Development of a reliable *in vitro* maturation system for zebrafish oocytes

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Abstract

In zebrafish oocytes, it has been reported that a 60 or 75% Leibovitz L-15 medium or simple balanced saline solution containing 17α , 20β -dihydroxy-4-pregnen-3-one (DHP) is effective for nuclear maturation. However, most of the oocytes that matured under these conditions were not fertilized and did not hatch. Thus, these *in vitro* maturation methods could not support the cytoplasmic maturation of zebrafish oocytes. Therefore, we tried to develop a reliable *in vitro* maturation method for zebrafish oocytes, which supports their ability to be fertilized and to develop till hatching. When zebrafish oocytes at stage III were cultured in 50–100% Leibovitz L-15 medium supplemented with DHP, the highest rates of cleavage (24%) and hatching (12%) were obtained from oocytes matured in 90% Leibovitz L-15 medium. When we examined the suitable pH (7.5–9.5) of the 90% medium, higher rates of cleavage (45%) and hatching (33%) were obtained in oocytes matured at pH 9.0 than at pH 7.5, 8.5, or 9.5 (cleavage rate, 16–29%; hatching rate, 8–21%). In oocytes matured in 90% Leibovitz L-15 medium at pH 9.0, high rates of cleavage (70%) and hatching (63%) were obtained when oocytes were cultured for 270 min with 0.5 mg/ml BSA. Thus, 90% Leibovitz L-15 medium at pH 9.0 containing 0.5 mg/ml BSA was effective for normal maturation of zebrafish oocytes. This method will become a powerful tool for understanding the mechanism of *in vitro* maturation in zebrafish oocytes and for the practical use of immature oocytes.

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Introduction

In some mammals, a method for *in vitro* maturation from the germinal vesicle (GV) stage to metaphase II has been established and used to understand the mechanism regulating oogenesis and folliculogenesis. In the 1960s and 1970s, it was reported that *in vitro* matured oocytes could be fertilized *in vitro* in many mammals including humans (Edwards *et al.* 1969), mice (Cross & Brinster 1970, Iwamatsu & Chang 1972, Mukherjee 1972), rabbits (Brackett *et al.* 1972), guinea pigs (Yanagimachi 1974), hamsters (Whittingham & Bavister 1974), rats (Niwa & Chang 1975, Niwa *et al.* 1976), cows (Iritani & Niwa 1977), and pigs (Iritani *et al.* 1978). Moreover, live young were obtained from *in vitro* matured oocytes in mice (Mukherjee 1972), rabbits (Mills *et al.* 1973), humans (Steptoe & Edwards 1976), cows (Hanada 1985), and pigs (Mattioli *et al.* 1989). From the studies on *in vitro* fertilization (IVF) and *in vitro* maturation of mammalian oocytes, it has been recognized that nuclear maturation of oocytes does not guarantee their ability to be fertilized and to develop to term and that the maturation of the cytoplasm is essential (Eppig 1996).

In fish, including rainbow trout (*Oncorhynchus mykiss*; Jalabert 1976, Nagahama *et al.* 1980), goldfish

(*Carassius auratus*; Jalabert 1976), yellow perch (*Perca flavescens*; Goetz & Theofan 1979), amago salmon (*Oncorhynchus rhodurus*; Nagahama *et al.* 1980), medaka (*Oryzias latipes*; Iwamatsu *et al.* 1987), and zebrafish (*Danio rerio*; Selman *et al.* 1993, 1994), 17α , 20β -dihydroxy-4-pregnen-3-one (DHP) is effective as a maturation-inducing hormone for the *in vitro* maturation of fully grown oocytes at the GV stage (stage III). In addition, it has been reported that gonadotropin, activin, epidermal growth factor, and transforming growth factor- α promote *in vitro* maturation of zebrafish oocytes (Pang & Ge 1999, 2002a, 2002b). It has also been reported that cyclin B (Kondo *et al.* 1997) and transforming growth factor- β 1 (Kohli *et al.* 2003, 2005) play roles in the maturation of zebrafish oocytes. In some fish, the pH of ovarian fluid is not neutral but alkaline (Fauvel *et al.* 1993, Lahnsteiner *et al.* 1995, Linhart *et al.* 1995). Moreover, Patiño *et al.* (2005) found that alkaline conditions facilitated the *in vitro* maturation of oocytes from the GV stage (stage III) to MII stage (stage V) in the Atlantic croaker (*Micropogonias undulatus*). However, none of these studies examined the ability of oocytes to be fertilized and to develop till hatching after *in vitro* maturation.

