24時間室温保存したマウス卵母細胞子からの産仔の作出

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—Original—

Production of Offspring from One-Day-Old Oocytes Stored at Room Temperature

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Abstract. To determine the feasibility of preserving oocytes without freezing, we stored mouse oocytes in several media at different temperatures for one day. Confocal microscopy of the metaphase-II spindle in these stored oocytes revealed gross abnormalities in both the spindle and the arrangement of chromosomes. The abnormal spindles could not be rescued by transplanting the aged spindle-chromosome complex into a fresh enucleated oocyte. A diploid parthenogenetic development showed that some of the oocytes stored at room temperature could still develop into blastocysts (10–57%). However, oocytes stored in a refrigerator (5%) or incubator (0%) lost the potential almost entirely. Fertilization of room-temperature-preserved oocytes with fresh spermatozoa by ICSI or IVF resulted in, respectively, 4 and 10%, full-term births. These results suggest that when oocytes are stored at room temperature for one day, most have irreversible damage not only to their cytoplasm but also to the spindle. However, since at least a few percent of stored oocytes retained the potential for full-term development, it may be possible to overcome these problems and develop a simple method for preserving mammalian oocytes without freezing.
Key words: Aging, Preservation, Offspring, Room temperature

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After ovulation, the mammalian oocyte quickly loses its potential to give rise to a developmentally competent embryo. The *in vivo* aging of postovulatory oocytes has been studied extensively in the past 30 years. Aged oocytes often showed signs of accelerated development such as rapid fertilization, pronuclear formation and blastocyst development compared to non-aged oocytes [1–3]. Under *in vivo* conditions, ovulated mouse eggs maintained maximum fertilization competency for only 4–6 h [4]. *In vitro* fertilization (IVF) of further aged oocytes led to polyspermy [5] or aneuploidy due to premature centromere separation [6] and more importantly, resulted in a severe decrease of newborns [7, 8], with those that were carried to full term often being malformed [9]. As the time following ovulation progresses, aged unfertilized eggs as well as aged fertilized embryos exhibit varying degrees of fragmentation and cell death [10, 11]. Recently, it has been demonstrated that these eggs and embryos undergo molecular changes characteristic of apoptosis or programmed cell death, a process of cell selection that is observed in many cell types [12–14]. Gordo *et al.* [15] identified several critical molecules involved in

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the regulation of Ca^{2+} homeostasis and the balance between anti- and pro-apoptotic proteins that were lost or inactivated during postovulatory aging of oocytes.

Despite inherent difficulties, gamete cryopreservation has revolutionized animal husbandry as well as reproductive medicine. Frozen-thawed spermatozoa and oocytes can often regain their viability and can be used for fertilization [16–18]. 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 can sometimes be insufficient for maintaining suitable temperatures. Thus, it would be ideal to be able to store spermatozoa and oocytes for a several days at room temperature [19]. We have previously demonstrated that mouse spermatozoa can be freeze-dried and stored at room temperature for up to one month without losing their genetic and reproductive potential [20]. However, oocytes are more fragile and difficult to store without freezing.

For these reasons, only a few investigations of room temperature storage of oocytes have been conducted [21] and none have succeeded in producing viable offspring from such aged oocytes *in vitro*. In the present study, we demonstrate that although most *in vitro* aged oocytes have irreversible damage to the metaphase-II spindle, at least some retained full developmental potential after storage for one day at room temperature.

Materials and Methods

Animals

Oocytes and spermatozoa were harvested from B6D2F1 (C57BL/6 × DBA/2) mice. CD-1 females mated with vasectomized males of the same strain were used as surrogate mothers of IVF/ICSI embryos. All animals were maintained in accordance with the guidelines of the Laboratory Animal Service at RIKEN CDB. The protocol for animal handling and treatment was reviewed and approved by the Animal Care and Use Committee at the RIKEN CDB.

Oocyte collection and storage temperature

Female mice were induced to superovulate by consecutive injections of eCG (5 IU) and hCG (5 IU) 48 h apart. Fourteen hours after hCG injection, cumulus-oocyte complexes (COC) were collected from oviducts and stored in either modified HEPES-buffered CZB medium [22], KSOM medium (Specialty media) or THY [23] at 4 C (in a refrigerator), at 27 C (room temperature) covered in aluminum foil on a desk, or at 37 C (in an incubator under 5% CO_2 in air) for one day.

Observation of oocyte and spindle morphology by light and confocal laser microscopy

Stored and control fresh oocytes were freed from the cumulus cells by adding 0.1% bovine testicular hyaluronidase (ICN Biochemicals, Costa Mesa, CA) to COC containing medium, for a final concentration of 300 USP units/mg. The oocytes were rinsed twice with CZB medium and then observed by phase-contrast or confocal scanning laser microscopy. The oocytes were assigned to six categories according to their gross cellular morphology: shrunken (Fig. 1A), fragmented (Fig. 1B), distorted (Fig. 1C), two-cell-like (Fig. 1D), water-bubble-containing (Fig. 1E), and normal (Fig. 1F). Oocytes judged as normal or water-bubble-containing were whole-mounted on glass slides and fixed with 2.5% (v/v) glutaraldehyde for 10 min. Fixed samples were then stained with 0.25% (w/v) acetolacmoid [24] and examined by phase-contrast microscopy. For a more detailed examination, another group of oocytes were observed by confocal microscopy. Aged oocytes were fixed in PBS-PVA containing 3.5% (w/v) paraformaldehyde and 0.2% (v/v) Triton X-100 for 30 min. Fixed oocytes were washed twice in PBS-PVA and stored overnight in PBS supplemented with 3% (w/v) bovine serum albumin (PBS-BSA; Sigma) and 1% (v/v) Triton X-100 (Nacalai Tesque, Inc. Kyoto, Japan) at 4 C. For staining of α-tubulin, oocytes were incubated in anti-α-tubulin-conjugated FITC (1:100, SIGMA) for 60 min at room temperature. After being washed three times in PBS-BSA for 10 min each, DNA was stained with 400 μg/ml of propidium iodide (Sigma Chemical Co.). Following complete washing, oocytes were mounted on slides using Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA) and observed under a Bio-Rad Radiance 2100 confocal scanning laser microscope (BIO-RAD).

