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Full term development of normal mice after transfer of IVF embryos derived from oocytes stored at room temperature for 1 day

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Summary

Early studies have shown that some mouse cumulus–oocyte complexes (COCs) stored at room temperature for 24 h still retained full developmental potential. In this study, we stored denuded mouse oocytes (DOs) at room temperature (25 °C) for 24 h and activated these oocytes with 10 mM $SrCl_2$ or fertilized the oocytes by IVF. We found that nearly half of the DOs stored at room temperature for 1 day can be fertilized normally by IVF and that two foster mothers gave birth to seven pups. Embryos from stored oocytes were cultured in CZB medium with or without 1 μg/ml 17β-estradiol (E_2). The numbers of embryo that developed to morula/blastocyst stage after parthenogenetic activation and IVF were significantly increased when E_2 was added to the culture ($p < 0.05$). These results suggest that E_2 might improve mouse embryo development *in vitro.* The birth of seven agouti pups and their healthy growth indicated that the storage of DOs at room temperature for 1 day may be a practical procedure for mammalian reproduction.

Keywords: Embryo development, 17β-Estradiol, IVF, Oocyte, Pup

Introduction

Mammalian oocytes are usually fertilized soon after ovulation. If fertilization does not occur in time, unfertilized oocytes that remained in the oviduct (aging *in vivo*) or cultured in a suitable medium (aging *in vitro*) overall undergo time-dependent aging (Yanagimachi & Chang, 1961; Whittingham & Siracusa, 1978). It is well known that fertilization or artificial activation of aged oocytes could result in abnormal development (Juetten, 1983; Tarin *et al.*, 1999, 2002; Gordo *et al.*, 2002). Despite inherent difficulties, gamete cryopreservation has revolutionized mammalian husbandry and reproductive medicine. It has been reported that frozen–thawed gametes can often regain their viability and be used for fertilization (Watson, 1990; Wakayama *et al.*, 1998; Nakagata, 2000). Conventional storage of gametes in liquid nitrogen (−196 °C) is complicated. It requires the constant supply of liquid nitrogen and, during transportation, dry ice might sometimes be insufficient for maintaining suitable temperatures. Thus, it would be ideal if we could store gametes for 1 day, or even several days, at room temperature. Previous investigations have already shown that mouse spermatozoa can be freeze-dried and stored at room temperature for up to 1 month without losing their genetic and reproductive potential (Wakayama & Yanagimachi, 1998) and mouse cumulus–oocyte complexes (COCs) stored at room temperature for 24 h can still retain full developmental potential (Wakayama *et al.*, 2004).

Oocytes matured both *in vivo* and *in vitro* are enclosed with cumulus cells (CCs). The CCs stay with the oocyte matured *in vivo* for a variable period after ovulation, depending upon the species (Yanagimachi & Chang, 1961; Longo, 1974, 1980; Tan, 1985, 1988), but they will always stay with oocytes matured *in vitro* until artificially removed. The roles of the surrounding CCs in maturation, ovulation and fertilization of oocytes

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have been extensively studied (Eppig, 1982, 1991; Buccione *et al.*, 1990; Tanghe *et al.*, 2002), yet little is known about their roles in oocyte aging. Early studies have shown that ovulated oocytes with CCs aged *in vitro* displayed similar morphological alterations as those aged *in vivo* (Longo, 1980; Webb *et al.*, 1986; Tan, 1988) and spontaneous reduction in MPF activity (Kikuchi *et al.*, 1995, 2000; Wu *et al.*, 1997) and cytoskeletal alteration (Kim *et al.*, 1996) have been reported in the aging process of oocytes with CCs matured *in vitro.* Abbott *et al.* (1998) indicated that *in vitro* culture of mouse oocytes free of CCs could retard the spontaneous activation of cell-cycle progression that normally occurred in unfertilized eggs *in vivo* (Xu *et al.*, 1997; Abbott *et al.*, 1998). Recently, Miao *et al.* (2005) also demonstrated that CCs could accelerate the progression of *in vitro* aging of mouse oocytes.