Only a few studies have examined the ability of matured oocytes to be fertilized and to develop till hatching. In the zebrafish, Li *et al.* (1993) reported an *in vitro* maturation method for zebrafish oocytes at stage III in which oocytes could be matured and fertilized, and the fertilized eggs could develop to term. But this *in vitro* maturation method has been reproduced neither by other researchers nor by themselves, and its use has not become widespread. At present, the viability of zebrafish oocytes at stage III is assessed by morphological observation and/or trypan blue (TB) staining (Isayeva *et al.* 2004, Plachinta *et al.* 2004). In the medaka, Iwamatsu (1973) reported that immature oocytes after luteinizing hormone (LH) surge could mature, be fertilized, hatch, and develop into adult fish with an *in vitro* schedule. Thus, immature medaka oocytes after LH surge can be made to undergo cytoplasmic maturation *in vitro*. However, hatched juveniles have not been obtained from *in vitro* matured medaka oocytes derived from oocytes before LH surge. Thus, it is not clear whether the current *in vitro* maturation method for fully grown fish oocytes at the GV stage before LH surge supports the cytoplasmic maturation of the oocytes.

In this study, we first examined whether the current *in vitro* maturation method for zebrafish oocytes supports the cytoplasmic maturation of the oocytes by examining their ability to be fertilized and to develop till hatching. Because the results showed that the method did not support cytoplasmic maturation sufficiently, we tried to develop a reliable *in vitro* maturation system for zebrafish oocytes by examining their ability to be fertilized and to develop till hatching.

Results

The ability of zebrafish oocytes to mature, to be fertilized (cleave), and to develop till hatching

Figure 1 shows the ability of stage III oocytes to mature as assessed based on a translucent appearance and the ability to be fertilized and to develop till hatching following insemination after being cultured with the current *in vitro* maturation method: in 60 or 75% Leibovitz L-15 medium (pH 7.5) for 270 min at 26 °C (Selman *et al.* 1993, 1994). Most oocytes had matured (82 and 87% respectively) as assessed by their appearance. From TB staining, high proportions of oocytes were considered alive (72 and 76% respectively). However, the rates of cleavage (3 and 7% respectively) and hatching (0 and 3% respectively) after IVF were quite low. Therefore, the cytoplasm of most oocytes judged to be mature by their appearance or to be alive by TB staining did not mature to support the ability of oocytes to be fertilized and to develop till hatching.

Figure 1 The ability of stage III zebrafish oocytes to mature, to be fertilized, and to develop to term after being cultured in 60% Leibovitz L-15 medium containing DHP (pH 7.5; 60% LM) or 75% Leibovitz L-15 medium containing DHP (pH 7.5; 75% LM) for 270 min at 26 °C. Maturation was assessed by a translucent appearance (open bars), viability was assessed by trypan blue (TB) staining (hatched bars), and the ability to be fertilized and to develop to term was assessed by cleavage to the two-cell stage (shaded bars) and by hatching (closed bars) respectively. Mature oocytes that had ovulated but not spawned (*in vivo*) or immature oocytes collected from ovaries were used (*in vitro*). Numbers in parentheses indicate the number of oocytes examined. *Significantly different from control ($P < 0.05$).

The effect of the osmolality of the culture medium on the cytoplasmic maturation of zebrafish oocytes

Figure 2 shows the ability of stage III oocytes to mature, to be fertilized (cleave), and to develop till hatching after being cultured in various concentrations (50–100%) of Leibovitz L-15 medium (pH 7.5). In 50, 60, and 75% Leibovitz L-15 media, the rates of cleavage (0, 2, and 6% respectively) and hatching (0, 0, and 3% respectively) were quite low. In 80, 90, and 95% Leibovitz L-15 media, the rates of cleavage (12, 24, and 12% respectively) and hatching (9, 12, and 9% respectively) increased slightly. The highest hatching rate was obtained with 90% Leibovitz L-15 medium, but the rate remained low (12%) and was not significantly different from the rates obtained with 75, 80, 95, and 100% Leibovitz L-15 media.

The effect of the culture period in 90% Leibovitz L-15 medium (pH 7.5) on the cytoplasmic maturation of zebrafish oocytes

As a preliminary experiment, stage III oocytes were cultured in 90% Leibovitz L-15 medium (pH 7.5) for 180–220 min. After 180 min, none of them had matured morphologically (0%, 0/40). After 190 min, a small proportion (10%, 4/40) of oocytes had matured morphologically. After 220 min, most oocytes (98%, 39/40) had matured morphologically. Therefore, IVF was conducted after culture for periods ranging from 220 to 360 min.

