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Development of Normal Mice After Microinjection of Round Spermatids Into Oocytes Stored at Room Temperature for One Day

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ABSTRACT Early studies have shown that some mouse cumulus–oocyte complexes (COCs) stored at room temperature for 24 hr still retained full developmental potential. In this study, we stored mouse COCs and denuded oocytes (DOs) at room temperature for 24 hr and activated these oocytes with 10 mM $SrCl_2$ or injected the oocytes with round spermatids. We found that DOs were better than COCs when stored at room temperature for 1 day and more normal oocytes were obtained when COCs were stored in more H-CZB medium at room temperature for 1 day. The rates of normal oocytes were significantly different after preservation with three schemes (90.01%, 55.81%, and 86.70%, $P < 0.05$). Our results also indicated that oocytes stored at room temperature for 1 day were fertilized normally (extrusion of the second polar body and formation of male and female pronuclei [PN]) after microinjection of round spermatid nuclei, and that the existence of cumulus cells (CCs) during oocyte storage did not significantly influence the early cleavage but had a detrimental effect on later embryo development and full-term development. After fertilization, most embryos developed to two-cell stage after being cultured for 24 hr, and the development rates of four- to eight-cell embryos between two experiments were similar. However, the rates of morula/blastocyst formation were significantly different (47.44% and 26.27%, respectively, $P < 0.05$). The birth of four healthy pups from stored DOs indicated that the storage of DOs at room temperature for 1 day might become a practical procedure in mammalian reproduction. *Mol. Reprod. Dev. 75: 795–800, 2008.* © 2007 Wiley-Liss, Inc.

Key Words: cumulus; round spermatid; fertilization; embryo development; pup

INTRODUCTION

Mammalian oocytes are normally 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) usually undergo time-dependent aging (Yanagimachi and Chang, 1961; Whittingham and 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 animal husbandry as well as reproductive medicine. It has been reported that frozen-thawed spermatozoa and oocytes can often regain their viability and be used for fertilization (Watson, 1990; Wakayama et al., 1998; Nakagata, 2000). Long-term storage of spermatozoa and oocytes in liquid nitrogen (−196°C) is possible, but requires the constant replacement of liquid nitrogen, and during transport, even over short distances, dry ice may sometimes be insufficient for maintaining suitable temperatures. Thus, it would be ideal if we can 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 a month without losing their genetic and reproductive potential (Wakayama and Yanagimachi, 1998), and mouse cumulus–oocyte complexes (COCs) stored at room temperature for 24 hr 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 oocyte matured in vivo for a variable period after ovulation, depending upon the species (Yanagimachi and 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

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in maturation, ovulation, and fertilization of oocytes 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. indicated that in vitro culture of mouse oocytes free of CCs could retard the spontaneous activation of cell cycle progression that normally occurred in in vivo unfertilized eggs (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.

The microfertilization technique has enabled us to use immature male germ cells (spermatogenic cells) as substitute gametes. Normal offspring have been born by microfertilization with round spermatids in mouse (Ogura et al., 1994; Kimura and Yanagimachi, 1995b; Liu et al., 1997), rabbit (Sofikitis et al., 1994), human beings (Tesarik et al., 1995), hamster (Haigo et al., 2004), and by microfertilization with secondary spermatocytes in mouse (Kimura and Yanagimachi, 1995a).

In this article, we compared the development of mouse oocytes with or without CCs stored at room temperature (25°C) for 24 hr after microfertilization with round spermatids. We also tested whether the mouse embryos developed from round spermatids and oocytes stored at room temperature for 24 hr could go through full-term development.

MATERIALS AND METHODS

Animals

Kunming female (a white strain) and C57BL/6 male (a black strain) mice, 2–4 months old, were used in this study. They were kept in an air-conditioned room (23°C, 50% relative humidity) under 14 hr light and 10 hr dark cycles.

