

# Effects of Oxygen Tension on Survival and Growth In Vitro of Bovine Oocytes from Early Antral Follicles

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**Abstract:** The purpose of this study was to assess the effect of oxygen condition on the survival and growth in vitro of bovine oocyte-cumulus/granulosa cell complexes isolated from early antral follicles. Complexes comprised of a growing oocyte enclosed in cumulus layers and a piece of mural granulosa cells were isolated from early antral follicles, 0.5–0.7 mm in diameter, and cultured for 14 days in TCM199 supplemented with 5% fetal bovine serum and 4 mM hypoxanthine in an atmosphere of 5% O<sub>2</sub> or 20% O<sub>2</sub>. Mean oocyte diameter at the beginning of culture was about 95 μm. Within 4 days formation of the antrum-like structure was evident in virtually all complexes, regardless of high or low oxygen tension, but many of the complexes failed to retain this structure after 8 days. At low oxygen tension, complexes retain the antrum-like structure for slightly longer periods than those cultured at high oxygen tension, yet no significant difference was found. Accordingly, the percentage of oocytes that survived in culture was higher at low oxygen tension. Nevertheless, the oocytes (105.9 ± 8.4 μm) were smaller than those grown at high oxygen tension (112.6 ± 10.5 μm) (P < 0.05). Supplementation of estradiol-17β was effective in increasing the percentage of follicles that retained the cavity, especially at low oxygen tension, and of oocytes recovered as being enclosed with cumulus cells after 14-day cultures, but no promotion of oocyte growth caused by estradiol-17β was observed. Taken together, a reduced oxygen condition may be beneficial in improving the survival rate of oocytes, but

not in promoting growth in the culture system used in this study.

**Key words:** Bovine, Growing oocytes, Granulosa cell, Culture, Oxygen tension

Primordial follicles in mammalian ovaries are a large source of oocytes, though most of them undergo degeneration before recruitment in the growing pool. These primordial follicles would be a potentially valuable resource, if we could rescue them before they are lost in ovaries and if we could provide them with environments that will support their growth and differentiation to a growth stage equivalent to that of oocytes in preovulatory follicles, but it is very difficult to utilize them with the current culture technology, though it is not impossible [1]. A more applicable approach may be culturing of oocytes that have already entered the growing phase, because most them will degenerate in ovaries before completion of the lengthy process of growth to the final size. Various culture systems have been devised for growing oocytes in several species, including mice, rats, pigs, cattle, sheep and humans; see [2] for reviews. Among these studies, several different culture systems have been tested for mouse oocytes, and it has become evident that mouse oocytes can be fully-grown *in vitro* from the mid-growth phase at a moderate frequency, and achieve the competence to undergo meiotic maturation and fertilization [3–5]. Some of them are competent to undergo preimplantation embryo development, and even further development [1, 3, 5]. Except for studies on the mouse, most well established culture systems are probably

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those for bovine growing oocytes [6–14]. In one case [10], it was clearly demonstrated that an oocyte competent to undergo embryo development to term was developed after 14 days of culture, starting with the material isolated from early antral follicles. Nevertheless, overall efficiency of oocyte growth *in vitro* is low.

There is a body of evidence suggesting that oocyte growth is regulated by nutrients and molecular signals provided from associating granulosa cells [15, 16]. Low molecular weight molecules are transferred between oocytes and granulosa cells through gap junctions [17, 18]. In addition to the communication through gap junctions, both granulosa cells and oocytes produce paracrine factors [19, 20], which are important in the regulation of oocyte growth and follicular development. For example, growth differentiation factor-9, which is produced by oocytes, promotes granulosa cell proliferation [21–23], and regulates granulosa cell differentiation [24–28]. Moreover, oocytes seem to promote the formation of follicular antrum [29, 30], so that there is a bidirectional regulatory loop between the oocytes and their companion granulosa cells [31, 32]. In order to establish optimal culture conditions to support oocyte growth, normal proliferation and differentiation of granulosa cells surrounding oocytes should be the most essential components of the culture systems. Abnormal differentiation of either granulosa cells or oocytes during cultures would eventually result in degeneration or aberrant growth of oocytes.