Figure 3 shows the ability of stage III oocytes to mature, to be fertilized (cleave), and to develop till hatching after being cultured for various periods. The rates of

Figure 2 The ability of stage III zebrafish oocytes to mature, to be fertilized, and to develop to term after being cultured in various concentrations of Leibovitz L-15 medium containing DHP (pH 7.5) for 270 min at 26 °C. Maturation was assessed by a translucent appearance (open bars), and the ability to be fertilized and to develop to term was assessed by cleavage to the two-cell stage (shaded bars) and hatching (closed bars) respectively. Numbers in parentheses indicate the number of oocytes examined. Values with different subscripts within the same criterion were significantly different ($P < 0.05$).

cleavage (4%) and hatching (2%) were low when the culture period was 220 min, but increased as the culture period was extended; the highest rates of cleavage and hatching were obtained after 260 min of culture (29 and 20% respectively), and both rates decreased when the culture period was extended further (270–360 min). In this condition, the highest rates of cleavage and hatching remained low. Moreover, the period that supported the high rates of fertilization and term development was limited to 10 min.

The effect of the pH of 90% Leibovitz L-15 medium on the cytoplasmic maturation of zebrafish oocytes

Figure 4 shows the ability of stage III oocytes to mature, to be fertilized (cleave), and to develop till hatching after being cultured in 90% Leibovitz L-15 medium at various pH (7.5–9.5). The rates of cleavage (45%) and hatching (33%) for oocytes matured in a medium at pH 9.0 were higher than that obtained at pH 7.5 (the cleavage rate being 29% and the hatching rate being 20%). Alkaline

conditions (pH 9.0) appear to facilitate the cytoplasmic maturation of zebrafish oocytes.

The effect of BSA and the culture period on the cytoplasmic maturation of zebrafish oocytes

Figure 5 shows the ability of stage III oocytes to mature, to be fertilized (cleave), and to develop till hatching after being cultured for maturation in 90% Leibovitz L-15 medium (pH 9.0) for various periods (220–480 min) without or with BSA. Without BSA (Fig. 5A, control), relatively high rates of cleavage and hatching were obtained after culture for periods ranging from 220 to 300 min. However, the rates of cleavage (27–46%) and hatching (24–39%) did not increase substantially. On the other hand, with BSA (Fig. 5B), the rates of cleavage and hatching reached 64 and 60% respectively with 270 min of culture. As was the case without BSA, high rates of cleavage and hatching were obtained with BSA (Fig. 5B) after a wide range of culture periods (from 220 to 300 min; Fig. 5B).

Figure 3 The ability of stage III zebrafish oocytes to mature, to be fertilized, and to develop to term after being cultured in 90% Leibovitz L-15 medium containing DHP (pH 7.5) for various periods at 26 °C. Maturation was assessed by a translucent appearance (open bars), and the ability to be fertilized and to develop to term was assessed by cleavage to the two-cell stage (shaded bars) and hatching (closed bars) respectively. Numbers in parentheses indicate the number of oocytes examined. Values with different subscripts within the same criterion were significantly different ($P < 0.05$).

Figure 4 The ability of zebrafish stage III oocytes to mature, to be fertilized, and to develop to term after being cultured in 90% Leibovitz L-15 medium containing DHP with different pH for 260 min at 26 °C. Maturation was assessed by a translucent appearance (open bars), and the ability to be fertilized and to develop to term was assessed by cleavage to the two-cell stage (shaded bars) and by hatching (closed bars) respectively. Numbers in parentheses indicate the number of oocytes examined. *Significantly different from control (pH 7.5; $P < 0.05$).

Confirming the fertilization of *in vitro* matured zebrafish oocytes

To confirm that hatched juveniles definitely originated from fertilized oocytes, and not parthenogenetically activated oocytes, *in vitro* matured leopard-type zebrafish oocytes were inseminated with wild-type sperm. Figure 6A shows a juvenile derived from a leopard-type oocyte inseminated with leopard-type sperm (control). The fish had the typical appearance of leopard-type

zebrafish (2/2). On the other hand, the juveniles derived from leopard-type oocytes and wild-type sperm had the appearance of wild-type zebrafish (16/16; Fig. 6B). This indicates that *in vitro* matured oocytes that resulted in hatching after insemination were definitely fertilized.