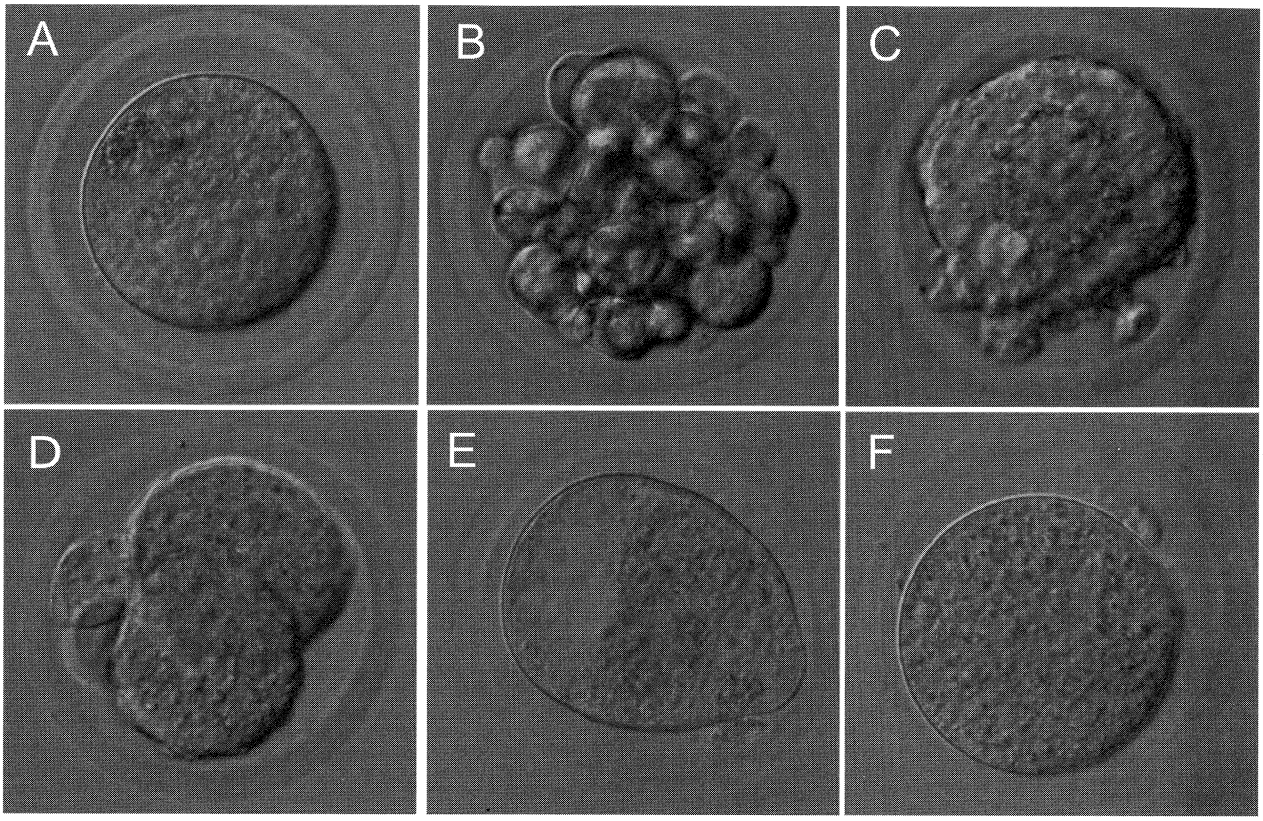

Fig. 1. Classification of stored oocytes. Six different types of oocytes were observed after storage at several temperatures for one day. (A) "shrunken", the size is reduced and the cell-surface is not smooth; (B) "fragmented"; (C) "distorted", the oocyte is not round and sometimes exhibits pronuclear-like structures in the cytoplasm; (D) "two-cell like"; (E) "water-bubble-containing", the oocyte contains a water bubble inside the cytoplasm; (F) "normal".

Oocyte activation and inhibition of second polar body extrusion

After treatment with hyaluronidase, oocytes were rinsed twice and then incubated for 6–7 h in Ca^{2+}-free CZB containing both 10 mM Sr^{2+} and 5 μg/ml cytochalasin B. Sr^{2+} was used to activate oocytes, and cytochalasin B was added to prevent extrusion of the second polar body [25]. Oocytes with at least one pronucleus were considered to be activated, and these were washed and cultured in KSOM until they reached the morula/blastocyst stage.

Fertilization by IVF or ICSI

To prepare sperm, the caudae epididymides were removed from a mature male mouse (B6D2F1, 8–9 weeks old). Blood and adipose tissue were removed from the surface and the caudal (enlarged) part was excised with a pair of fine scissors. This was compressed to release a dense mass of spermatozoa from which drops (~ 2 μl) were taken and placed in a dish containing 0.4 ml TYH [23] and cultured in a CO_2 incubator (37 C) for 20–30 min. The concentration of the spermatozoa was approximately 3–5 $\times$ 10^6/ml at this stage. For IVF, 5 to 10 μl of sperm suspension were collected and introduced into each drop containing one-day-aged oocytes according to the method described by Toyoda *et al.* [23]. ICSI was carried out using a modified version of a technique described by Kimura and Yanagimachi [26]. The sperm suspension (2–3 μl) was mixed thoroughly with 20 μl of HEPES-buffered CZB medium containing 12% (w/v) polyvinyl pyrrolidone (PVP, Mr: 360 kDa) in a micromanipulation chamber. A single spermatozoon was drawn tail first into the injection pipette and moved back and forth until the head-midpiece junction was at the opening of the injection pipette. The head was separated from the midpiece and tail by applying one or more

mechanical pulses using a piezoelectric element [26]. After discarding the midpiece and tail, the head was redrawn into the pipette and injected into an oocyte. The sperm-injected oocytes were transferred into CZB medium under mineral oil and incubated at 37 C in a humidified atmosphere of 5% CO_2 in air.

