Culture Media Affect Follicle Survival and Oocyte Maturation in Preantral Mouse Follicle Cultures

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Abstract: *To investigate the effect of media on mouse follicular development and oocyte maturation in vitro, we compared eight culture media. Early preantral follicles were mechanically dissected from the ovaries of 14-day-old mice and cultured for 10 days. The tested media were: α-MEM, D-MEM/F-12, D-MEM with high glucose (4.5 g/L) (D-MEM[H]) or low glucose (1 g/L) (D-MEM[L]), Waymouth, M199, IMDM, and RPMI1640. All of the media were supplemented with 5% FBS, ITS, 100 mIU/ ml rhFSH, 1 mIU/ml rhLH and, 0.5% gentamicin. Compared to the other media (P < 0.05), a higher percentage of follicles survived and antral-like cavity formation was seen after 10 days of culture when the follicles were cultured in α-MEM, Waymouth, D-MEM/F-12, or D-MEM(L). Among the four media with higher follicle survival, the oocyte diameter on day 10 of culture was largest in α-MEM and smallest in Waymouth. The highest percentages of MII oocytes were obtained when follicles were cultured in α-MEM, D-MEM(L), and D-MEM/F-12. In contrast, in Waymouth, no MII oocytes were obtained. The media used for mouse preantral follicle growth in vitro affected follicle survival and oocyte maturation. Of the media that we tested, α-MEM, D-MEM(L), and D-MEM/F-12 were superior for mouse preantral follicle cultures.*
Key words: Preantral follicle, In vitro growth, Culture medium, Mouse

Introduction

In vitro follicle culture systems were developed to grow immature oocytes from early follicle stages into fertilizable oocytes. They are also often used as a tool for investigating folliculogenesis and oogenesis. There are two methods for isolating follicles: (1) enzymatic isolation of oocyte-granulosa cell complexes [1–3], and (2) mechanical dissection of preantral follicles. Currently, cultures of mechanically isolated intact follicles are the only model of oocyte-granulosa-theca cell interactions during follicular development [4, 5].

When mechanically isolated mouse follicles are cultured in microdroplets under oil for 12 days, nucleus-matured oocytes are produced [6]. These oocytes, derived from *in vitro* growth of preantral follicles, develop into blastocysts following *in vitro* fertilization and can undergo gestation and birth after embryo transfer. However, a few papers have reported the production of live offspring after *in vitro* maturation and fertilization of oocytes recovered from preantral mouse follicles cultured *in vitro* [1, 7–10].

To our knowledge, five types of culture media have been used for *in vitro* growth of mouse follicles: α-minimum essential medium (α-MEM) [1, 4–6], MEM [11–13], Opti-MEM [14], Waymouth's MB 752/1 Medium (Waymouth) [2, 3], and Medium 199 (M199) [15]. Of these five media, α-MEM is used most often for *in vitro* culture of mouse preantral follicles; however, which medium is best for *in vitro* growth of preantral follicles is not known, nor is it clear whether different cultures have an influence on follicular growth and oocyte maturity.

The present study investigated the effects of different media on follicular development *in vitro* and on oocyte maturation after stimulation with hCG and EGF. The parameters that we analyzed were follicle survival, antral-like cavity formation, oocyte growth, and meiotic maturation of *in vitro* grown oocytes.

Materials and Methods

Mice

F1 hybrid female mice (C57BL/6 × DBA/2; 14 days old) were used for the collection of follicles. Early preantral follicles were mechanically dissected from 14-day-old mouse ovaries. These prepubertal mice were obtained from C57BL/6 females mated with DBA/2 males. All of the mice were purchased from CLEA Japan (Shizuoka, Japan). They were fed a standard diet *ad libitum* and were maintained in a temperature and light-controlled room at 25°C, 12 h light and 12 h darkness, with the light starting at 0600 h. The experiments were approved by the Committee for Ethics on Animal Experiments of the Prefectural University of Hiroshima, Japan.

Isolation of preantral follicles

The 14-day-old B6D2F1 mouse ovaries were placed in Leibovitz L-15 medium (Invitrogen Life Technologies; GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, Utah, USA) at 37°C. Early preantral follicles were mechanically dissected from the ovaries with fine, 26G needles. For our study, we selected follicles with a diameter of 100–130 μm, which were enclosed by an intact basal membrane and had at least a few thecal cells. In a preliminary experiment, the theca cells were detected by cytochemical staining with alkaline phosphatase, the activity of which is localized specifically to the cells [16].