Discussion

The present study showed in the zebrafish that oocytes assessed as matured morphologically or as alive with TB staining do not always have the ability to be fertilized and to develop till hatching. The results shown in Fig. 1 indicate that the cytoplasm of the oocytes that had undergone nuclear maturation had not matured to support the ability to be fertilized and to develop till hatching. Like in mammals, therefore, the nuclear maturation of oocytes in fish does not guarantee their ability to be fertilized and to develop till hatching, and the maturation of the cytoplasm is essential. To assess the ability of stage III zebrafish oocytes to mature normally, it is therefore necessary to examine the rates of cleavage and hatching of oocytes after IVF.

Leibovitz L-15 medium was used as the basic medium for oocyte maturation because this amino acid-rich medium was considered reliable. To clarify the components essential for reliable *in vitro* maturation of oocytes, the use of simple balanced saline solutions (Kondo *et al.* 1997, Sakai *et al.* 1997, Kohli *et al.* 2003, 2005, Patiño *et al.* 2005) might be suitable. However,

Figure 5 The ability of zebrafish stage III oocytes to mature, to be fertilized, and to develop to term after being cultured in 90% Leibovitz L-15 medium containing DHP (pH 9.0) without (A, control) and with 0.5% BSA (B) for various periods at 26 °C. Maturation was assessed by a translucent appearance (open bars), and the ability to be fertilized and to develop to term was assessed by cleavage to the two-cell stage (shaded bars) and by hatching (closed bars) respectively. Numbers in parentheses indicate the number of oocytes examined. *Significantly different from control (A) ($P < 0.05$).

Figure 6 Photographs of juveniles of zebrafish derived from (A) a leopard-type oocyte and leopard-type sperm and (B) a leopard-type oocyte and wild-type zebrafish sperm. Bars indicate 1 cm.

we used Leibovitz L-15 medium, because the aim of our study was to find conditions suitable for normal cytoplasmic maturation.

The most suitable concentration of Leibovitz L-15 medium was 90%, at which the osmolality was 0.29 Osm/kg (Fig. 2). This osmolality would be close to the isotonic value of zebrafish oocytes, although the isotonic osmolality of zebrafish oocytes has not been determined. The osmolality is close to that of the culture medium (0.27 Osm/kg, 90% TCM199) for medaka oocytes (Iwamatsu *et al.* 1987).

The pH of the maturation medium markedly influenced normal maturation of zebrafish oocytes, and the optimum pH was 9.0 (Fig. 4). Only a few studies have used alkaline culture media for the maturation of fish oocytes/follicles. Goetz & Nagahama (1985) reported in human chorionic gonadotropin (hCG)-treated goldfish that there was a significant increase in the spontaneous ovulation *in vitro* of fully grown follicles as the pH of culture media increased from 7.3 to 8.9. Patiño *et al.* (2005) reported in the Atlantic croaker that, in a culture medium containing hCG, the rates of spontaneous ovulation of fully grown follicles and GV breakdown of intrafollicular oocytes were significantly higher at pH 8.5 than 7.5. However, no attention has been paid to the pH of culture media for cytoplasmic maturation of fish oocytes. On the other hand, it has been reported that the pH of ovarian fluid is high in various species of fish, such as turbot (*Scophthalmus maximus*; pH 8.1; Fauvel *et al.* 1993), salmonid fish (pH 8.4–8.8; Lahnsteiner *et al.* 1995), Atlantic croaker (*Micropogonias undulatus*; pH 8.5; Patiño *et al.* 2005), and common carp (*Cyprinus carpio* L.; pH 9.0; Linhart *et al.* 1995). Therefore, alkaline conditions would be physiological for immature teleost oocytes and thus might be suitable for the *in vitro* maturation of oocytes.

When oocytes were matured in 90% Leibovitz L-15 medium at pH 9.0, the duration for which they retained the ability to be fertilized and to develop till hatching was extended. By incorporating BSA in the culture medium, the duration was not affected but the ability of oocytes to be fertilized and to develop till hatching was improved significantly. Here, most of the fertilized oocytes developed till hatching. Therefore, the addition of BSA to a maturation medium would be effective for

acquiring the fertilizing ability in zebrafish oocytes. Sakai *et al.* (1997) reported that BSA was effective in maintaining the fertilizing ability of ovulated zebrafish oocytes at stage V for 1 h. It was also reported that ovarian fluid of coho salmon (*Oncorhynchus kisutch*) was effective in maintaining the fertilizing ability of ovulated zebrafish oocytes for 6 h (Corley-Smith *et al.* 1996). Thus, the ovarian fluid of fish might contain BSA-like proteins that affect the interaction between oocytes and sperm, and BSA might have similar effects on the interaction.