In vitro and in vivo development

Between 5 and 7 h after IVF or ICSI, oocytes were examined using an inverted microscope. Stored oocytes often failed to extrude the second polar body; therefore, those with two distinct pronuclei and one second polar body were considered to be normally fertilized zygotes (Fig. 3E). These oocytes were selected and continuously cultured for up to 72 h to evaluate embryonic development. When the embryos reached the morula/blastocyst stages (72 h after IVF or ICSI), they were transferred to the uteri of CD-1 albino surrogate mothers, which had been mated with vasectomized CD-1 males three days before. All surrogate mothers were euthanatized at 19.5 dpc, and their uteri were examined for the presence of fetuses and implantation sites. Live fetuses were raised by lactating CD-1 foster mothers.

Exchange of metaphase II spindles from one-day-old oocytes to fresh oocytes

Enucleation of the spindle from a fresh oocyte was performed essentially as described previously [27]. Briefly, a group of fresh oocytes was transferred to a droplet of HEPES-CZB containing 5 μg/ml cytochalasin B, which had been previously placed under mineral oil in the operation chamber on the microscope stage. The metaphase II chromosome-spindle complex, identified as a translucent spot in the ooplasm, was drawn into the pipette along with a small amount of accompanying ooplasm. After oocytes in one group were enucleated, they were transferred into cytochalasin B-free CZB medium and kept there for up to 2 h at 37.5 C [27]. They were then placed into an injection drop on the operation chamber. Enucleation of old oocytes was performed in the same manner as for fresh oocytes. When the metaphase II spindle complex was drawn into the enucleation/injection pipette from the stored oocyte, the operation chamber was moved to position the pipette over the injection drop containing enucleated fresh oocytes. The spindle was then injected into an enucleated fresh oocyte as described previously [28].

Statistical analysis

Each experiment was carried out at least five times. The data were analyzed by the chi-squared test.

Results

Classification of one-day-old mouse oocytes stored at different temperatures

As shown in Table 1, the morphology of fresh oocytes just after ovulation was almost normal. On the other hand, the incidence of abnormal oocytes varied according to storage temperature, but not storage medium. When oocytes were stored at 27 C (room temperature) or 37 C in a CO_2 incubator, 61 to 97% (average, 82.7%) of oocytes were externally judged as normal (Table 1, Fig. 1F). When oocytes were stored at 4 C, none were judged as normal (Table 1), and 76% of oocytes had a water bubble in the cytoplasm (Fig. 1E). The proportion of shrunken and fragmented oocytes (Figs. 1A and B) across all storage temperatures was about 18%, which was the same as fresh control (17%); therefore, these types of abnormalities probably already were present at the time of oocyte collection from the oviduct and were not associated with oocyte storage. When oocytes were stored in the 37 C incubator, 7 to 27% of oocytes exhibited two-cell like morphology (Fig. 1D), suggesting that they may have been spontaneously activated this morphology was only rarely observed at other temperatures.

Microscopic observation of aged oocytes

To observe the morphology of the metaphase II spindle, a total of 147 stored oocytes were examined by light and confocal scanning laser microscopy. As shown in Fig. 2, when oocytes were observed by light microscopy, most oocytes stored at 4 C showed grossly misaligned chromosomes (Fig. 2C). Oocytes stored at 27 C and 37 C showed relatively less misalignment (Fig. 2B), but their chromosomal arrangement was different from that of control fresh oocytes (Fig. 2A). Observation of aged oocytes by confocal microscopy after immunostaining of α-tubulin afforded a much more detailed view of these

Table 1. Abnormality of oocytes after storage at different temperatures for 24 h

Storage temperature (C)	Storage medium	No. of oocytes collected	No. (%) of oocyte with different configuration					
			Shrinkage	Fragment	Distoration	2-cell like	Water bubble	Normal
Fresh (Control)	–	175	18 (10.3)	11 (6.3)	0 (0.0)	0 (0.0)	0	146 (83.4)
4	KSOM	200	22 (11.0)	21 (10.5)	1 (0.5)	0 (0.0)	156 (78.0)	0 (0.0)
	CZB-H	218	19 (8.7)	21 (9.6)	24 (11.0)	1 (0.4)	153 (70.1)	0 (0.0)
	TYH	158	20 (12.6)	4 (2.5)	6 (3.8)	0 (0.0)	128 (81.0)	0 (0.0)
	Total	576	61 (10.6)	46 (7.9)	31 (5.4)	1 (0.2)	437 (75.9)	0 (0.0)
27	KSOM	253	5 (1.9)	50 (19.7)	5 (1.9)	0 (0.0)	0 (0.0)	219 (86.5)
	CZB-H	231	13 (5.6)	30 (12.9)	4 (1.7)	0 (0.0)	0 (0.0)	225 (97.4)
	TYH	345	14 (4.0)	43 (12.5)	13 (3.4)	0 (0.0)	0 (0.0)	275 (79.7)
	Total	829	32 (3.8)	123 (14.8)	22 (2.6)	0 (0.0)	0 (0.0)	719 (86.7)
37	KSOM	153	17 (11.1)	22 (14.3)	0 (0.0)	21 (13.7)	0 (0.0)	93 (60.8)
	CZB-H	138	9 (6.5)	6 (4.3)	0 (0.0)	9 (6.5)	0 (0.0)	114 (82.6)
	TYH	156	18 (9.7)	10 (5.4)	0 (0.0)	39 (20.9)	0 (0.0)	119 (63.9)
	Total	435	44 (10.1)	38 (8.7)	0 (0.0)	69 (15.8)	0 (0.0)	326 (74.9)

abnormalities. Stored oocytes exhibited not only chromosomal misalignment but also abnormal spindles that were elongated or either partially or completely disrupted (45%, 45% and 10%, respectively; n=11) (Figs. 2E and F). These spindle abnormalities were seen even in oocytes with normally aligned chromosomes.