Preparation of Spermatids

The spermatogenic cells were collected and suspended in 0.9% NaCl solution, and the seminiferous tubules of a C57BL/6 male mouse were minced as described previously (Kimura and Yanagimachi, 1995b; Liu et al., 1997; Jiang et al., 2005). The round spermatids used in this study were immature haploid cells that had a decondensed nucleus. One aliquot of spermatogenic cell suspension was mixed with ~10 aliquots of modified HEPES–CZB medium containing 12% (w/v) polyvinylpyrrolidone (PVP, Mr 360 kDa; Sigma-Aldrich, CO) in a micro-manipulation chamber.

Oocyte Collection and Storage

Kunming female mice were induced to superovulate with injection of 7.5 IU pregnant mare serum gonadotropin (PMSG; Tianjin Animal Hormone Factory), and followed by 7.5 IU human chorionic gonadotropin (hCG; Ningbo Animal Hormone Factory) 48 hr later. Approximately 13–14 hr after hCG injection, COCs were collected from oviducts. Three storage methods were used.

Method 1. COCs were placed in modified HEPES-buffered CZB (H-CZB) medium, and treated with 0.1% hyaluronidase in order to disperse CCs. Then 10 denuded oocytes (DOs) were stored in a 20 μl H-CZB medium drop at room temperature covered with aluminum foil in a dish for 24 hr. Also medium drops were covered in mineral oil to prevent evaporation.

Method 2. COCs from one mouse were stored in a 200 μl H-CZB medium drop at room temperature covered with aluminum foil in a dish for 24 hr. Also medium drops were covered in mineral oil to prevent evaporation.

Method 3. COCs from three to four mice were stored in 3–5 ml H-CZB medium at room temperature covered with aluminum foil in a dish for 24 hr. The morphology of oocytes stored by Methods 1, 2, and 3 were evaluated under a stereomicroscope.

Parthenogenetic Activation

After 24 hr storage, survived oocytes were cultured in Ca^{2+}-free CZB medium supplemented with 5.56 mM D-glucose containing 10 mM $SrCl_2$ for 5 hr 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) for 4 days at 37°C in a humidified atmosphere containing 5% CO_2.

Microinjection of Round Spermatids (ROSI) Into Oocytes

Identification and injection of round spermatids were performed as previously reported (Kimura and Yanagimachi, 1995b; Miki et al., 2004). About 60 min before spermatid injection, survived oocytes stored by Methods 1 and 3 were activated by treatment with 10 mM $SrCl_2$ in Ca^{2+}-free CZB medium for 20 min at 37°C in a humidified atmosphere containing 5% CO_2 (Miki et al., 2004; Jiang et al., 2005). The spermatid nuclei, together with a small volume of the cytoplasm, were injected into oocytes by using a Piezo-driven micromanipulator. All the procedures were performed at room temperature within 20 min. The injected oocytes were then kept at room temperature for about 10 min before they were incubated at 37°C. Oocytes that survived were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Embryo Transfer to Foster Mothers

After 24 hr in culture, two-cell-stage embryos were transferred into the oviducts of day 0.5 pseudo-pregnant females. The uteri of pseudo-pregnant females were examined for fetuses and implantation sites on day 19 or 20.

TABLE 1. Abnormality of Oocytes After Storage at Room Temperature for 24 hr

Storage method	No. of oocytes collected	No. of oocytes with different configuration (%)					
		Death	Fragment	Shrinkage	Distortion	Two-cell like	Normal
Method 1**	1372	109 (7.94)*	4 (0.29)	7 (0.51)	12 (0.87)	5 (0.36)	1235 (90.01)[a]
Method 2**	387	145 (37.48)	15 (3.88)	2 (0.52)	9 (2.33)	0 (0)	216 (55.81)[b]
Method 3**	1549	171 (11.04)	3 (0.19)	13 (0.84)	18 (1.16)	1 (0.06)	1343 (86.70)[c]

Values with different superscripts within a column differ significantly ($P < 0.05$).
*Dead oocytes just from oviducts were included in this group.
**Method 1: 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 at room temperature covered in aluminum foil in a dish for 1 day. And medium drops were covered in mineral oil to prevent evaporation. Method 2: COCs from one mouse were stored in a 200 μl H-CZB medium drop at room temperature covered in aluminum foil in a dish for 1 day. And medium drops were covered in mineral oil to prevent evaporation. Method 3: COCs from three to four mice were stored in 3–5 ml H-CZB medium at room temperature covered in aluminum foil in a dish for 1 day.

