Growth of Bovine Oocyte-Granulosa Cell Complexes Cultured Individually in Microdrops of Various Sizes

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Abstract. In mammalian embryo culture, the embryo:medium volume ratio can substantially affect embryo developmental performance. In the present study, we tested the possibility of improving the growth of bovine oocytes by reducing the medium volume, from a typical volume used in mouse follicle culture to a minimum possible level. A total of 282 complexes, each containing a growing oocyte 87–100 μm in diameter, were individually placed in microdrops of 2, 5, 10 or 20 μl and cultured for 13 days in a modified TCM-199 supplemented with 4% polyvinylpyrrolidone (molecular weight: 360 kDa). Oocyte diameter was measured every other day to trace the growth of each oocyte. Half the medium was replaced every other day or every day, and comparison revealed that daily replacement was more favorable for culture of these microdrops. The highest survival rate, 95%, occurred in the 20-μl microdrops, where most oocytes continued to grow throughout the culture period. In comparison, in the 5- and 10-μl microdrops, more oocytes died, and growth slowed towards the end of culture. In the 2-μl microdrops, which had the highest death rate, growth virtually ceased after 9 days. The surviving oocytes were usually accompanied by a characteristic dome-like structure of the granulosa cell mass, except in the 2-μl microdrops. In conclusion, the 20-μl microdrops allowed oocyte growth at an acceptable level, and any further reduction of the volume only had a negative impact on oocytes.

Key words: Bovine, Growth, In vitro, Microdrop, Oocyte-granulosa cell complex

complexes consisting of an oocyte and cumulus/granulosa cells was performed as previously described by Harada et al. [22] with some modifications [12]. Briefly, early antral follicles measuring 0.3–0.7 mm in diameter were isolated from the surface of the ovaries. The follicles were opened using forceps and a blade to dissect out the oocyte-cumulus cell complexes associated with a group of mural granulosa cells, hereafter referred to as oocyte-granulosa cell complexes or simply complexes. The collected complexes were each transferred to a microdrop of the collection medium, a HEPES-buffered modified Minimum Essential Medium (pH 7.2) prepared with Earle’s balanced salt solution, essential amino acids, 20 mM HEPES (Gibco, Grand Island, NY, USA), 5 mM sodium bicarbonate, 1 mM sodium pyruvate (Wako, Osaka, Japan), 4% polyvinylpyrrolidone (PVP; w/v, molecular weight 360 kDa) and 0.08 mg/ml kanamycin sulfate (Sigma-Aldrich, St. Louis, MO, USA). Only oocytes with a healthy appearance were used.

Oocyte growth in microdrops

The culture medium used for oocyte growth was TCM-199 (Gibco) supplemented with 0.1 mg/ml sodium pyruvate, 4% PVP, 0.08 mg/ml kanamycin, 0.1 μg/ml estradiol-17β, 4 mM hypoxanthine (Sigma), 5% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 10 ng/ml bovine follicle stimulating hormone (FSH; NIDDK, Washington, DC, USA). Each complex was transferred into microdrops of 2, 5, 10 or 20 μl overlaid with paraffin oil (Nacalai, Kyoto, Japan) in culture dishes (Falcon 1007; Becton Dickinson Labware, Bedford, MA, USA). The cultures were housed in an incubator at 38.5°C under an atmosphere of 5% CO2 in air. The oocytes we re cultured at 38.5°C for 24 h under an atmosphere of 5% CO2 in air. At the end of the culture period, the oocytes were mechanically denuded of granulosa cells using a small bore pipette with the help of hyaluronidase (Sigma). The oocytes, all uniquely identified, were then mounted on a slide and fixed with acetic alcohol. The following day, the chromatin was stained with acetic orcein and examined under an inverted microscope. Oocytes were classified according to the five stages of meiotic maturation: germinal vesicle, diakinesis or prometaphase I, metaphase I, anaphase I or telophase I and metaphase II.

Data presentation and statistical analysis

The following characteristics were compared among the different volume groups: survival rate, oocyte diameter, morphology of the complexes and meiotic competence of the obtained oocytes. The distribution of oocyte diameter is shown in the figures using notched boxes and whisker plots. Comparison of oocyte diameters on Day 13 among the experimental groups was performed using a t-test. Statistical analysis of the percentage of oocyte recovery and cavity formation among the experimental groups was performed using Fisher’s exact test. Regression analysis was used to evaluate the relationship between the oocyte sizes on Days 1 and 13. Differences were considered significant at P<0.05.