Development of parthenogenetic diploid embryos

The spindle abnormalities seen in stored oocytes seemed to disrupt the equal segregation of sister chromosomes in the oocyte and the second polar body that occurs upon fertilization. The resulting aneuploid embryos would not be able to develop normally. To examine the developmental capacity of the cytoplasm of stored oocytes, the embryos must first have a normal karyotype. Therefore, we produced parthenogenetic diploid embryos from stored oocytes (Table 2). These stored oocytes were activated with Sr^{2+} under the existence of a cytokinesis inhibitor, cytochalasin B, thus giving rise to diploid embryos without the loss of any chromosomes. After storage at 37 C, many oocytes (up to 64%) lysed during the activation process. All surviving oocytes formed more than 3 pronuclei (Fig. 3C), but none of them developed to the eight-cell stage. Among the few oocytes stored at 4 C that were judged to have normal gross cellular morphology, 12% lysed during activation. Most activated oocytes had one pronucleus (83%) instead of two (Fig. 3A), and 81% of embryos stopped development at the one-cell stage. Only 17 embryos (0 to 13%) developed to the morula/blastocyst stages. On the other hand, when oocytes were stored at room temperature, 81% of oocytes survived after activation, and 65% of treated oocytes had two pronuclei (Fig. 3B). Seventy-two hours after activation, 64 embryos (10 to 57%) developed to the morula/blastocyst stages. In all experiments, the storage medium did not affect significantly the development of parthenogenetic diploid embryos.

Fertilization and development of aged oocytes

The parthenogenetic experiments revealed that TYH medium was best for one-day storage at room temperature (Table 2). Therefore, this medium was used for fertilization experiments. When one-day-old stored oocytes were inseminated with fresh spermatozoa, 43% of oocytes were judged as fertilized (Table 3 and Fig. 3E). Seventy-two hours after insemination, 38 embryos (42%) developed to the morula/blastocyst stages. These were transferred into surrogate females, and nine of them (7.9%) developed to full term. On the other hand, 88% of stored oocytes survived after injection of a fresh sperm head, and 84% of those, namely 74% of injected oocytes in Table 3, were successfully fertilized. Fifty-six embryos (66%) developed to the morula/blastocyst stages 72 h