Statistical Analysis

The data were analyzed by using Chi-square (χ^2) test (SPSS 13.0). $P \leq 0.05$ was considered significant.

RESULTS

Classification of Mouse Oocytes Stored at Room Temperature

As shown in Table 1, the incidence of abnormal oocytes (Fig. 1A–E) varied according to the storage methods. The percentages of normal oocytes (Fig. 1F) stored with the three methods were significantly different. The rates of normal oocytes stored by Methods 1, 2, and 3 were 90.01%, 55.81%, and 86.70%, respectively ($P < 0.05$).

Parthenogenetic Development

Most parthenogenetic embryos (87.29% vs. 84.87% vs. 91.57%, $P > 0.05$) developed to two-cell stage after being cultured for 24 hr, and there was no difference among the development rates of four- to eight-cell embryos of the three methods (64.95% vs. 54.62% vs. 57.30%, $P > 0.05$), while the rates of morula/blastocyst formation were significantly different (40.89%, 15.13%, and 24.16%, respectively, $P < 0.05$) (Table 2). The results suggest that CCs do not significantly influence the early cleavage but affect the later development of parthenogenetic embryos developed from oocytes stored for 24 hr.

Fertilization and Development of Oocytes Stored at Room Temperature for 24 hr

Oocytes (1,484) from Methods 1 and 3 were successfully manipulated. One-day-old oocytes could be fertilized by round spermatid nuclei injection. As shown in Figure 2A, most microfertilized eggs (80.41% vs. 85.34%, $P > 0.05$) (Table 3) formed 2PN. The shape and size of two pronuclei (PN) in oocytes stored at room

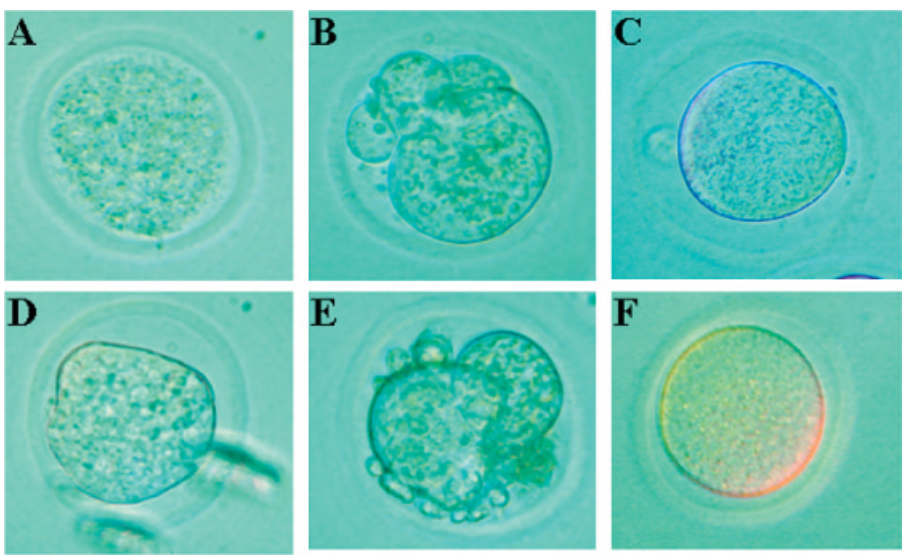

Fig. 1. Classification of stored oocytes. Six different types of oocytes were observed after storage at room temperature for 1 day by three methods (400×). **A**: Dead; (**B**) fragmented; (**C**) shrunken, the size is reduced and the cell-surface is not smooth; (**D**) distorted, the oocyte is not round and sometimes exhibits pronuclear-like structures in the cytoplasm; (**E**) two-cell like; (**F**) normal.