Culture conditions

Fifty follicles were cultured in a 35 mm collagen type I-coated dish (BD BioCoat; BD Falcon, Bedford, MA, USA) containing 2 ml of culture medium without an overlay of mineral oil. Three replicates were performed for each experimental group. The culture dishes were maintained in an incubator at 37°C with 5% CO_2 in air for 10 days. Half of the medium was changed every 2 days.

Culture media

The following eight media, purchased from Invitrogen Life Technologies (GIBCO) were used for the follicle culture: α-minimum essential medium (α-MEM), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (1:1) Mixture (D-MEM/F-12), Dulbecco's Modified Eagle Medium with high glucose (4.5 g/L) (D-MEM[H]) or low glucose (1 g/L) (D-MEM[L]), Waymouth's MB 752/1 Medium (Waymouth), Medium 199 (M199), Isocove's Modified Dulbecco's Medium (IMDM), and RPMI1640.

All of the media were supplemented with 5% FBS (Hyclone), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium (ITS; Sigma, St. Louis, MO, USA), 100 mIU/ml recombinant human FSH (rhFSH, Gonal-F; Serono, Geneva, Switzerland), 1 mIU/ml recombinant human LH (rhLH, Luveris; Serono) and 0.5% gentamicin (Sigma).

Evaluation of follicle survival and antral-like cavity formation

Development of *in vitro* cultured follicles was evaluated morphologically, and follicle survival was recorded according to Cortvrindt *et al.* (1996) [6]. In this culture system, survival was defined as those follicles that retained their oocyte completely embedded within the granulosa cell mass; this was expressed as a percentage of the follicles initially included at the start of culture. Antral-like cavity formation was defined as the thecal cells attached to the bottom of the dish, a ring of large mural granulosa cells that enclosed the antral-like cavity, and a cumulus oophorus located centrally with small cumulus cells in close contact with the oocyte.

Measurement of oocyte diameters

After 10 days of culture, ten follicles from each culture were picked at random from the groups with more than 70% follicle survival. The follicle cells were then mechanically removed from the oocytes. The oocyte diameter was measured using a calibrated ocular micrometer at × 400 magnification.

Fluorescence detection of chromatin configuration

After measuring the oocyte diameter, the chromatin configuration of the germinal vesicle stage (GV) oocytes was investigated by labeling the oocytes with 20 μg/ml Hoechst 33342 (Sigma) and mounting them on slides. The oocytes were examined with an epifluorescence microscope equipped with a UV filter (Nikon, Tokyo, Japan). Images were recorded with a CCD camera (ORCA-cooled CCD; Hamamatsu Photonics, Shizuoka, Japan) and analyzed with Image Pro Plus software (Media Cybernetic, Silver Spring, MD, USA).

Ovulation induction (mucification) and oocyte maturation

To induce maturation of follicular oocytes after 10 days of culture, 1.5 IU/ml recombinant human chorionic gonadotropin (rhCG; Ovidrel, Serono) and 5 ng/ml recombinant human epidermal growth factor (rhEGF; Upstate Biotechnology, Lake Placid, NY, USA) were added to the culture medium [17]. After 15–17 h, the oocytes were denuded with hyaluronidase, fixed and
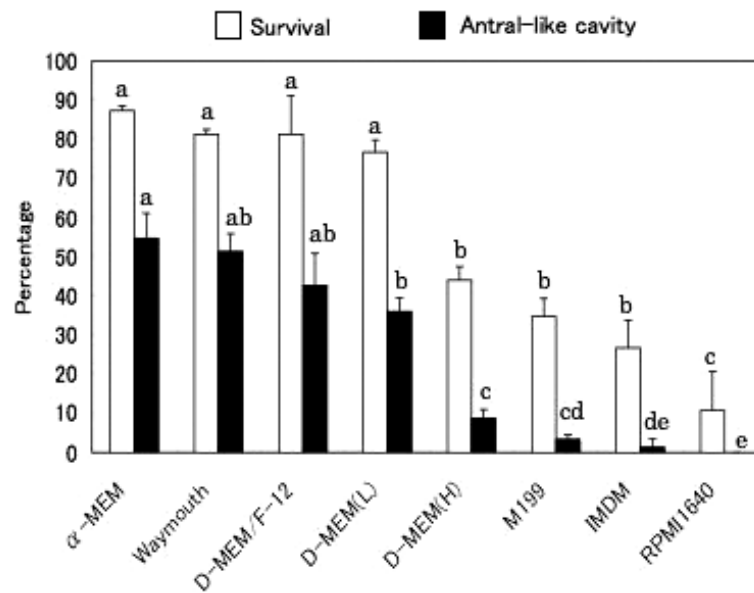
Fig. 1. Follicle survival and antral-like cavity formation in preantral follicle culture using eight different media. Follicles were cultured for 10 days in α-MEM, Waymouth, D-MEM/F-12, D-MEM (L; low glucose, H; high glucose), M199, IMDM or RPMI1640. Each column shows the mean ± standard deviation (SD). Different letters indicate significant differences (a–e: $P < 0.05$).

stained with aceto-lacmoid. Nuclear maturation was assessed by the presence of the first polar body and metaphase chromosome.