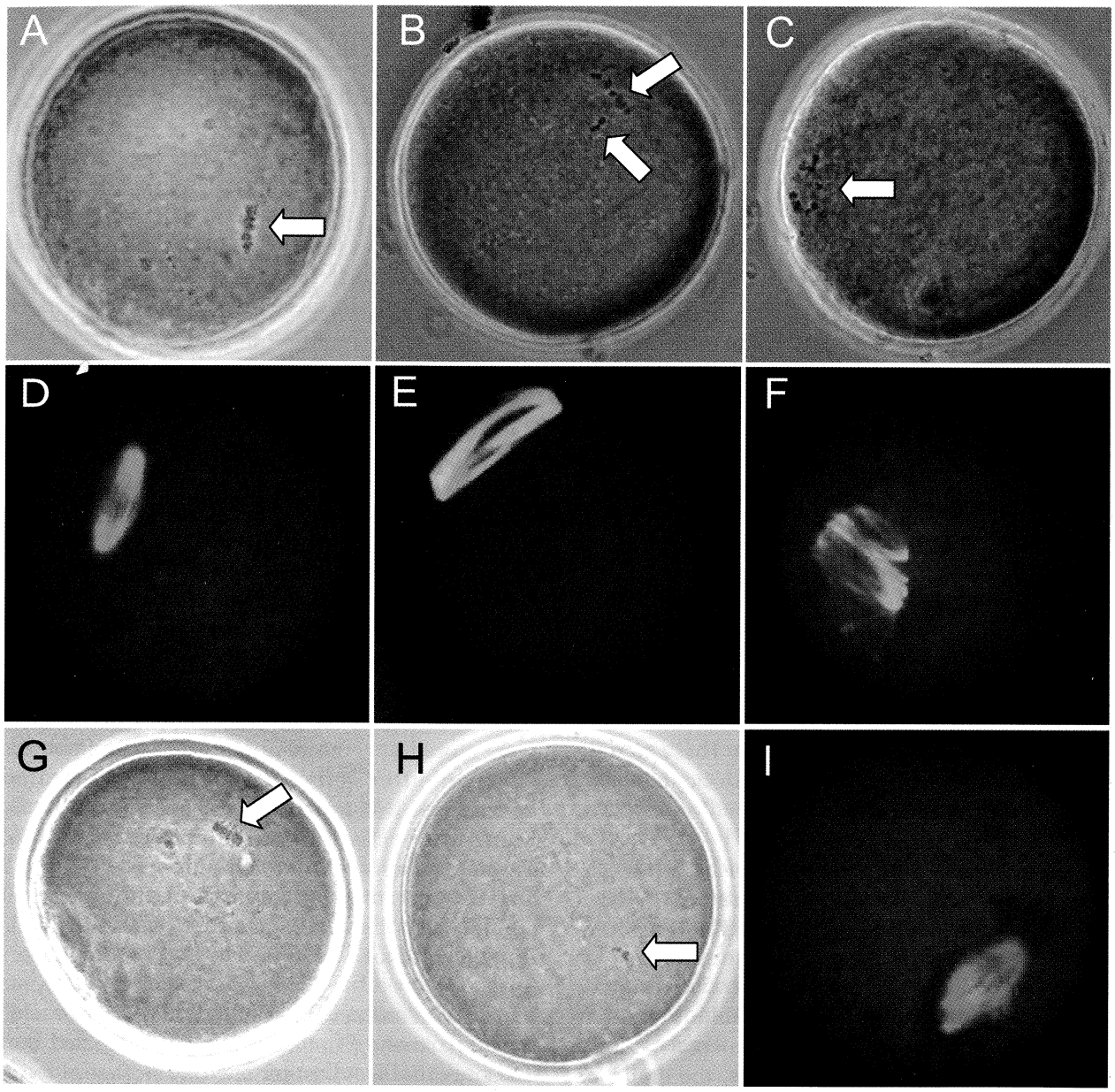

Fig. 2. Alignment of chromosomes and spindles of oocytes at the second metaphase after one-day storage at various temperatures. Stored, reconstructed and fresh oocytes were examined by light and confocal microscopy. (A and D) fresh oocytes; (B, C, E and F) stored oocytes. Most stored oocytes had misaligned chromosomes (B and C), and abnormal spindles such as elongated (E) or disrupted (F). The spindles of stored oocytes were transferred into enucleated fresh oocytes (G, H, I). A reconstructed oocyte showed relatively normal chromosomal alignment (G). However, a few chromosomes were found to remain in the cytoplasm of enucleated aged oocytes (H). Confocal images show that all reconstructed oocytes exhibit abnormal spindle formation (I).

after ICSI, and three (4%) developed to full term after embryonic transfer (Fig. 3F).

MII transfer into fresh oocytes

To rescue the spindle configuration of stored oocytes, MII chromosomes and spindles of stored oocytes at room temperature were transferred into fresh enucleated oocytes. Due to the difficulty of the technique, the success rate of spindle transplantation into fresh oocytes was very low

Table 2. Preimplantation development of mouse diploid parthenogenetic oocyte stored at 4, 27 and 37 C for 24 h

Storage temperature (C)	Storage medium	No. of oocytes treated	No. of oocytes				No. (%)* of embryos developing to each stage			
			Dead	Activated						
				Total	1 PN	2 PN	1-cell	2-cell	8-cell	Mol-Bla
Fresh	KSOM	100	10	90 (90)	0	90	0	0	1	89 (99)
4	KSOM	146	8	138 (95)	133	5	114	5	18	1 (1)
	CZB-H	155	36	119 (77)	107	12	87	14	2	16 (13)
	TYH	60	0	60 (100)	60	0	60	0	0	0 (0)
	Total	361	44	317 (88)	300	17	291	19	20	17 (5)
27	KSOM	118	20	92 (83)	0	92	36	14	16	26 (28)[a]
	CZB-H	102	39	63 (62)	4	59	40	3	10	6 (10)[b]
	TYH	97	2	56 (98)	0	56	6	11	7	32 (33)[a]
	Total	317	61	211 (81)	4	207	82	28	33	64 (20)
37	KSOM	97	76	21 (22)	0	21	12	7	2	0 (0)
	CZB-H	98	79	19 (19)	0	19	19	0	0	0 (0)
	TYH	65	61	4 (93)	0	4	1	1	1	0 (0)
	Total	260	216	44 (17)	0	44	32	8	3	0 (0)

Percentages with different superscripts in the same stages were significantly different ($P < 0.05$).
Number of embryos developing to each stage was represented by the results at the end of culture.
*: Percentage of activated oocytes.