TABLE 2. Preimplantation Development of Mouse Parthenogenetic Oocytes Stored at Room Temperature for 24 hr

Storage method	No. of oocytes treated	Dead	Activated (%)	No. of embryos developed to each stage (%)		
				Two-cell	Four- to eight-cell	Mol-Bla
Method 1	337	14	291 (86.35)	254 (87.29)[a]	189 (64.95)[a]	119 (40.89)[a]
Method 2	216	93	119 (55.09)	101 (84.87)[a]	65 (54.62)[a]	18 (15.13)[b]
Method 3	299	118	178 (59.53)	163 (91.57)[a]	102 (57.30)[a]	43 (24.16)[b]

Values with different superscripts within a column differ significantly ($P < 0.05$).

temperature for 24 hr were also similar to those in fresh oocytes after round spermatid injection (ROSI). These results suggest that most mouse oocytes stored at room temperature for 24 hr can be fertilized by round spermatid injection. Most of these embryos (93.59% vs. 84.85%, $P > 0.05$) developed to two-cell (Fig. 2B) stage after being cultured for 24 hr, and the rates of embryos developed to four- to eight-cell (Fig. 2C,D) in the two methods were not significantly different (62.82% vs. 54.55%, $P > 0.05$), while the rates of morula/blastocyst (Fig. 2E,F) formation (47.44% and 26.27%, respectively) were significantly different ($P < 0.05$). The results indicate that CCs do not significantly influence the 2PN formation and early cleavage but obviously exert a detrimental effect on the later development of mouse ROSI embryos developed from oocytes stored at room temperature for 1 day.

Birth of Pups After Embryo Transfer

None of the 15 foster females with embryos from oocytes stored by Method 3 gave birth to a pup. Nevertheless, with embryos from Method 1, one of the nine fosters gave birth to four pups, three males (agouti) and one female (agouti) on day 20. Moreover, all pups had black eyes and pigmented coat (Table 4, Fig. 3).

Donors of round spermatid nuclei were C57BL/6 (black coat) and foster mothers were Kunming mice (white coat), which had never been exposed to pigmented males, therefore, the offspring should be derived from the microfertilized eggs. All the pups grow normally up to now (120 days).

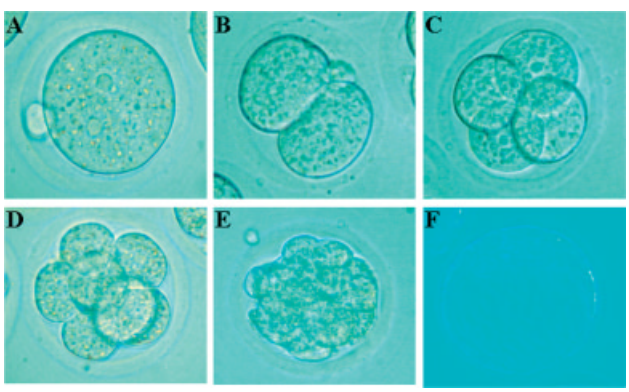

Fig. 2. In vitro development of mouse embryos derived from oocytes stored at room temperature for 1 day and round spermatids microinjection (400×). **A**: Zygote with a male and a female pronuclei; (**B**) two-cell embryo; (**C**) four-cell embryo; (**D**) eight-cell embryo; (**E**) morula; (**F**) blastocyst.

DISCUSSION

Cryopreservation of gametes has been very successful in human beings and some mammalian species (Polge, 1952; Wakayama et al., 1998; Nakagata, 2000), but it needs constant supply of liquid nitrogen, which is inconvenient and also expensive. So people are now paying more attention to find new and inexpensive methods for gamete preservation. In this study, three schemes were designed to determine whether CCs influence the development potential of oocytes preserved at room temperature. We found that the rates of normal oocytes were significantly different after preservation with the three schemes (90.01%, 55.81%, and 86.70%) as shown in Table 1. It is showed that DOs were better than COCs when stored at room temperature for 1 day and more normal oocytes were obtained when COCs were stored in more H-CZB medium at room temperature for 1 day. A recent study reported that many oocytes with CCs aged in vivo and in vitro showed a partial cortical granule (CG) release (Miao et al., 2005). This result was further substantiated by the observation that the t_{50} for chymotrypsin-mediated zona pellucida (ZP) dissolution increased significantly in the ZP from oocytes aged in vivo or in vitro with CCs for up to 24 hr after hCG administration compared to the ZP from freshly ovulated oocytes (Miao et al., 2005). However, oocytes aged in vitro without CCs showed little CG exocytosis (Miao et al., 2005). If COCs were preserved at room temperature, CCs would accelerate the progression of in vitro aging of mouse oocytes. Since COCs stored at room temperature still had metabolic activity, more H-CZB medium would be more useful to maintain metabolic balance.