Statistical analysis

The percentage data from each replicate were arcsine-transformed before being subjected to one-way analysis of variance (ANOVA). All of the experiments were replicated at least three times. Differences among the means in different groups were compared with Tukey's multiple comparison test using GraphPad PRISM (GraphPad Software; La Jolla, CA, USA). The differences were considered to be significant when $P < 0.05$.

Results

Follicle survival and antral-like cavity formation

The results of follicle survival and antral-like cavity formation in the eight media are summarized in Fig. 1. A higher percentage of follicles survived after 10 days of culture when the follicles were cultured in α-MEM (87%), Waymouth (81%), D-MEM/F-12 (81%), or D-MEM(L) (77%) than when they were cultured in the other media (10–44%) ($P < 0.05$). In addition, there was a higher percentage of antral-like cavity formation when
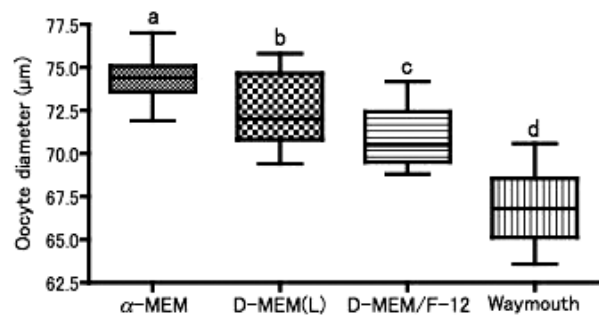

Fig. 2. Oocyte diameter of *in vitro*-grown oocytes on day 10 of culture in four different media. Follicles were cultured in α-MEM, D-MEM(L), D-MEM/F-12, or Waymouth. Data are presented by using box and whisker plots as the percentile distribution of oocytes. Different letters indicate significant differences (a–d: $P < 0.05$).

the follicles were cultured in α-MEM (55%), Waymouth (51%), D-MEM/F-12 (43%), or D-MEM(L) (36%) than when they were cultured in the other media (0–9%) ($P < 0.05$).

Oocyte growth and chromatin configuration

As shown in Fig. 2, on day 10, the mean diameter of oocytes cultured in α-MEM (74.3 ± 1.1 μm) were larger
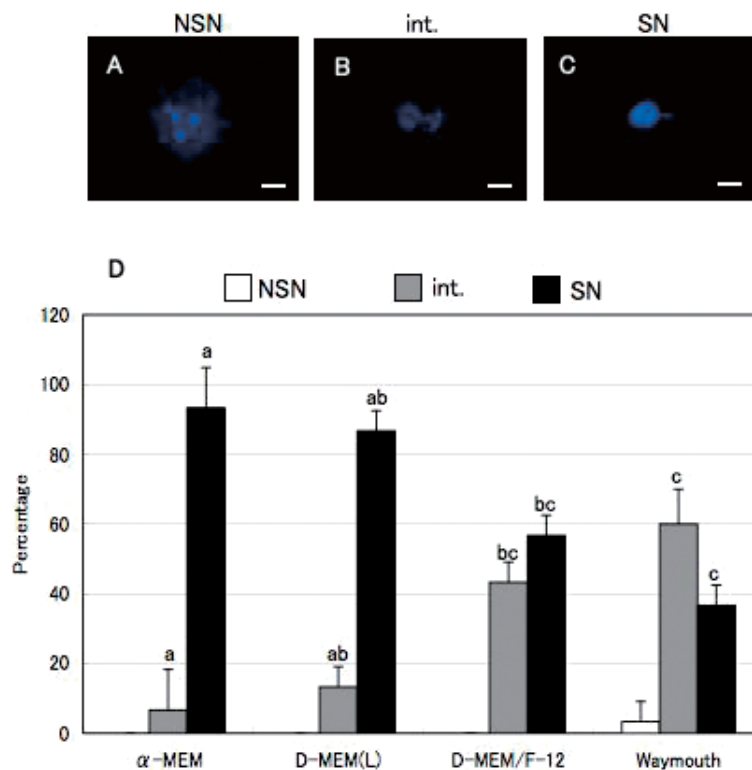
Fig. 3. Three chromatin configuration patterns in *in vitro*-grown oocytes on day 10 of culture in four different media. (A) NSN, (B) int., and (C) SN types of chromatin configurations. (D) Distribution of chromatin configuration patterns on day 10 of culture in four different media. Follicles were cultured in α-MEM, D-MEM(L), D-MEM/F-12, or Waymouth. Each column shows the mean ± SD. Different letters indicate significant differences (a–c: $P < 0.05$). Scale bars represent 10 μm.