The oxygen condition in the culture is probably one of the factors most affecting the health of cells *in vitro*. Oxygen tension affects the proliferation of various cell types [33–36], oocyte maturation [37] and preimplantation embryo development [38–42]. The oxygen concentration also affects *in vitro* growth of mouse oocytes, though the optimal concentration appears to depend on the culture system [43, 44]. A relatively hypoxic culture environment suitable for oocytes surrounded by granulosa cell layers [43] may be too anaerobic for oocytes enclosed within intact follicles [44]. Care should therefore be taken when comparing the effects of oxygen tension in experimental groups wherein quite different numbers of granulosa cells or other somatic cells are in contact with the oocytes. In this study it was determined whether the oxygen concentration affects the morphology and survival of bovine oocyte-cumulus/granulosa cell complexes, and the growth of oocytes. In some experiments the effect of oxygen tension was examined under conditions where the proliferation of granulosa

cells was stimulated by estradiol-17 $\beta$ . Complexes of growing oocytes enclosed in cumulus cell layers were obtained from early antral follicles as each of the complexes was associated with a group of mural granulosa cells [7]. Complexes embedded in collagen gels were cultured for 14 days in serum-supplemented medium with a high or low oxygen tension.

## Materials and Methods

### *Collection of growing oocytes from early antral follicles*

To isolate and culture growing oocytes, the methods described by Harada *et al.* [7] were used. Bovine ovaries were obtained at a local abattoir, and transported to the laboratory in phosphate-buffered saline. From the surface of the ovaries early antral follicles, 0.5–0.7 mm in diameter, were isolated, and only healthy-looking follicles were used for this study. Oocyte-cumulus cell complexes, each of which was attached by a group of mural granulosa cells, were dissected from the follicles with fine forceps. Hereinafter these complexes are referred to as oocyte-cumulus/granulosa cell complexes (OCGs). No theca cells were included in the complexes. Each OCG was transferred to a microdrop of HEPES-buffered TCM199 (Sigma Chemical Co., St Louis, MO, USA) under paraffin oil, and the diameter of oocytes exclusive of the zona pellucida was measured to the nearest 0.5  $\mu$ m with an ocular micrometer attached to an inverted phase contrast microscope. The average oocyte diameter was about 95  $\mu$ m after isolation from early antral follicles.

### *Culture of OCGs*

OCGs were embedded in collagen gels by the method based on the manufacturer's instructions (Nitta Gelatin, Tokyo, Japan) and modifications designed to culture porcine preantral follicles [45]. Briefly, the collagen solution (0.3%, Cellmatrix Type I; Nitta Gelatin) was mixed with 10 times-concentrated TCM199 without NaHCO<sub>3</sub>, and 0.05 N NaOH solution containing 22 mg/ml NaHCO<sub>3</sub> and 47.7 mg/ml HEPES, at a ratio of 8:1:1. The mixture, approximately 600  $\mu$ l, was placed in the center of a culture dish (#1008, Falcon; Becton Dickinson Labware, Lincoln Park, NJ, USA), and OCGs were transferred into the mixed gel solution. After incubation for a few minutes, 4 ml of the culture medium described below was added to the dish. The standard culture medium used in this study was TCM 199 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 0.1 mg/ml sodium pyruvate (Wako Pure Chemical Industries Ltd., Osaka, Japan), 0.08 mg/ml kanamycin

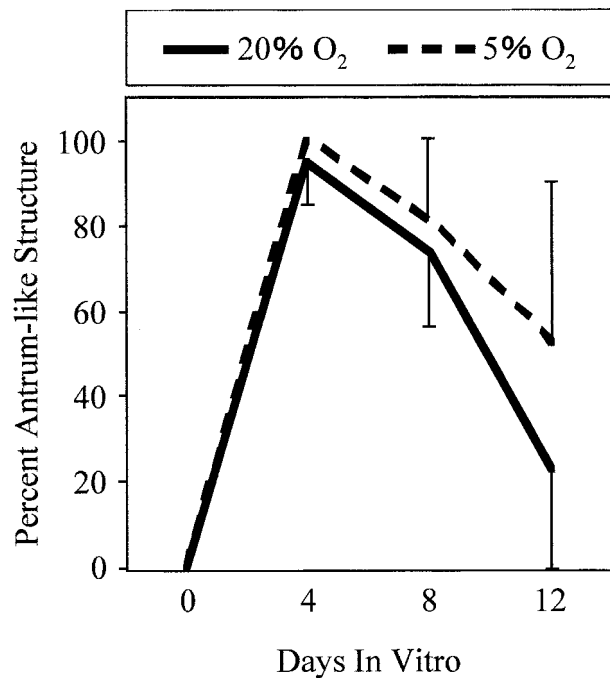
(Sigma) and 5% fetal bovine serum (HyClone, Logan, UT, USA). The medium was also supplemented with 4 mM hypoxanthine (GIBCO, Grand Island, NY, USA), because the structure of complexes is maintained better in the presence of 2–4 mM hypoxanthine [7, 46, and our unpublished observations]. OCGs were cultured at 38.5°C under an atmosphere of either 5% CO<sub>2</sub> and 95% air, or 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>. In the second series of experiments, estradiol-17β (0.1 μg/ml, Sigma) was added to the culture medium to promote granulosa cell proliferation. Estradiol-17β was first dissolved in ethanol at a concentration of 0.1 mg/ml, and diluted just before use to make the final concentration. Cultures were fed every 4 days by replacement of approximately half of the medium. Formation of the antrum-like cavity was examined after 4, 8 and 12 days of culture. At the end of 14-day cultures, complexes were isolated from collagen gels with forceps, and all oocytes were collected, whether or not they were enclosed by cumulus cells. Only the oocytes whose morphology was observed to be normal under a phase-contrast microscope were examined for the diameter as described above.