In vitro fertilization (IVF) has been used frequently for the treatment of sterility in humans; to improve the quality and increase production in cattle (Suzuki *et al.*, 1996). In laboratory animals, IVF has been used for analysis of fertilization events and to ameliorate defects in the reproductive ability of mutant mice (Suzuki *et al.*, 1987, 1988, 1996; Yokoyama *et al.*, 1995) or aged mice (Suzuki *et al.*, 1994). Early studies have shown that the addition of E_2 to oocyte maturation medium could increase the fertilization and cleavage rates of the *in vitro* matured human oocytes (Tesarik & Mendoza 1995). Although 1 μg/ml E_2 is often used routinely in *in vitro* maturation (IVM) and in *in vitro* fertilization (IVF) (Beker *et al.*, 2002), yet little is known about its roles in embryos development.

In this study, mouse oocytes without CCs were stored at room temperature (25 °C) for 24 h and were used for *in vitro* fertilization with fresh spermatozoa. We also tested the effect of E_2 in CZB medium on subsequent embryo development.

Materials and methods

Animals

Kunming female (white) and C57Bl/6 male (black) mice, 2–4 months old, were used in this study. Kunming mice were originally derived from ICR mice (CD-1) (Yu *et al.*, 2006). They were kept in an air-conditioned room (23 °C, 50% relative humidity) under 14-h light and 10-h dark cycles.

Oocyte collection and storage

Kunming female mice were superovulated by injection of 7.5 IU pregnant mare serum gonadotropin (PMSG, Tianjin Animal Hormone Factory), followed by 7.5 IU human chorionic gonadotropin (hCG, Ningbo Animal Hormone Factory) 48 h later. Approximately 13–14 h after hCG injection, COCs were collected from oviducts. COCs were placed in modified HEPES-buffered CZB (H-CZB) medium and treated with 0.1% hyaluronidase in order to disperse cumulus cells. Then 10 oocytes were stored in a 20 μl H-CZB medium drop for 24 h at room temperature (25 °C). Medium droplets were covered with mineral oil to prevent evaporation.

Sperm collection and capacitation

Spermatozoa were obtained from C57Bl/6 male mice. After the male mice were killed, one caudae epididymis was removed and the spermatozoa were gently squeezed out. Then the spermatozoa were placed into a 400 μl HTF drop in the incubator (5% CO_2 in air, 37 °C) for 1.5 h for capacitation.

Parthenogenetic activation

Fresh oocytes and categories of survived oocytes after 24 h storage were cultured in Ca^{2+}-free CZB medium supplemented with 5.56 mM D-glucose containing 10 mM $SrCl_2$ for 5 h at 37 °C in a humidified atmosphere containing 5% CO_2, then washed and cultured in modified CZB medium (Cummins *et al.*, 1998; Kishikawa *et al.*, 1999; Takeda *et al.*, 2005) with or without 1 μg/ml E_2 (Beker *et al.*, 2002; Zhang, 2004) at 37 °C in a humidified atmosphere containing 5% CO_2 for further culture.

In vitro fertilization

Both fresh and stored oocytes were inseminated in 400 μl microdroplets of sperm suspension in HTF medium (2×10^6 motile sperm/ml) overlaid with mineral oil for 5 h at 37 °C in a humidified atmosphere containing 5% CO_2. They were then washed and transferred into fresh CZB medium with or without 1 μg/ml E_2 (Beker *et al.*, 2002; Zhang, 2004) for further culture.

In vitro embryo culture

In vitro culture (IVC) of embryos was carried out in 20 μl microdroplets of CZB medium with or without 1 μg/ml E_2(Beker *et al.*, 2002; Zhang, 2004) under mineral oil (10 embryos per drop) at 37 °C in a humidified atmosphere containing 5% CO_2. Embryos were cultured in CZB medium with or without 1 μg/ml E_2 (without glucose) for 48 h. On day 2 of culture, the embryos were checked for further development. Cleaved embryos were transferred to fresh culture medium supplemented with glucose (5 mg/ml) and incubated for another 2 days to the morula/blastocyst stage.

Table 1 Activation rates of fresh oocytes and storage oocytes

Type of oocytes	No. of oocytes collected	Death	No. of activated oocytes (%)
Storage oocytes	274	38	231 (84.31)[a]
Fresh oocytes	114	3	103 (90.35)[a]

[a]Values with same superscripts within a column do not differ significantly ($p > 0.05$).

Table 2 Fertilization rates of fresh oocytes and storage oocytes

Type of oocytes	No. of oocytes collected	Death	No. of activated oocytes (%)
Storage oocytes	1269	94	598 (47.12)[a]
Fresh oocytes	198	5	179 (90.40)[b]

[a,b]Values with different superscripts within a column differ significantly ($p < 0.05$).