than those of oocytes cultured in D-MEM(L) (72.4 ± 2.0 μm), D-MEM/F-12 (70.9 ± 1.7 μm), and Waymouth (66.9 ± 2.1 μm).

We observed three types of chromatin configurations in GV oocytes (Fig. 3, A–C), as described by Bouniol-Baly *et al.* (1999): (A) chromatin distributed diffusely around the nucleolus (non-surrounded nucleolus: NSN), (C) chromatin compacted in a ring around the nucleolus (surrounded nucleolus: SN), and (B) an intermediate (int.) chromatin configuration stage between NSN and SN [18]. We determined the nuclear stage of GV oocytes cultured in four of the media according to this classification (Fig. 3, D). Most of the oocytes in α-MEM (93%) and D-MEM(L) (87%) exhibited SN chromatin. The percentages of oocytes with SN chromatin in D-MEM/F-12 (57%) and Waymouth (37%) were significantly lower than that in α-MEM.

Response of cultured follicles to addition of hCG and EGF

There was a higher percentage of mucification in α-MEM, Waymouth, D-MEM/F-12 and D-MEM(L) (69–83%) than in the other media. The highest percentage of MII oocytes was obtained when follicles were cultured in α-MEM (66%), followed by D-MEM(L) (58%) and D-MEM/F-12 (47%). When the follicles were cultured in Waymouth, none of the oocytes reached maturation (MII) after induction with hCG and EGF (Table 1).

Discussion

Our study found that the type of culture medium affects follicle survival, as measured on day 10 of culture, as well as subsequent oocyte maturation after stimulation with hCG and EGF. In most reports of *in vitro* culture of mechanically isolated preantral follicles, α-MEM was used. However, there is little published

Table 1. Mucification and oocyte maturation after stimulation with rhCG and rhEGF

	No. of follicles stumulated with hCG/EGF (No. of replicates)	No. (%, mean ± SD) of mucified follicles	No. (%, mean ± SD) of oocytes after hCG / EGF	
			GVBD	MII
α-MEM	120 (3)	100 (83.3 ± 1.4)[a]	96 (96.1 ± 4.5)[ab]	66 (66.0 ± 5.8)[a]
Waymouth	120 (3)	91 (75.8 ± 2.9)[a]	70 (77.2 ± 11.3)[b]	0 (0 ± 0.0)[e]
D-MEM/F-12	120 (3)	89 (74.2 ± 12.8)[a]	89 (100 ± 0.0)[a]	42 (46.9 ± 5.6)[abc]
D-MEM(L)	120 (3)	83 (69.2 ± 6.3)[a]	81 (97.8 ± 3.9)[ab]	48 (57.5 ± 5.7)[ab]
D-MEM(H)	150 (3)	65 (43.3 ± 4.6)[b]	60 (92.2 ± 6.9)[ab]	15 (23.6 ± 7.3)[abcd]
M199	150 (3)	45 (30.0 ± 9.2)[b]	44 (97.6 ± 4.1)[ab]	6 (12.7 ± 11.1)[cde]
IMDM	150 (3)	35 (23.3 ± 3.1)[bc]	33 (94.4 ± 9.6)[ab]	1 (2.8 ± 4.8)[de]
RPMI1640	150 (3)	16 (10.7 ± 9.9)[c]	15 (97.0 ± 5.3)[ab]	4 (20.2 ± 17.8)[bcde]

The percentages of GVBD and MII are per mucified follicles. a–e; Values with different superscripts in the same column are significantly different ($P < 0.05$).

information regarding which medium is best for *in vitro* culture of preantral mouse follicles [19].