In this study, BSA was proven effective for the cytoplasmic maturation of zebrafish oocytes as for mammalian oocytes. Zebrafish oocytes that matured in 90% Leibovitz L-15 medium (pH 9.0) in the presence of BSA looked yellow, whereas those that matured in medium without BSA looked translucent (data not shown). Since yellowish oocytes at stage V are regarded as good quality oocytes (Westerfield 1995), BSA would be effective for cytoplasmic maturation (Fig. 5). In the medaka, it has also been reported that BSA is effective for the maturation of oocytes after LH surge (Iwamatsu 1973). In this study, we did not use endogenous proteins in fish ovarian fluid but used an exogenous protein, BSA. Therefore, the rates of cleavage and hatching could increase further if the essential component of fish ovarian fluid was used for the maturation of zebrafish oocytes. In bovine embryos, it has been suggested that BSA is endocytosed; then, after intracellular hydrolysis, its constituent amino acids contribute to nitrogen metabolism in the embryos (Orsi & Leese 2004). In zebrafish oocytes, the mechanism of the effect of BSA on maturation is not known, but it is possible that BSA is also endocytosed into fish oocytes. Further studies are necessary to clarify the mechanism by which BSA exerts a beneficial effect on the cytoplasmic maturation of zebrafish oocytes.

Unfortunately, only a small proportion (~10%) of oocytes were ovulated from the follicle in the present *in vitro* maturation system (data not shown). It has been reported that hCG plus activin (Pang & Ge 1999, 2002a) and epidermal growth factor plus transforming growth factor- α (Pang & Ge 1999) are effective for the *in vitro* maturation of zebrafish oocytes. Using these substances

for *in vitro* maturation with the proper timing, it may be possible to increase the ovulation rate.

Since juveniles having the wild-type appearance were obtained from wild-type sperm and *in vitro* matured leopard-type oocytes (Fig. 6B), sperm would have entered through the micropyle normally to fertilize *in vitro* matured oocytes. Therefore, the micropyle would have been constructed normally. If so, the present *in vitro* maturation system (alkaline solution containing BSA) may be close to physiological condition for the maturation of zebrafish oocytes.

Because the *in vitro* maturation method developed here was proven reliable, the method is effective for the practical use of immature zebrafish oocytes, and it may be applied to other teleost species as well. The present protocol may also become a powerful tool for understanding the mechanism regulating oogenesis and folliculogenesis in zebrafish. Since fish oocytes are large cells and have a large volume of cytoplasm, fish oocytes could be a suitable model for isolating factors controlling the cytoplasmic maturation of vertebrates' oocytes, especially at the molecular level.

Materials and Methods

Collection of zebrafish oocytes

About 20–40 mature zebrafish (wild type unless otherwise noted) purchased from a local fish dealer were maintained in 60-l aquaria under 14 h light:10 h darkness at 28 °C. To obtain immature oocytes at late stage (stage III), female zebrafish at 0–3 h before the end of the light period were decapitated under anesthesia with 0.2 mg/ml tricaine in distilled water (tricaine solution). Ovaries were recovered and placed in a culture medium at 25 °C. Follicles were separated manually with fine needles; their size varied from 0.34 to 0.69 mm. In this study, the follicles 0.65–0.69 mm in diameter, having an intrafollicular oocyte with a tough, dark ooplasm and a distinct GV, which were visible when viewed with transmitted light under a dissecting microscope, were collected and used as immature oocytes at stage III. To obtain mature oocytes at stage V, the belly of mature females was squeezed under anesthesia with the tricaine solution about 30 min before the end of the dark period.

All experiments were approved by the Animal Ethics Committee of the College of Agriculture, Kochi University.

Assessment of the ability of zebrafish oocytes to mature, to be fertilized (cleave), and to develop till hatching

It has been reported that a 60 or 75% Leibovitz L-15 medium containing 1 µg/ml DHP (pH 7.5) is effective for the maturation of stage III zebrafish oocytes (Selman *et al.* 1993, 1994). Thus, we first cultured stage III oocytes in 4 ml of a 60 or 75% Leibovitz L-15 medium supplemented with 100 µg/ml gentamycin in a culture dish (35 × 10 mm). To make the two media, Leibovitz L-15 medium (100%) was diluted with distilled water. To induce maturation of the oocytes, 5 µl ethanol containing DHP (0.8 µg/µl) was added to 4 ml of the culture medium; the final concentration of DHP being 1 µg/ml. After incubation at 26 °C for 270 min, the maturation and viability of the oocytes were examined. In this study, we considered oocytes to have matured to stage V when their visible remnants of GV completely disappeared and they became maximally translucent, while they were not ovulated from the follicular membrane. To examine the proportion of viable oocytes, cultured oocytes were stained for 10 min in 60 or 75% Leibovitz L-15 medium containing 0.2% TB at 25 °C. Numbers of total oocytes and viable oocytes (unstained) were counted under a dissecting microscope.