#### *Data presentation and statistical analysis*

Oocyte size is shown in figures as notched boxes and whisker plots prepared with Statview software (Abacus concepts, Inc., Berkeley, CA, USA) as described by Eppig and O'Brien [1]. Comparison of oocyte diameters was performed with *t*-test. The incidence of antrum-like structures is shown as the mean percentages of at least three independent experimental replicates, and the variation between experiments is shown with the standard deviation of the mean. For statistical analysis, data were subjected to arcsin transformation and ANOVA. Groups were compared by means of Fisher's protected least significant difference posthoc test with Statview (Abacus Concepts). When *P*<0.05, the difference was considered significant.

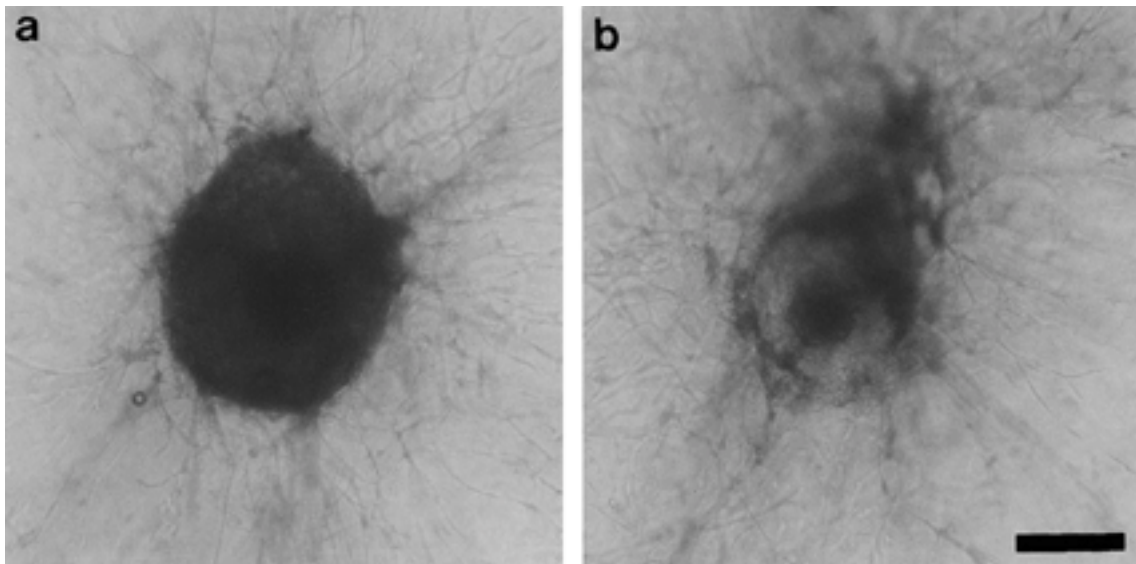
### **Results**

In the first set of experiments, OCGs were cultured for 14 days in hypoxanthine supplemented medium, at high or low oxygen tension. Since the complexes were obtained from early antral follicles, layers of cumulus cells surrounding oocytes and pieces of mural granulosa cells were clearly distinctive. The groups of mural granulosa cells attached to each complex were usually so small for the cavity to form unless the granulosa cells proliferated and the complex became of