In the present study, there was a higher percentage of follicle survival when the follicles were cultured in α-MEM, Waymouth, D-MEM/F-12, and D- MEM(L). Within the four media with higher follicle survival on day 10 of culture, the oocyte diameter was larger in α-MEM than in the other three media. The components of D-MEM(L) are very similar to those of α-MEM. Both media are developed from minimum essential medium (MEM). There was a higher percentage of MII oocytes in α-MEM after stimulation with hCG and EGF, followed by oocytes in D-MEM(L) and D-MEM/F-12. However, in Waymouth, none of the oocytes reached MII. Follicle survival and antral-like cavity formation in Waymouth were higher or the same as in the other three media, but the oocyte diameter was significantly smaller. It has been reported that the percentage of oocytes that reach MII *in vitro* depends upon the oocyte diameter [20, 21]. The very low maturation rate in Waymouth could be due to the smaller oocyte diameter.

In the present study, the percentage of GV oocytes in Waymouth with SN chromatin was significantly lower than in α-MEM or D-MEM(L). Pesty *et al.* (2007) reported that during *in vitro* growth of mouse follicles in α-MEM, all of the follicular oocytes displayed NSN chromatin at the beginning. In contrast, after 12 days of culture, all of the oocytes displayed the SN chromatin [22]. These results are consistent with those reported for *in vivo* oocytes, suggesting that the SN chromatin corresponds to a more advanced stage of oocyte development that is closer to ovulation than the NSN chromatin [18, 23–25]. The presence of SN chromatin at the GV stage is associated with a higher meiotic and developmental competence in fully grown oocytes [21,

26, 27].

One major difference among the culture media tested in this study was glucose concentration. The media with low glucose concentrations (1 g/L) were α-MEM, D-MEM(L), and M199, and the media with high glucose concentrations were RPMI1640 (2 g/L), D-MEM(H) (4.5 g/L), IMDM (4.5 g/L), and Waymouth (5 g/L). Follicle survival rates in the media with low glucose concentrations were relatively higher (except for M199), and in media with high glucose concentrations it was relatively lower (except for Waymouth). In the present study, we used the same medium (D-MEM) with two different glucose concentrations: (L) and (H). The follicle survival rate in D-MEM(L) was higher than in D-MEM(H). In contrast, the follicle survival rate was higher in Waymouth, but the oocyte diameter was smaller than in any other medium, and none of the cultured oocytes reached MII. Waymouth was designed as a defined medium for cultivation of mouse L929 cells [28], and is both qualitatively and quantitatively different from the other media. Waymouth has a glucose concentration 5-fold higher than that of the other media in which there was a higher rate of follicle survival. It seems that the balance of the increase of granulosa cells and the growth of the oocytes was disturbed by the high concentration of glucose.

Cortvrindt *et al.* (1996) reported that oocytes do not survive in reduced oxygen (5%) when the FSH concentration in the medium is 100 mIU/ml [6]. The percentage of oxygen also influences the generation of free radicals and much attention has been paid to the ability of ascorbic acid to act as an antioxidant [29], because both tissue remodeling and steroidogenesis are processes that produce reactive oxygen species. It is likely that ascorbic acid serves as an antioxidant

within the ovaries. In the present study, the follicle survival and maturation rates were the highest in α-MEM, which has a higher concentration of ascorbic acid (0.25 mM).

Ascorbic acid accumulation has been described at all stages of follicular development [30–32]. Granulosa cells, under the influence of FSH, actively take up ascorbic acid [33], and ascorbic acid is required as a cofactor in collagen synthesis and, hence, basal lamina expansion. Furthermore, ascorbic acid functions as an antioxidant, preventing cell death. During follicular development, the follicles become responsive to gonadotrophins and develop rapidly. Therefore, the presence of ascorbic acid in α-MEM likely supports follicular development and promotes oocyte maturity.

In most follicle culture systems, supplementation of the medium with FSH is essential for the survival and development of follicles [4, 6]. In the present study, all of the media were supplemented with 100 mIU/ml rhFSH. As we described, the selection of medium for *in vitro* growth of preantral follicles was important for follicle survival and follicular development even if FSH was present in the medium.

In conclusion, we found that the type of medium used for *in vitro* growth of preantral follicles affected follicle survival, follicular development, and oocyte maturity. When preantral follicles were cultured in α-MEM, D-MEM with low glucose, or D-MEM/F-12 for 10 days, most of the follicles survived and developed, and matured oocytes were obtained. Follicle survival, antral-like cavity formation, and oocyte diameters on day 10 of culture were higher in α-MEM than in any of the other media. Furthermore, the highest percentage of MII oocytes was obtained with this medium.

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