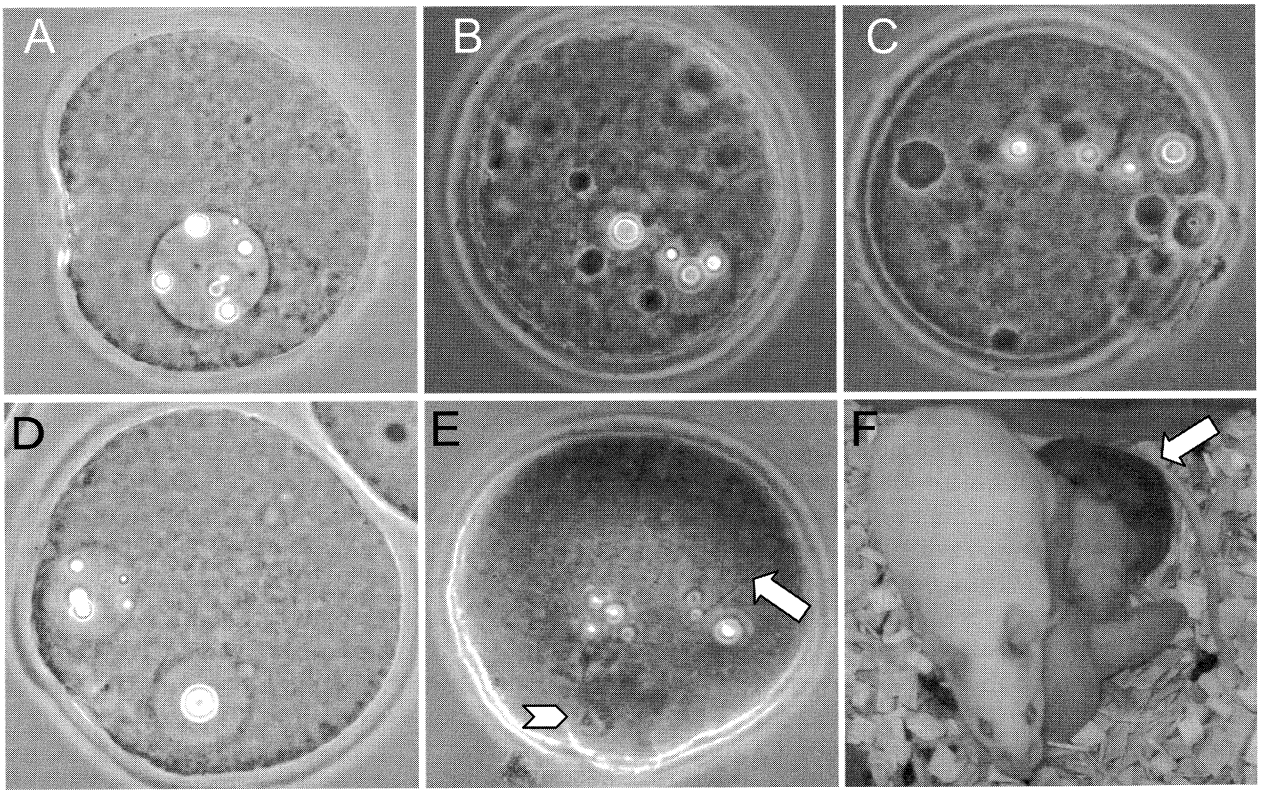

Fig. 3. Pronuclear formations observed in oocytes stored at various temperatures after IVF, ICSI or parthenogenetic activation and newborns after transfer of fertilized embryos. Stored oocytes were activated by Sr^{2+} with cytochalasin B (A to D), or fertilized by IVF (E). Only one pronucleus was formed in the oocyte stored at 4 C (A). Two pronuclei were formed in oocytes stored at room temperature (B) and in fresh oocytes (D). More than three pronuclei were formed in oocytes stored at 37 C (C). Stored oocytes were fertilized with fresh spermatozoa by IVF (E). An arrow indicates the tail of the spermatozoon, and an arrowhead indicates the second polar body. Offspring derived from oocytes stored at room temperature for one day (F). Offspring were taken from a surrogate mother by Caesarian section and raised by a foster mother.

Table 3. *In vitro* and *in vivo* development of oocytes fertilized by IVF or ICSI after storage at 27 C for 24 h

Storage period	Method of fertilization	No. of oocytes used	No. of oocytes fertilized	No. (%) of embryos developing to each stage				No of transferred embryos (recipients)	No. of offsprings (%) [female:male]
				1-cell	2-cell	4- to 8-cell	Morulae/ Blastocyst		
Fresh	ICSI	28	25 (89)	1	0	0	24 (96)	24 (2)	12 (50) [5:7]
24 h	IVF	208	90 (43)	0	52	0	38 (42)	38 (3)	9 (10) [8:1]
24 h	ICSI	115	85 (74)	14	15	0	56 (66)	56 (4)	3 (4) [3:0]

(24/63, 38%). Light and confocal microscopy of 16 reconstructed oocytes revealed that relatively normal chromosomal alignment was observed in a few oocytes (Fig. 2G). In the other reconstructed oocytes abnormality of chromosomal alignment (Fig. 2I) was comparable to non-reconstructed stored oocytes (Figs. 2E and F). After reconstruction, we also observed enucleated stored oocytes and found that a few chromosomes were left inside the cytoplasm (Fig. 2H). The remaining eight reconstructed oocytes were injected with fresh spermatozoa. Seven oocytes survived, but none of them developed to the morula/blastocyst stage. These results suggest that fresh oocyte cytoplasm cannot rescue stored oocyte spindles.

Discussion

Gamete preservation has been very successful in humans and some farm animals [16, 17, 29]. It has been also used as a means of maintaining large numbers of transgenic mouse strains [30]. Conventional gamete preservation is very expensive in the long term because of the need for a constant supply of liquid nitrogen. Maintaining rapidly increasing numbers of transgenic mouse strains is becoming more expensive every year. It is very important to find new and inexpensive methods of gamete preservation. Such methods would also be useful for short-term gamete preservation, such as that required for transportation over short distances [20] or for synchronization of embryo stage to the surrogate mother. In these cases, temporal gamete preservation without freezing would be useful. For the reasons, we are studying simple methods of maintaining gametes at room temperature [16, 20]. This report describes the first successful attempt, to our knowledge, to obtain live offspring from one-day-old mouse oocytes stored at room temperature, without freezing.

Abnormalities in the metaphase II spindle of stored oocytes, including misaligned chromosomes, or dispersed, elongated, or completely disrupted spindles, were commonly observed (Figs. 2E and F) by light and confocal microscopy. Such abnormalities, we thought, would disrupt the ability of aged oocytes to extrude normally the second polar body, resulting in aneuploidy after fertilization. To determine whether the poor developmental capacity of the stored oocyte was caused by cytoplasmic deterioration or spindle/nuclear damage, or both, the developmental capacity of stored oocytes after parthenogenetic activation and CB treatment was firstly examined. The oocytes were prevented from extruding the second polar body, thus resulting in a normal number of chromosomes. Therefore, it might be possible to reduce the effect of abnormal chromosomal arrangement on embryonic development. When oocytes were stored at 4 C in a refrigerator, most oocytes had a water bubble in the cytoplasm, and none were considered normal, but some of these oocytes (<13%) did develop to the morula/blastocyst stages. These results demonstrate that even some oocytes with water bubbles are able to develop to the morula/blastocyst stages. On the other hand, when oocytes were stored at 37 C in an incubator, 64 to 83% of oocytes were externally normal, but none developed to the morula/blastocyst stage after parthenogenetic activation. Tsuchiya *et al.* [21] reported that, when oocytes were stored at 5C for one day, 79% of stored oocytes were normal and 30% of stored oocytes developed to the morula/blastocyst stages after *in vitro* fertilization. However, they did not specifically categorize the oocytes by gross morphology as we did; therefore, their designation of "normal" may differ certainly

from ours. The contradiction between Tsuchiya's and our results might reflect the difference of storage media, since they used PBS with 33.3% FCS and we used TYH, CZB-H, and KSOM media with 0 to 0.5% BSA. Although our results showed no effect of storage media on the quality of stored oocytes, a high concentration of serum in the medium may be important for oocyte preservation at 4 C. Since they did not transfer these embryos to surrogate females, the developmental potential of the embryos was unclear.