To conduct IVF, the follicular membrane of matured oocytes at stage V was removed with a pair of fine forceps in the culture medium under a dissecting microscope (Fig. 7). The defolliculated oocytes were put on the lid of a culture dish containing ~200 µl of the culture medium. A male zebrafish was killed by decapitation under anesthesia with tricaine solution. Two testes were recovered from the male and placed in 100 µl of Hank's medium (Westerfield 1995) in a culture dish covered with paraffin oil at 25 °C. Sperm were obtained by tearing the testes with forceps and a needle in the medium and the sperm suspension was cooled with ice (0 °C) to preserve activity. For the insemination of defolliculated oocytes, 10 µl of the sperm suspension was added to the culture medium (~200 µl) containing defolliculated oocytes. After 1 min, inseminated oocytes were transferred to 4 ml E3 medium (Brand *et al.* 2002), and incubated at 26 °C. We also used oocytes matured *in vivo* as a control. The oocytes were observed under a microscope at 2 h after insemination for cleavage to the two-cell stage and at 72 h for hatching.

Figure 7 Photographs of a matured oocyte having the follicular membrane (arrows) peeled off mechanically with forceps. (A) An oocyte with the follicular membrane partly peeled off and (B) a defolliculated oocyte. Bars indicate 0.5 mm.

The effect of the culture condition and the culture period on the cytoplasmic maturation of zebrafish oocytes

To examine the effect of the osmolality of the culture medium (pH 7.5) on the cytoplasmic maturation, stage III oocytes were cultured in 4 ml of 50, 60, 75, 80, 90, 95, and 100% Leibovitz L-15 medium containing 1 µg/ml DHP (pH 7.5) for 270 min at 26 °C. The osmolality of each solution measured with a freezing point depression osmometer (OM801; Vogel, Giessen, Germany) was 0.16, 0.19, 0.24, 0.27, 0.29, 0.30, and 0.32 Osm/kg respectively.

To examine the effect of the culture period in 90% Leibovitz L-15 medium (pH 7.5) on the cytoplasmic maturation, stage III oocytes were cultured in 90% Leibovitz L-15 medium containing DHP (pH 7.5) for various periods ranging from 220 to 360 min at 26 °C.

To examine the effect of the pH of 90% Leibovitz L-15 medium on the cytoplasmic maturation, stage III oocytes were cultured in 90% Leibovitz L-15 medium containing DHP at various pH for 260 min at 26 °C. The pH was adjusted to 7.5, 8.5, 9.0, and 9.5 with NaOH.

To examine the effect of BSA and the culture period on the cytoplasmic maturation, stage III oocytes were cultured in 90% Leibovitz L-15 medium containing DHP (pH 9.0) in the presence or absence of 0.5% BSA for various periods ranging from 220 to 480 min at 26 °C.

The ability of cultured oocytes to be matured and fertilized and the ability of fertilizing eggs to develop till hatching were examined as described above.

Confirmation of the fertilization of zebrafish oocytes

Zebrafish oocytes could develop till hatching parthenogenetically (Streisinger *et al.* 1981, Westerfield 1995, Corley-Smith *et al.* 1996). To confirm the fertilization of *in vitro* matured oocytes, *in vitro* maturation and IVF were conducted using leopard-type (recessive) zebrafish oocytes. The stage III oocytes were collected from leopard-type zebrafish, and cultured in 90% Leibovitz L-15 medium containing DHP (pH 9.0) and 0.5% BSA for 270 min at 26 °C. The oocytes were inseminated with leopard-type (control) or wild-type (dominant) zebrafish sperm, as described previously. Hatched fry were raised in 10-l aquaria under 14 h light:10 h darkness for 60 days at 28 °C, and the pattern of the appearance of the juveniles was observed.

Statistical analysis

The significance of the difference in the rates of maturation, cleavage, and hatching after each treatment was analyzed with a χ^2 -test. Differences at $P < 0.05$ were considered statistically significant.