Embryo transfer to foster mothers

After 96 h in culture, morulae/blastocysts embryos were transferred into the uteri of day 3 pseudopregnant females. The uteri of pseudopregnant females were examined for fetuses on day 19 or 20.

Statistical analysis

The data were analysed using the chi-squared test (SPSS 13.0). A value of $p < 0.05$ was considered significant.

Results

Comparison of oocyte activation and fertilization rates

The parthenogenetic activation rates of fresh and storage oocytes were 90.35% and 84.31% respectively ($p > 0.05$) (Table 1). Furthermore, as shown in Table 2, the fertilization rates of fresh and storage oocytes were significantly different (90.40% and 47.12%, respectively, $p < 0.05$).

Parthenogenetic development of storage and fresh oocytes

Most parthenogenetic embryos from storage oocytes (93.58% and 87.70%, $p > 0.05$) developed to the 2-cell stage after cultured in CZB medium or CZB medium + E_2 for 24 h. There was significant difference between the development rates of 4–8-cell embryos in the two media (59.63% and 68.85%, $p < 0.05$). In addition, the development rates of morula/blastocyst were significantly different (41.18% and 48.36%, respectively, $p < 0.05$) (Table 3). There was also significant difference between the development rates of blastocysts derived from activated fresh oocytes cultured in the two media (6.33% and 18.60%, $p < 0.05$) (Table 4).

Development of IVF embryos from storage oocytes

As shown in Fig. 1*a*, 47.12% of IVF eggs formed two pronuclei (2PN) after IVF. The shape and size of 2PN in storage oocytes were similar to those in fresh oocytes after IVF. These results suggest that a large proportion of mouse oocytes stored at room temperature for

Table 3 The effects of E_2 on development of parthenogenetic oocytes stored at room temperature for 1 day

Culture medium	No. of activated oocytes	No. (%) of embryos developed to each stage		
		2-cell	4–8-cell	Morula–Blastocyst
CZB	109	102 (93.58)[a]	65 (59.63)[a]	42 (41.18)[a]
CZB + E_2	122	107 (87.70)[a]	84 (68.85)[b]	59 (48.36)[b]

[a,b]Values with different superscripts within a column differ significantly ($p < 0.05$).

Table 4 The effects of E_2 on development of parthenogenetic fresh oocytes

Culture medium	No. of activated oocytes	No. (%) of embryos developed to each stage			
		2-cell	4–8-cell	Morula	Blastocyst
CZB	79	63 (79.75)[a]	58 (73.42)[a]	51 (64.56)[a]	5 (6.33)[a]
CZB + E_2	86	75 (87.21)[a]	69 (80.23)[a]	64 (74.42)[a]	16 (18.60)[b]

[a,b]Values with different superscripts within a column differ significantly ($p < 0.05$).

Table 5 The effects of E_2 on development of fertilized embryos from storage oocytes

Culture medium	No. of oocytes with 2PN	No. (%) of embryos developed to each stage			No. of recipients	No. of live offspring (recipients)
		2-cell	4–8-cell	Morula–Blastocyst		
CZB	342	309 (90.35)[a]	207 (60.53)[a]	167 (48.83)[a]	7	1 (1)
CZB + E_2	256	243 (94.92)[a]	174 (67.97)[b]	141 (55.08)[b]	6	6 (1)

[a,b]Values with different superscripts within a column differ significantly ($p < 0.05$).