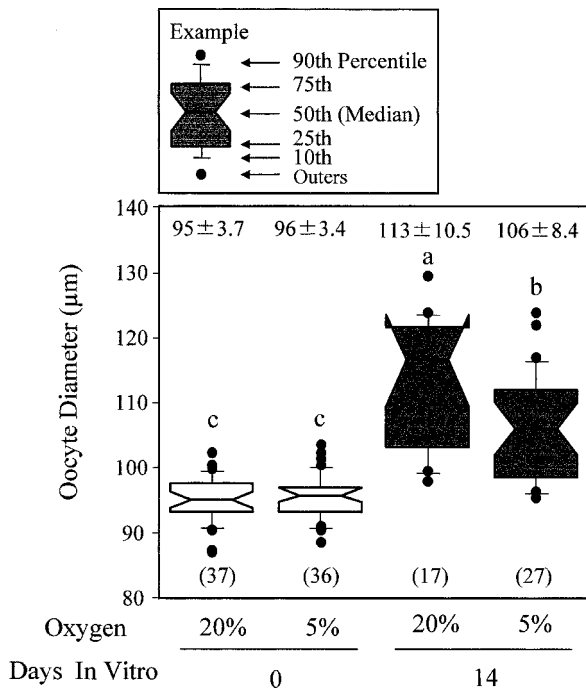


**Fig. 1.** Formation and loss of the antrum-like structure in the bovine oocyte-cumulus/granulosa cell complexes in 20% O<sub>2</sub> or 5% O<sub>2</sub>. Data are expressed as the mean ± SD percentage of complexes exhibiting an antrum-like cavity in three independent experiments having 10–14 complexes in each group.

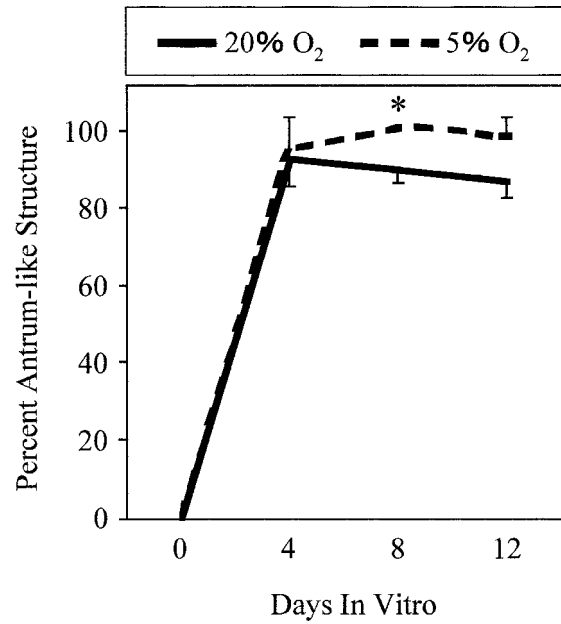
a size comparable to that of late-preantral follicles. Within 4 days in culture, virtually all OCGs formed a completely enclosed fluid-filled cavity. After formation of the antrum-like structure, some complexes failed to retain the cavity, presumably due to the reduced number of cells, within a few days (Fig. 1). The loss of antrum-like structure usually became manifested by the disappearance of "mural" granulosa cells (Fig. 2). This failure appeared to occur earlier at high oxygen tension, where 74% and 23% of the complexes retained the cavity after 8 and 12 days of culture, respectively. In the low oxygen condition, 81% and 53% of the complexes retained the cavity after 8 and 12 days of culture, respectively, and more normal oocytes were observed after a 14-day culture in a 5% O<sub>2</sub> atmosphere than those cultured in an atmosphere of 20% O<sub>2</sub> (75% vs. 46%). As shown in Fig. 3, an increase in oocyte size during culture, from 95.3 ± 3.5 μm (n=73), is evident in both groups. Interestingly, more oocytes became larger than 115 μm at high oxygen tension than those at low oxygen tension, despite the lower surviving rate of the oocytes. The mean diameter ± SD after the culture in