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References

- Brackett BG, Mills JA & Jeitles GG Jr 1972 *In vitro* fertilization of rabbit ova recovered from ovarian follicles. *Fertility and Sterility* **23** 898–909.
- Brand M, Granato M & Nusslein-Volhard C 2002 Keeping and raising zebrafish. In *Zebrafish*, pp 30–34. Eds C Nusslein-Volhard & R Dahm. New York: Oxford University Press.
- Corley-Smith GE, Lim CJ & Brandhorst BP 1996 Production of androgenetic zebrafish (*Danio rerio*). *Genetics* **142** 1265–1276.
- Cross PC & Brinster RL 1970 *In vitro* development of mouse oocytes. *Biology of Reproduction* **3** 298–307.
- Edwards RG, Bavister BD & Steptoe PC 1969 Early stages of fertilization *in vitro* of human oocytes matured *in vitro*. *Nature* **221** 632–635.
- Eppig JJ 1996 Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. *Reproduction Fertility Development* **8** 485–489.
- Fauvel C, Omnes MH, Suquet M & Normant Y 1993 Reliable assessment of overripening in turbot (*Scophthalmus maximus*) by a simple pH measurement. *Aquaculture* **117** 107–113.
- Goetz FW & Nagahama Y 1985 Effects of pH on *in vitro* ovulation of goldfish (*Carassius auratus*) oocytes. *Journal of Experimental Zoology* **235** 81–85.
- Goetz FW & Theofan G 1979 *In vitro* stimulation of germinal vesicle breakdown and ovulation of yellow perch (*Perca flavescens*) oocytes. Effects of 17 α -hydroxy-20 β -dihydroprogesterone and prostaglandins. *General and Comparative Endocrinology* **37** 273–285.
- Hanada A 1985 *In vitro* fertilization in cattle – with particular reference to sperm capacitation by ionophore A23187. *Japanese Journal of Animal Reproduction* **31** 21–26.
- Iritani A & Niwa K 1977 Capacitation of bull spermatozoa and fertilization *in vitro* of cattle follicular oocytes matured in culture. *Journal of Reproduction and Fertility* **50** 119–121.
- Iritani A, Niwa K & Imai H 1978 Sperm penetration *in vitro* of pig follicular oocytes matured in culture. *Journal of Reproduction and Fertility* **54** 379–383.
- Isayeva A, Zhang T & Rawson DM 2004 Studies on chilling sensitivity of zebrafish (*Danio rerio*) oocytes. *Cryobiology* **49** 114–122.
- Iwamatsu T 1973 Studies on oocyte maturation in the Medaka, *Oryzias latipes* – improvement of culture medium for oocytes *in vitro*. *Japanese Journal of Ichthyology* **20** 218–224.
- Iwamatsu T & Chang MC 1972 Sperm penetration *in vitro* of mouse oocytes at various times during maturation. *Journal of Reproduction and Fertility* **31** 237–247.
- Iwamatsu T, Takahashi SY, Sakai N, Nagahama Y & Onitake K 1987 Induction and inhibition of *in vitro* oocyte maturation and production of steroids in fish follicles by forskolin. *Journal of Experimental Zoology* **241** 101–111.
- Jalabert B 1976 *In vitro* oocyte maturation and ovulation in rainbow trout (*Salmo gairdneri*), northern pike (*Esox lucius*), and goldfish (*Carassius auratus*). *Journal of the Fisheries Research Board of Canada* **33** 974–988.
- Kohli G, Hu S, Clelland E, Muccio TD, Rothenstein J & Peng C 2003 Cloning of transforming growth factor- β 1 (TGF- β 1) and its type II receptor from zebrafish ovary and role of TGF- β 1 in oocyte maturation. *Endocrinology* **144** 1931–1941.
- Kohli G, Clelland E & Peng C 2005 Potential targets of transforming growth factor-beta 1 during inhibition of oocyte maturation in zebrafish. *Reproductive Biology and Endocrinology* **3** 53–63.
- Kondo T, Yanagawa T, Yoshida N & Yamashita M 1997 Introduction of cyclin B induces activation of the maturation-promoting factor and breakdown of germinal vesicle in growing zebrafish oocytes unresponsive to the maturation-inducing hormone. *Developmental Biology* **190** 142–152.
- Lahnsteiner F, Weismann T & Patzner RA 1995 Composition of the ovarian fluid in 4 salmonid species: *Oncorhynchus mykiss*, *Salmo trutta flaccustris*, *Salvelinus alpinus* and *Hucho hucho*. *Reproduction, Nutrition, Development* **35** 465–474.