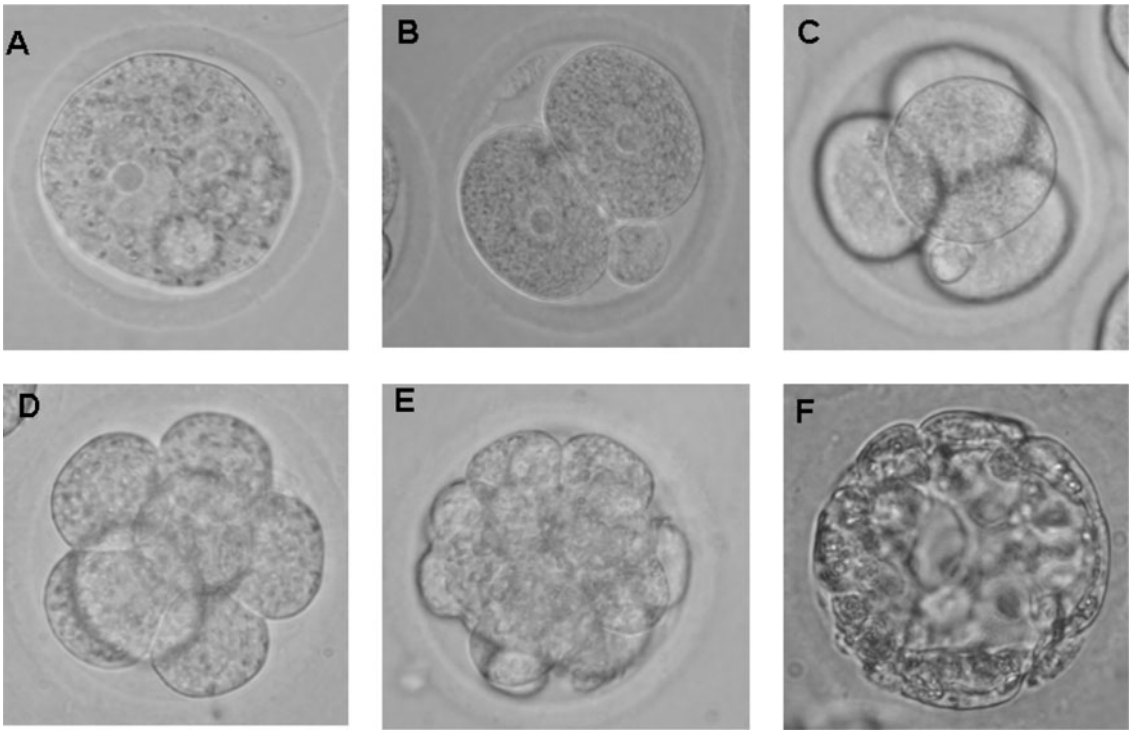

Figure 1 *In vitro* development of mouse embryos derived from IVF of oocytes stored at room temperature for 1 day (×400). (*a*) zygote with a male and a female pronuclei; (*b*) 2-cell embryo; (*c*) 4-cell embryo; (*d*) 8-cell embryo; (*e*) morula; (*f*) blastocyst.

24 h can be fertilized by IVF. Most of these embryos (90.35% vs. 94.92%, $p > 0.05$) developed to the 2-cell stage (Table 5, Fig. 1*b*) after being cultured in the two media for 24 h, while the rates of embryos developed to the 4–8-cell stage (Table 5, Fig. 1*c*,*d*) in the two media were significantly different (60.53% vs. 67.97%, $p < 0.05$). In addition, the rates of morula/blastocyst (Table 5, Fig. 1*e*,*f*) (48.83% and 55.08%, respectively) were also significantly different ($p < 0.05$).

Birth of pups after embryo transfer

One out of seven foster females transferred with embryos cultured in CZB medium gave birth to a female pup (agouti) (Table 5, Fig. 2*a*). One out of six foster females with embryos cultured in CZB with E_2 medium gave birth to six pups, two males (agouti) and four females (agouti) on day 20 (Table 5, Fig. 2*b*). All pups had black eyes and a pigmented coat. The donors of spermatozoa were C57Bl/6 (black coat) and foster mothers were Kunming mice (white coat), who had never been exposed to pigmented males, therefore, the offspring were derived from the IVF eggs. All the pups developed normally to date (134 days and 147 days at the time of submission).

Discussion

Cryopreservation of gametes has been very successful in humans and some mammalian species (Polge, 1952; Wakayama *et al.*, 1998; Nakagata 2000), but it needs a constant supply of liquid nitrogen, which is inconvenient and also expensive. New inexpensive methods for gamete preservation are therefore of interest at the current time. Wakayama *et al.* (2004) found that mouse COCs stored at room temperature for 24 h can retain full developmental potential. In this experiment, storage of denuded oocytes at room temperature for 1 day decreased fertilization rate