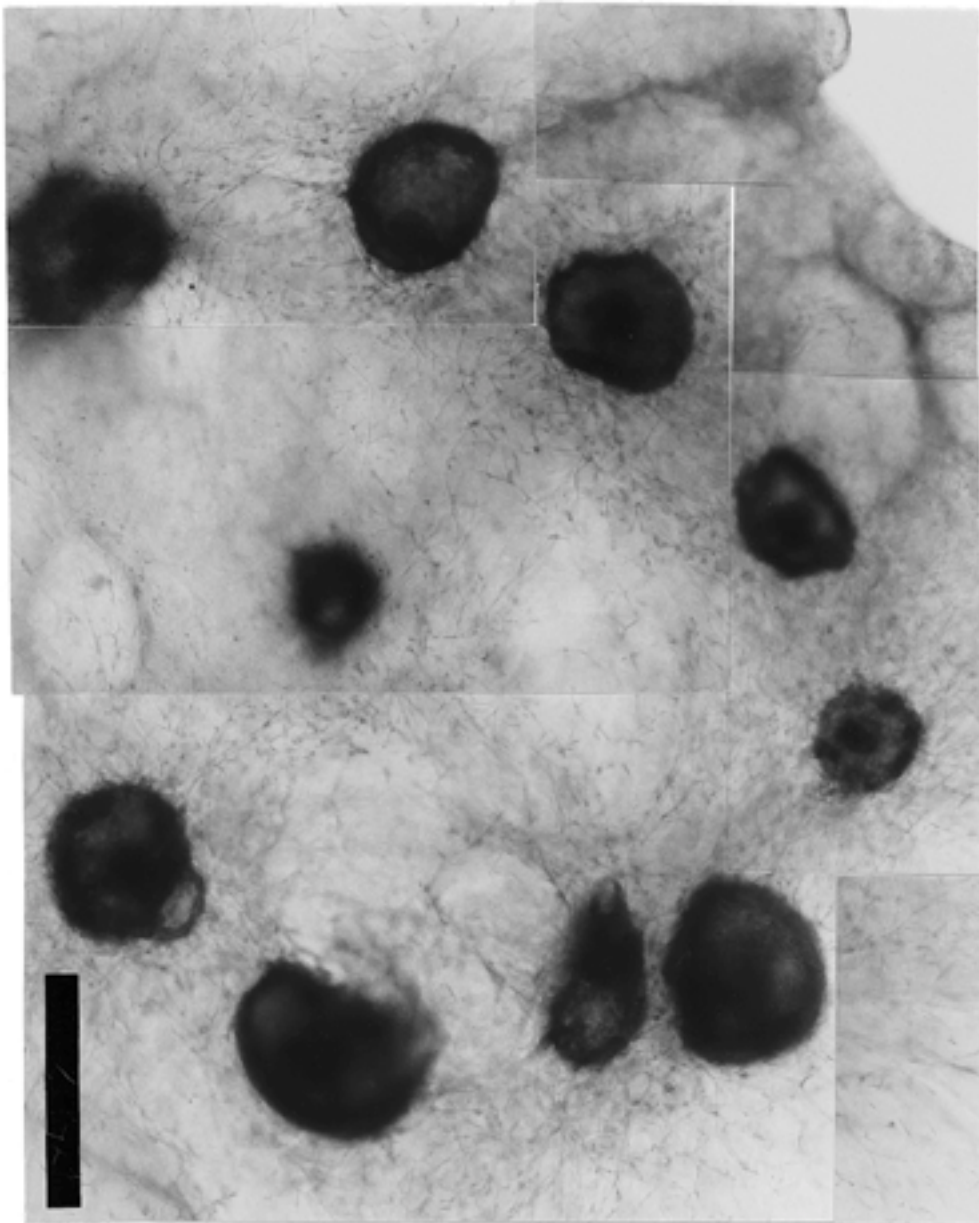
**Fig. 2.** Oocyte-cumulus/granulosa cell complexes cultured for 8 days in an atmosphere of 5% O<sub>2</sub> (a) or 20% O<sub>2</sub> (b). Note the intact “mural” granulosa cell layers (a), and the exposed oocyte-cumulus cell complex (b). Bar indicates 200 μm.



**Fig. 3.** Comparison of oocyte diameter after recovery from 14-day cultures in the standard medium in an atmosphere of 20% O<sub>2</sub> or 5% O<sub>2</sub>. The numbers above the box plots indicate the mean ± SD diameter (μm) of the oocytes in that group, and numbers in parentheses below the box plots are the number of oocytes. Different letters above the box plots indicate significant difference ( $P < 0.05$ ).



