats¹³⁾, goats³⁾ and rabbits¹⁴⁾. In mice, 5% O₂ was reported to improve the blastocyst rate in oocytes collected from Tuck No. 1 strain²⁶⁾ and F1 (C57 BL×DBA)⁵⁾, but to have no advantage in oocytes from MF 1 strain¹⁷⁾, Swiss outbred strain¹⁾ and a randomly bred Swiss strain⁵⁾. The contradiction in the effect of O₂ tension for IVC on the development of mouse embryos is no doubt due in part to the differences in animal strain, as well as to the culture medium components⁴⁾.

An overall improvement of around 20% in the efficiency of blastocyst formation was observed by conducting IVM under 5%. Extending the culture period for IVM increased the live cell numbers in the blastocysts, regardless of the O₂ tension in the gas atmosphere. However, further experiments are needed to optimize the culture period for IVM under 5% O₂ since excessive extension of the culture period for IVM may cause the aging of oocytes.

In conclusion, the present results demonstrate that IVM and IVC under 5% O₂ can improve the efficiency of IVP of mouse embryos compared with those under 20% O₂. The results also suggest that IVM under 20% O₂ may delay oocyte nuclear maturation and/or the fertilizability of oocytes and impair the developmental capacity of oocytes.

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