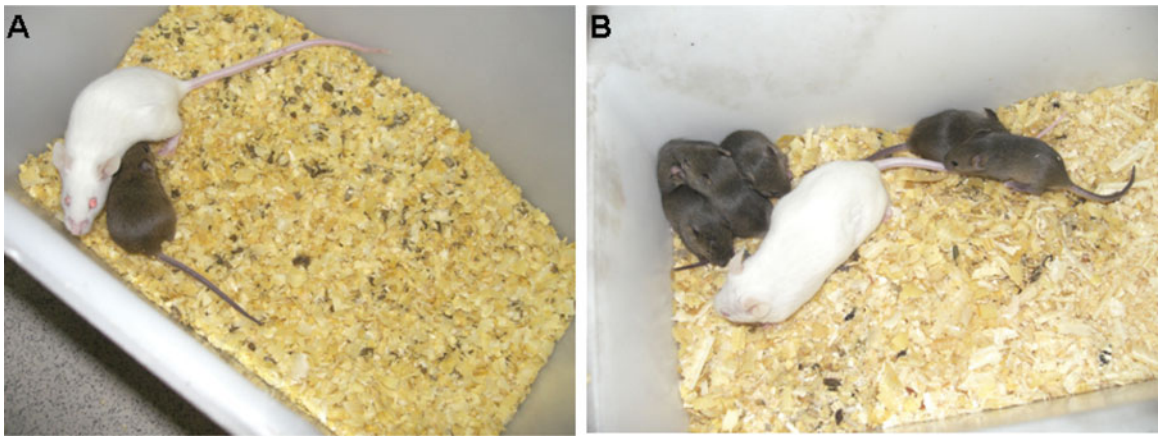

Figure 2 The foster mothers (white) and pups (agouti). (*a*) One pup from embryos cultured in CZB medium without E_2; (*b*) Six pups from embryos cultured in CZB with E_2.

when compared with that of fresh oocytes (90.40% and 47.12%, respectively, $p < 0.05$). On the other hand, there was no significant difference in the activation percentages of fresh oocytes and storage oocytes when they were activated by Sr^{2+} (90.35% and 84.31%, respectively, $p > 0.05$). Miao *et al*. (2005) showed that although oocytes aged *in vitro* without CCs showed a less degree of cortical granule (CG) exocytosis, they displayed an unusually long t_{50} for zona pellucida (ZP) dissolution. Downs *et al*. (1986) found that serum maintained the ability to fertilize mouse oocytes matured *in vitro* by preventing the hardening of the ZP. Our oocytes were stored in H-CZB medium without serum. The results of fertilization and activation suggested that, although most DOs stored at room temperature for 1 day were still normal, the ZP of stored DOs became hard during storage at room temperature, so their fertilization rate became low. Serum might prevent the hardening of the ZP during DOs that were stored at room temperature in H-CZB medium.

Tesarik *et al*. (1995) reported that the addition of E_2 to oocyte maturation medium did not produce any apparent effects on either germinal vesicle breakdown or further progression of meiosis, but it did increase the fertilization and cleavage rates of the *in vitro* matured oocytes (Tesarik *et al*., 1995). Guler *et al*. (2000) found that E_2 played an important role in the nuclear and cytoplasmic maturation of sheep oocytes *in vitro* in defined conditions. Zhang *et al*. (2004) found that the 2-cell block of reconstructed oocytes of hamster could be overcome by adding E_2 to M199. In this experiment, some mouse oocytes stored at room temperature for 24 h can be fertilized normally by IVF. Most of these embryos (90.35% vs. 94.92%, $p > 0.05$) developed to 2-cell stage after being cultured in the two media for 24 h, while the rates of embryos developed to the 4–8-cell stage in the two media were significantly different (60.53% vs. 67.97%, $p < 0.05$). In addition, the rates of morula/blastocyst formation (48.83% and 55.08%, respectively) were significantly different ($p < 0.05$). There was also significant difference between the rates of blastocysts derived from activated fresh oocytes cultured in the two media (6.33% and 18.60%, $p < 0.05$). E_2 is the most potent steroidal estrogen (female sex hormone) and is produced endogenously by all mammalian species. It is produced in the ovary, placenta and possibly the adrenal cortex. It can diffuse through the plasma membrane and bind with the receptor. The receptor with bound ligand is then translocated into the nucleus, where its DNA-binding domain binds to response elements, allowing E_2 to stimulate transcription of target genes. We therefore proposed a hypothesis that E_2 would improve mouse embryos development *in vitro* through stimulating transcription of some important genes.

In conclusion, we have demonstrated that some DOs stored at room temperature for 1 day can be fertilized normally by IVF and that E_2 might improve mouse embryo development *in vitro.* The birth of seven agouti pups in this study and their healthy growth indicate that the storage of DOs at room temperature for 1 day may become a practical procedure in mammalian reproduction.

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