**Fig. 4.** Formation and loss of the antrum-like structure by the bovine oocyte-cumulus/granulosa cell complexes in 20% O<sub>2</sub> or 5% O<sub>2</sub> in the presence of estradiol-17β. Estradiol-17β (0.1 μg/ml) was added to the medium of the same composition as used in the experiments shown in Fig. 1. Data are expressed as the mean ± SD percentage of complexes exhibiting an antrum-like cavity in three independent experiments having 10–14 complexes in each group. An asterisk (\*) indicates significant difference ( $P < 0.05$ ).

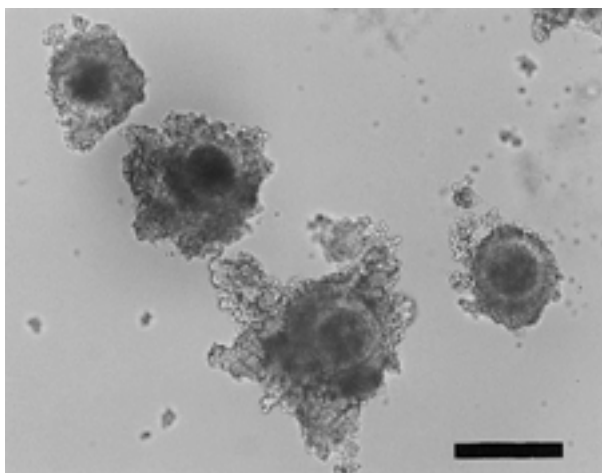


**Fig. 5.** Oocyte-cumulus/granulosa cell complexes cultured for 14 days in medium supplemented with estradiol-17 $\beta$  at low oxygen tension. Note the antrum-like structure in the complexes embedded in the collagen gel. Bar indicates 1 mm.

high or low oxygen tension was  $112.6 \pm 10.5 \mu\text{m}$  and  $105.9 \pm 8.4 \mu\text{m}$ , respectively.

From the observations obtained in the first experiment, the loss of antrum-like morphology appeared to be due, at least in part, to the decreased number of granulosa cells. Therefore, in the second experiment cultures were performed with medium supplemented with estradiol-17 $\beta$ , which acts to promote

granulosa cell proliferation [47]. Consistent with the observation described above, the formation of antrum-like cavities was evident within 4 days (Fig. 4). It should be noted, however, that the incidence of the loss of antrum-like cavities was delayed in the presence of estradiol-17 $\beta$  in both groups (Fig. 5). Especially the percentage of follicles with the cavity after 8 day-culture was significantly higher at reduced oxygen tension than



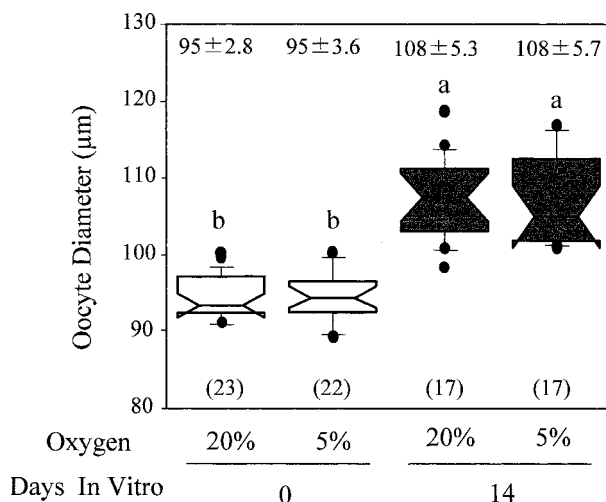
**Fig. 6.** Oocyte-cumulus cell complexes recovered from the complexes shown in Fig. 5. Bar indicates 200  $\mu\text{m}$ .

at high oxygen tension. When oocytes were recovered after the culture, almost all of them were still contained in the complexes (Fig. 6), and over 70% of oocytes in both oxygen conditions survived through the 14-day culture period. To our surprise, however, increase in oocyte size was not stimulated (Fig. 7), despite these improved culture conditions as judged by retention of antrum-like cavities and continued accompaniment of cumulus cells with oocytes. For some oocytes, the culture period was extended for 6 days beyond our standard 14-day culture. Twenty-four oocytes were cultured in either high or low oxygen conditions, and 18 oocytes (75%) were recovered in both oxygen conditions. Of these, 56% and 44% of oocytes at high or low oxygen tension, respectively, had already been spontaneously freed from surrounding cumulus cells. Average oocyte diameter was not increased during the extended culture period regardless of oxygen tension (about 106  $\mu\text{m}$ ).