The best results were obtained from oocytes stored at room temperature for one day. More than 80% of the oocytes were externally judged as normal, and 10 to 60% of parthenogenetic diploid embryos developed to the morula/blastocyst stages after treatment with Sr^{2+} and cytochalasin B. Therefore, oocytes stored at room temperature for one day were used for evaluation of the potency of fertilization and following development. Why room temperature is better than 4 C or 37 C for oocyte preservation? Probably, the cold temperature saves energy of stored oocytes and decreases the process of metabolism. However, as shown in Fig. 2C, when oocytes were stored at 4 C, they were irreversibly damaged by the polymerization of microtubules and could not continue the second meiosis after fertilization. Interestingly, we could not find any difference in the oocyte quality among storage media, however, the potential of *in vitro* development after parthenogenetic activation was significantly reduced when CZB-H medium was used. In general, Hepes buffered medium has been used and improved for *in vitro* culture without CO_2 gas, but it is also known that Hepes buffer adversely affects embryo development *in vivo* [31]. The oocytes stored in CZB-H for 24 h exhibited a quite normal surface, but it was damaged in capacity of development.

When the oocytes stored at room temperature were fertilized *in vitro* or subjected to ICSI with fresh spermatozoa, 42% and 66% of fertilized embryos developed to the morula/blastocyst stages, and 24% and 5% of transferred embryos developed to full term, respectively. To our knowledge, this is the first example of a full-term birth from one-day-old oocytes. Fertilization by IVF was more difficult than by ICSI, perhaps because hardening of the zonae pellucida in the aged oocyte [32–34] made the entry of spermatozoa difficult. The developmental potency of aged oocytes to the morula/blastocyst was not different between the two methods. However, IVF embryos developed to full term much more frequently than ICSI embryos after transfer. The aberrant MII spindle morphology in aged oocytes suggests that the stored oocyte may extrude the second polar body with an abnormal complement of chromosomes due to non-disjunction during meiosis II [35] after fertilization. The resulting embryos are aneuploidy and, therefore, only 24% of morula/blastocysts derived from IVF could develop to full term. On the other hand, when stored oocytes were fertilized by ICSI, many were damaged by the injection of spermatozoa under microscopic control and were thus more likely to extrude an abnormal second polar body. Our preliminary experiments showed that triploid embryos developed to the morula/blastocyst stage with the same degree of diploid parthenogenetic embryos (unpublished observation). Therefore, aneuploid or triploid embryos fertilized by ICSI developed to the morula/blastocyst stages, but only a few embryos reached to term. Similar abnormalities of the oocyte spindle were reported when aged females or senescence-accelerated mice were used [36–38]. Thus, *in vitro* aged oocytes may be a valuable model to study aging-associated infertility.

The substitution of the cytoplasm of stored oocytes for that of a fresh one would appear to be a promising way to reduce the incidence of aneuploidy due to chromosomal misalignment and abnormal spindle formation. Our previous data and those of many others suggest that spindle transplantation itself does not increase the incidence of aneuploidy, and fresh mature oocyte cytoplasm can support normal meiotic division of a transferred spindle [26, 28, 38–41]. In one study, a first polar body undergoing apoptotic death and harboring widely dispersed chromosomes was transplanted into an enucleated oocyte. Even this grossly abnormal spindle could be rescued by nuclear transplantation, and the resulting reconstructed oocyte was able to develop to full term after fertilization [42]. However, in this study, only a few reconstructed oocytes showed a relatively normal chromosomal lineup, and confocal microscopy revealed abnormal spindles even in these (Fig. 2I). Furthermore, none of these oocytes developed to full term after ICSI. It may be

possible that spindle damage caused by *in vitro* aging cannot be rescued by nuclear transplantation due to irreversible damage such as non-disjunction or premature disjunction of sister chromosomes.

In conclusion, we demonstrated that although most *in vitro* aged oocytes have irreversible damage in the metaphase II spindle rather than in the cytoplasm, some retain full developmental potential after storage for one day at room temperature. Thus, it may be possible to develop simple methods to preserve oocytes for long periods of time without freezing.