Wakayama et al. (2004) have observed the abnormalities in metaphase II spindles of oocytes stored at room temperature for 1 day, including misaligned chromosomes, or dispersed, elongated, or completely disrupted spindles. In order to determine whether CCs influence the developmental capacity of the oocytes stored at room temperature for 1 day, in our study, oocytes stored by three methods were activated by 10 mM $SrCl_2$ in Ca^{2+}-free CZB medium supplemented with 5.56 mM D-glucose

TABLE 3. In Vitro Development of Oocytes Stored by Methods 1 and 3 Following ROSI

Storage method	No. of oocytes injected	No. of oocytes with 2PN (%)	No. of embryos developed to each stage (%)		
			Two-cell	Four- to eight-cell	Mol-Bla
Method 1	97	78 (80.41)[a]	73 (93.59)[a]	49 (62.82)[a]	37 (47.44)[a]
Method 3	116	99 (85.34)[a]	84 (84.85)[a]	54 (54.55)[a]	26 (26.27)[b]

Values with different superscripts within a column differ significantly ($P < 0.05$).

TABLE 4. In Vivo Development of Mouse Embryos Developed From Oocytes Stored by Methods 1 and 3 and Fertilized by ROSI

Storage method	No. of oocytes injected	No. of oocytes with 2PN (%)	Two-cell (%)	No. of recipients	No. of live offspring (recipients)
Method 1	624	537 (86.06)[a]	489 (91.06)[a]	9	4 (1)
Method 3	647	579 (89.49)[a]	507 (87.56)[a]	15	0

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

containing for 5 hr at 37°C in a humidified atmosphere containing 5% CO_2. We found that most parthenogenetic embryos developed to two-cell stage after being cultured for 24 hr, and there was no difference among the development rates of four- to eight-cell embryos in the three methods ($P > 0.05$), while the rates of morula/blastocyst formation were significantly different (40.89%, 15.13%, and 24.16%, respectively, $P < 0.05$). The results suggest that the existence of CCs during storage of oocytes is detrimental to embryo development.

Kimura and Yanagimachi (1995b) have demonstrated that round spermatids could not activate the oocytes, and the oocytes had to be activated artificially before spermatid injection. In this study, we pre-activated the 1-day-old oocytes stored by Methods 1 and 3 with 10 mM Sr^{2+}in Ca^{2+}-free CZB for 20 min before round spermatid injection. The percentages of normal fertilization are 80.41% and 85.34%, respectively, higher than those eggs which were post-activated or unactivated with Sr^{2+}(our unpublished data). It is demonstrated that oocytes stored at room temperature for 1 day were not naturally activated. Most embryos (93.59% vs. 84.85%) developed to two-cell stage after being cultured for 24 hr, and the development rates of four- to eight-cell embryos between the two experiments were similar. However, the rates of morula/blastocyst formation were significantly different (47.44% and 26.27%, respectively, $P < 0.05$). When 489 two-cell embryos from DOs stored for 1 day were transferred to nine foster mothers, four (0.82%) full-term pups were born and grew into healthy adults. But when 507 two-cell embryos from 1-day-old COCs were transferred to 15 foster mothers, no pup was born. These results demonstrated that mouse round spermatids are competent like mature sperm to transform into male pronuclei after being injected into MII oocytes stored at room temperature for 1 day, and pre-activation with Sr^{2+} for 20 min possibly benefited the formation of 2PN and development of embryos. Moreover, the existence of CCs during room temperature storage could decrease the later embryo development and the full-term developmental capacity of oocytes.

Fig. 3. The foster (white) and pups (agouti).

CONCLUSIONS

We demonstrate that DOs were better than COCs when stored at room temperature for 1 day and that the existence of CCs during oocyte storage had a detrimental effect on later embryo development and full-term development of the stored oocytes. The birth of four 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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