## Discussion

The results reported here show that more complexes comprised of a growing oocyte and granulosa/cumulus cells can survive at reduced oxygen tension. In spite of the prolonged retention of antrum-like structures, however, the reduced oxygen tension did not stimulate the growth of oocytes.

It may not be surprising that more oocytes survived at low oxygen tension in the standard medium, and that granulosa cell proliferation may have been promoted, for there is growing evidence suggesting that the



**Fig. 7.** Comparison of oocyte diameter after recovery from 14-day cultures in the estradiol-17 $\beta$  supplemented medium in an atmosphere of 20% O<sub>2</sub> or 5% O<sub>2</sub>. The numbers above the box plots indicate the mean  $\pm$  SD diameter ( $\mu\text{m}$ ) of the oocytes in that group, and numbers in parentheses below the box plots are the numbers of oocytes. Different letters above the box plots indicate significant difference ( $P < 0.05$ ).

reduced oxygen tension is preferable in cultures for various cell types [33–36]. Deleterious effects of free-radical mediated oxidative stress in general would easily damage cells at high oxygen tension. Our results may simply indicate that reduced oxygen tension is suitable for the survival and proliferation of granulosa cells, which may have resulted in higher viability of oocytes. It was also possible that more oocytes survived in 5% O<sub>2</sub> because the oxidative stress was reduced also in the oocytes. Since oocytes play an important role in controlling the growth of granulosa cells, it was possible that in this study that more oocytes might have participated in regulating their environment at lower oxygen tension through the interaction with companion granulosa cells. On the other hand, oocytes were recovered at high frequency when cultured in medium supplemented with estradiol-17 $\beta$  regardless of oxygen tension. In this case, the granulosa cells maintained in appropriate conditions could have provided more nutrients for oocytes. Alternatively, the increase in the number of granulosa cells associating the oocytes could have mitigated the deleterious oxidative stress, or both.

It has been suggested that oocytes also play a crucial role in the process of antrum formation [29, 30]. We found the formation of antrum-like cavities in virtually all

the complexes after 4 days. This observation therefore may also suggest the existence of an intimate interaction between granulosa cells and oocytes, though the nature of the oocyte factor involved is not known. At least for the first few days the interaction must have occurred in all groups, but about 70% of the complexes in the standard medium failed to retain the antrum-like structure after a 12-day culture. Loss of the cavity seemed to be due, at least in part, to a decrease in the number of granulosa cells comprising the complex. This may be partly supported by results showing that the addition of estradiol-17 $\beta$ , which obviously promoted granulosa cell proliferation, was effective in prolonging the period in which complexes retain the antrum-like structure. But it is not known whether estradiol-17 $\beta$  may have roles other than the promotion of granulosa cell proliferation, such as the prevention of apoptosis or stimulating the differentiation of granulosa/cumulus cells.

Finally it should be noted that in spite of the improvement in the survival rate of complexes, neither reduced oxygen tension nor estradiol supplementation stimulated the growth of oocytes, even though the oocytes were larger than those before the culture. In fact, average oocyte size was smaller at reduced oxygen tension than at high oxygen tension when both were maintained in the standard medium. In the presence of estradiol-17 $\beta$ , however, oocytes became similar in size to those cultured at reduced oxygen tension with the standard medium. Importantly, many of the 'small' oocytes which recovered after the culture at reduced oxygen tension and/or with estradiol-17 $\beta$  were accompanied by layers of cumulus cells. Herlands *et al.* [16] reported that the rate of oocyte growth is directly related to the number of granulosa cells coupled to it. Therefore, although we did not examine the coupling between oocytes and companion granulosa cells, it seems possible that maintenance of the association between cumulus cells and oocytes was not sufficient quantitatively to support oocyte growth under the culture conditions used. Perhaps culture conditions suitable for granulosa cell proliferation may not necessarily be those which can provide optimal support for oocyte growth. But it should be pointed out that the smaller average size of oocytes observed in groups with a higher survival rate might be simply due to the survival of many small oocytes, as mentioned by Eppig and O'Brien [1]. The quality of oocytes grown under the culture conditions presented here remains to be investigated. Only a small proportion of oocytes that grew to over 115  $\mu\text{m}$  have become competent to

undergo preimplantation embryo development (unpublished observations). Further studies are necessary to establish culture conditions under which normal growth and differentiation of oocytes and granulosa cells are compatible.

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