Results

Morphology of complexes in vitro

The complexes, when placed on a dish, started to grow outward by the next day. Figure 1 shows their typical morphology in the 2-, 5- and 20-μl microdrops during culture with a daily medium exchange. As the common primary morphology on Day 3, each complex had an oocyte at the centre, around which piled granulosa cell populations spread outward to become a thin cell layer on the periphery of the complex (Fig. 1A–C). Over the next few days, the complexes gradually developed a small cavity (or cavities) in a dense area of the granulosa cells. Then, development of a well-developed cavity within the next few days became a common characteristic among the complexes, except those in the 2-μl microdrops. Figure 1D–F shows the morphology on Day 8. Many complexes in the 2-μl microdrops still had no cavities; however, if they did, they were very small (Fig. 1D). In contrast, the complexes in the 20-μl microdrops usually developed a dome-like structure by Day 8 (Fig. 1F). Even on Day 13, the complexes in the 2-μl microdrops were not much larger than those on Day 3 (Fig. 1G). On the other hand, growth of the complexes apparently continued in the 5- and 20-μl microdrops (Fig. 1H, I) during the last 4 days, and of these two groups, the complexes of the latter were larger. A similar dome-like structure developed in the 10-μl microdrops, but the complexes were slightly smaller than those grown in the 20-μl microdrops (figure not shown).

There were some other complexes exhibiting undesirable but not uncommon morphology (Fig. 2). While the oocytes could survive without more than a single cell layer around them (Fig. 2A), some died despite being enveloped with sufficient granulosa cells (Fig. 2B). The dome-like structure sometimes formed only to subsequently deflate (Fig. 2C).
Replacement of half the medium every other day

A steady decrease in survival rate was found in all groups during the second week of culture (Fig. 3A). The most severe decrease, i.e., 7% on Day 13, was seen in the 2-μl microdrops, while the best rate, i.e., 65%, was seen in the 20-μl microdrops. Figure 3B shows the percentages of oocytes accompanied by at least one cavity. The rate fluctuated with time, but eventually became approximately equal to the rate of surviving oocytes in each group (Fig. 3A and B).

The changes in the size distribution of the oocytes in the above described experiments are shown in Fig. 4. The box plots represent the size of the oocytes that survived the whole culture period. Thus, for the two oocytes left in the 2-μl microdrops, no box plots were prepared. The mean diameters on Day 13 in the 5-, 10- and 20-μl microdrops were 110.0 ± 4.9, 108.6 ± 6.4 and 110.9 ± 5.3 μm, respectively. Oocyte growth in culture was obvious from about 93 μm on Day 1. No significant differences were found among the four groups.

Replacement of half the medium daily

When the frequency of medium exchange was doubled, oocyte viability, oocyte growth and dome/cavity formation in the complexes were all favorably affected. As shown in Fig. 5A, the oocytes degenerated more slowly than in the experiments depicted...
Bovine oocyte growth in microdrop in Fig. 3A. The 20-μl microdrops were particularly favorable for maintaining a high survival rate, which ended up being 95% (Fig. 5A). Because of the higher survival rates, the percentage of cavity formation was also elevated as the viable oocytes were usually accompanied by one or more cavities. The rate of cavity formation increased sharply toward Day 7, when it reached a plateau (Fig. 5B). The percentages on Day 13, in all except the 2-μl microdrops, were almost parallel to the survival rates (Fig. 5A and B). In the 2-μl microdrops, many complexes survived the 13-day culture period without forming cavities.

Figure 6 shows the size distributions of the oocytes from Days 1 to 13 in the experiments described above and depicted in Fig. 5. The mean oocyte diameter on Day 1 was approximately 94 μm, and the final sizes in the 2-, 5-, 10- and 20-μl microdrops were 105.5 ± 3.6, 109.1 ± 5.4, 109.3 ± 5.2 and 112.1 ± 5.6 μm, respectively. The average oocyte size in the 20-μl microdrops was significantly larger than those attained in the smaller microdrops. Only the growth of oocytes in the 20-μl microdrops continued satisfactorily until the end of the culture period, while in others, the growth slowed in the last few days.