- Li S, Mao Z, Han W, Sun Z, Yan W, Chen H & Yan S 1993 *In vitro* oocyte maturation in the zebra fish, *Brachydanio rerio*, and the fertilization and development of the mature egg. *Chinese Journal of Biotechnology* **9** 247–255.
- Linhart O, Kudo S, Billard R, Slechts V & Mikodina EV 1995 Morphology, composition and fertilization of carp eggs. *Aquaculture* **129** 75–93.
- Mattioli M, Bacci ML, Galeati G & Seren E 1989 Developmental competence of pig oocytes matured and fertilized *in vitro*. *Theriogenology* **31** 1201–1207.
- Mills JA, Jeitles GG Jr & Brackett BG 1973 Embryo transfer following *in vitro* and *in vivo* fertilization of rabbit ova. *Fertility and Sterility* **24** 602–608.
- Mukherjee AB 1972 Normal progeny from fertilization *in vitro* of mouse oocytes matured in culture and spermatozoa capacitated *in vitro*. *Nature* **237** 397–398.
- Nagahama Y, Kagawa H & Tashiro F 1980 The *in vitro* effects of various gonadotropins and steroid hormones on oocyte maturation in amago salmon *Oncorhynchus rhodurus* and rainbow trout *Salmo gairdneri*. *Bulletin of the Japanese Society of Scientific Fisheries* **46** 1097–1102.
- Niwa K & Chang MC 1975 Fertilization of rat eggs *in vitro* at various times before and after ovulation with special reference to fertilization of ovarian oocytes matured in culture. *Journal of Reproduction and Fertility* **43** 435–451.
- Niwa K, Miyake M, Iritani A & Nishikawa Y 1976 Fertilization of rat oocytes cultured *in vitro* from various stages of maturation. *Journal of Reproduction and Fertility* **47** 105–106.
- Orsi NM & Leese HJ 2004 Amino acid metabolism of preimplantation bovine embryos cultured with bovine serum albumin or polyvinyl alcohol. *Theriogenology* **61** 561–572.
- Pang Y & Ge W 1999 Activin stimulation of zebrafish oocyte maturation *in vitro* and its potential role in mediating gonadotropin-induced oocyte maturation. *Biology of Reproduction* **61** 987–992.
- Pang Y & Ge W 2002a Gonadotropin and activin enhance maturational competence of oocytes in the zebrafish (*Danio rerio*). *Biology of Reproduction* **66** 259–265.
- Pang Y & Ge W 2002b Epidermal growth factor and TGF α promote zebrafish oocyte maturation *in vitro*: potential role of the ovarian activin regulatory system. *Endocrinology* **143** 47–54.
- Patino R, Bolamba D, Thomas P & Kumakura N 2005 Effects of external pH on hormonally regulated ovarian follicle maturation and ovulation in Atlantic croaker. *General and Comparative Endocrinology* **141** 126–134.
- Plachinta M, Zhang T & Rawson DM 2004 Studies on cryoprotectant toxicity to zebrafish (*Danio rerio*) oocytes. *Cryo-Letters* **25** 415–424.
- Sakai N, Burgess S & Hopkins N 1997 Delayed *in vitro* fertilization of zebrafish eggs in Hank's saline containing bovine serum albumin. *Molecular Marine Biology and Biotechnology* **6** 84–87.
- Selman K, Wallace RA, Sarka A & Qi X 1993 Stages of oocyte development in the zebrafish, *Brachydanio rerio*. *Journal of Morphology* **218** 203–224.
- Selman K, Petrino TR & Wallace RA 1994 Experimental conditions for oocyte maturation in the zebrafish, *Brachydanio rerio*. *Journal of Experimental Zoology* **269** 538–550.
- Steptoe PC & Edwards RG 1976 Reimplantation of a human embryo with subsequent tubal pregnancy. *Lancet* **1** 880–882.
- Streisinger G, Walker C, Dower N, Knauber D & Singer F 1981 Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). *Nature* **291** 293–296.
- Westerfield M 1995 In *The Zebrafish Book. A guide for the laboratory use of zebrafish (Brachydanio rerio)*. Eugene: University Oregon Press.
- Whittingham DG & Bavister BD 1974 Development of hamster eggs fertilized *in vitro* or *in vivo*. *Journal of Reproduction and Fertility* **38** 489–492.
- Yanagimachi R 1974 Maturation and fertilization *in vitro* of guinea-pig ovarian oocytes. *Journal of Reproduction and Fertility* **38** 485–488.

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