Oocyte size and meiotic competence

Figure 7 shows scatter plots of the size distribution of individual oocytes on Days 1 and 13. In each group, a moderate but significant positive correlation was found, except in the 2-μl microdrops. A relatively high correlation was found in the 5-μl microdrops,
oocytes were more likely to progress to metaphase II. Since the 2-

μm requirement for a culture system targeting growth of bovine oocytes should be that the oocytes grow larger than 110 μm.

Fig. 7. Scatter plots of oocyte sizes on Days 1 and 13 and the meiotic competence of the resulting oocytes are shown. Data are pooled from five independent experiments including those shown in Figs. 5 and 6. The numbers of analyzed oocytes shown in each panel are as follows: A (2 μl)=34, B (5 μl)=15, C (10 μl)=30 and D (20 μl)=34.

Discussion

In the present study, only the 20-μl microdrops allowed an acceptable level of oocyte growth; this medium volume is essentially the same as that often used (20–30 μl) for the culture of mouse preantral follicles [8, 23–25]. Thus, using smaller microdrops than “usual” compromised the growth of bovine oocytes, and the 2-μl microdrops were particularly detrimental in every regard.

Given these circumstances, our observations may be primarily related to undernourishment and accumulation of cell waste such as ammonium [26] in the medium, which might eventually have had a negative impact on the cells’ interactions. This hypothesis is plausible because (i) the morphology of the complexes in the 20-μl microdrops was similar to that seen in a larger medium volume [12] and (ii) extending the interval of medium exchange markedly reduced the survival rate. Along with their proliferation, the granulosa cells possibly created an increasingly deteriorating environment in the extremely small 2-μl microdrops, which then diminished the overall activity of the cells. In fact, oocyte growth was apt to level off in the later stages of culture, which occurred earlier in the 2-μl microdrops than in the other microdrops.

Even in the 20-μl microdrops, many oocytes fell short of the 120 μm diameter; in this regard, no greater growth was accomplished than that in a previous study [12]. However, we have gathered new and detailed data on how oocytes reached this size on Day 13, and this enables us to discuss why they did not reach their potential full size. A proportion of oocytes possibly experienced slow growth due to the suboptimal culture conditions in a manner similar to that in the smaller microdrops. In addition, Fig. 6 suggests that 13 days was too short a time for oocytes <95 μm on Day 1 to complete their growth. It is probably best to extend the culture period on the presumption of continuous oocyte growth during the extended period. In the mouse, the appropriate length of the culture period is mostly determined by the oocyte size at the start of culture [27]. Considerable attention should also be paid to the initial oocyte size and culture period duration in bovine oocytes. The culture period could be shortened if the oocytes are somehow induced to grow faster—faster than the rate in vivo—although this seems to be quite unattainable at present.

Dome/cavity formation almost always accompanied the surviving oocytes after growth, except in the 2-μl microdrops, suggesting that an intimate relationship exists between cavity formation and the presence of the oocyte itself or oocyte-derived factor(s). Given that a similar dome-like structure has been documented for several mammals [28–30], this morphological alteration seems to be firmly programmed and almost inseparable from normal oocyte growth. However, a dome-like structure is not an absolute requirement for oocytes to complete their growth, as evidenced in the pioneering studies of Eppig et al. [6, 31], which showed that mouse oocytes can accomplish growth in vitro, both in size and function, without forming a dome-like structure. Similarly, in some cases, bovine complexes having small cavities, but not a dome, support sufficient growth of oocytes [12]. In the present study, the fact that many complexes did not have cavities in the 2-μl microdrops was probably due to an insufficient number of granulosa cells.

An oocyte size of about 110 μm seems to be a prerequisite for progression to metaphase II. This is in agreement with previous studies that suggest that competence first emerges at around this size in oocytes growing in vivo [32, 33]. In other words, the minimum requirement for a culture system targeting growth of bovine oocytes should be that the oocytes grow larger than 110 μm.
regardless of their size before culture.

Despite the original intention, our results did not support the hypothesis that merely reducing the amount of medium would improve oocyte growth. From a practical viewpoint, a volume less than 20 μl/per complex is not preferable, at least as long as a static culture medium is used. It might be possible to grow complexes more efficiently with a smaller amount of medium by placing them in a microfluidic environment equipped with a controlled dynamic medium flow, similar to the ones proposed recently for embryo culture [34, 35].

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