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References

1. **Fraser LR.** Rate of fertilization in vitro and subsequent nuclear development as a function of the post-ovulatory age of the mouse egg. *J Reprod Fertil* 1979; 55: 153–160.
2. **Ishikawa H, Omoe K, Enda A.** Compensatory development in preimplantation mouse embryos derived from delayed mating. *Biol Reprod* 1992; 47: 782–784.
3. **Ishikawa H, Omoe K, Endo A.** Growth and differentiation schedule of mouse embryos obtained from delayed matings. *Teratology* 1992; 45: 655–659.
4. **Lewis WH, Wright ES.** On the early development of the mosue egg. *Carnegie Inst Contrib Embryol* 1935; 25: 113–143.
5. **Santalo J, Badenas J, Calafell JM, Catala V, Munne S, Egozcue J, Estop AM.** The genetic risks of in vitro fertilization techniques: the use of an animal model. *J Assist Reprod Genet* 1992; 9: 462–474.
6. **Mailhes JB, Young D, London SN.** Postovulatory ageing of mouse oocytes in vivo and premature centromere separation and aneuploidy. *Biol Reprod* 1998; 58: 1206–1210.
7. **Marston JH, Chang MC.** The Fertilizable Life of Ova and Their Morphology Following Delayed Insemination in Mature and Immature Mice. *J Exp Zool* 1964; 155: 237–251.
8. **Tarin JJ, Perez-Albala S, Aguilar A, Minarro J, Hermenegildo C, Cano A.** Long-term effects of postovulatory aging of mouse oocytes on offspring: a two-generational study. *Biol Reprod* 1999; 61: 1347–1355.
9. **Witsch E.** Overripenessof the egg as a case of twinning and teratogenesis. *Cancer Res* 1952; 12: 763–786.
10. **Lanman JT.** Delays during reproduction and their effects on the embryo and fetus. 2. Aging of eggs. *N Engl J Med* 1968; 278: 1047–1054.
11. **Sakai N, Endo A.** Effects of delayed mating on preimplantation embryos in spontaneously ovulated mice. *Gamete Res* 1988; 19: 381–385.
12. **Exley GE, Tang C, McElhinny AS, Warner CM.** Expression of caspase and BCL-2 apoptotic family members in mouse preimplantation embryos. *Biol Reprod* 1999; 61: 231–239.
13. **Morita Y, Tilly JL.** Oocyte apoptosis: like sand through an hourglass. *Dev Biol* 1999; 213: 1–17.
14. **Perez GI, Tao XJ, Tilly JL.** Fragmentation and death (a.k.a. apoptosis) of ovulated oocytes. *Mol Hum Reprod* 1999; 5: 414–420.
15. **Gordo AC, Rodrigues P, Kurokawa M, Jellerette T, Exley GE, Warner C, Fissore R.** Intracellular calcium oscillations signal apoptosis rather than activation in in vitro aged mouse eggs. *Biol Reprod* 2002; 66: 1828–1837.
16. **Wakayama T, Whittingham DG, Yanagimachi R.** Production of normal offspring from mouse oocytes injected with spermatozoa cryopreserved with or without cryoprotection. *J Reprod Fertil* 1998; 112: 11–17.
17. **Nakagata N.** Cryopreservation of mouse spermatozoa. *Mamm Genome* 2000; 11: 572–576.
18. **Watson PF.** Artificial insemination and the preservation of semen. Marshail's Physiology of Reproduction Vol 2. Lamming EG (ed), London. Churchill Livingstone, 1990; 746–869.
19. **Tateno H, Wakayama T, Ward WS, Yanagimachi R.** Can alcohol retain the reproductive and genetic potential of sperm nuclei? Chromosome analysis of mouse spermatozoa stored in alcohol. *Zygote* 1998; 6: 233–238.
20. **Wakayama T, Yanagimachi R.** Development of normal mice from oocytes injected with freeze-dried spermatozoa. *Nat Biotechnol* 1998; 16: 639–641.
21. **Tsuchiya H, Ogonuki N, Kuwana T, Sankai T, Kanayama K.** Short-term preservation of mouse oocytes at 5 degrees C. *Exp Anim* 2001; 50: 441–443.
22. **Chatot CL, Lewis JL, Torres I, Ziomek CA.** Development of 1-cell embryos from different

strains of mice in CZB medium. *Biol Reprod* 1990; 42: 432–440.

23. **Toyoda Y, Yokoyama M, Hoshi T.** Studies on the fertilization of mouse eggs in vitro. *Jpn Anim Reprod* 1971; 16: 152–157.
24. **Wakayama T, Maruyama Y, Imamura K, Fukuta K.** Penetration of the zona pellucida of eggs of different species by spermatozoa of the Japanese filed vole, Microtus montebelli. *J Reprod Develop* 1993; 39: 319–323.
25. **Bos-Mikich A, Whittingham DG, Jones KT.** Meiotic and mitotic Ca^{2+} oscillations affect cell composition in resulting blastocysts. *Dev Biol* 1997; 182: 172–179.
26. **Kimura Y, Yanagimachi R.** Intracytoplasmic sperm injection in the mouse. *Biol Reprod* 1995; 52: 709–720.
27. **Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R.** Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 1998; 394: 369–374.
28. **Wakayama T, Yanagimachi R.** The first polar body can be used for the production of normal offspring in mice. *Biol Reprod* 1998; 59: 100–104.
29. **Polge C.** Fertilizing capacity of bull spermatozoa after freezing at 79 degrees C. *Nature* 1952; 169: 626–627.
30. **Nakagata N.** Use of cryopreservation techniques of embryos and spermatozoa for production of transgenic (Tg) mice and for maintenance of Tg mouse lines. *Lab Anim Sci* 1996; 46: 236–238.
31. **Mahadevan MM, Fleetham J, Church RB, Taylor PJ.** Growth of mouse embryos in bicarbonate media buffered by carbon dioxide, hepes, or phosphate. *J In Vitro Fert Embryo Transf* 1986; 3: 304–308.
32. **Wolf DP, Hamada M.** Age-dependent losses in the penetrability of mouse eggs. *J Reprod Fertil* 1976; 48: 213–214.
33. **Nogues C, Ponsa M, Vidal F, Boada M, Egozcue J.** Effects of aging on the zona pellucida surface of mouse oocytes. *J In Vitro Fert Embryo Transf* 1988; 5: 225–259.
34. **Eppig JJ, Wigglesowrth K, O'Brien MJ.** Comparison of embryonic developmental competence of mouse oocytes grown with and without serum. *Mol Reprod Dev* 1992; 32: 33–40.
35. **Zackowski JL, Martin-Deleon PA.** Second meiotic nondisjunction is not increased in postovulatory aged murine oocytes fertilized in vitro. *In Vitro Cell Dev Biol* 1988; 24: 133–137.
36. **Battaglia DE, Goodwin P, Klein NA, Soules MR.** Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women. *Hum Reprod* 1996; 11: 2217–2222.
37. **Volarcik K, Sheean L, Goldfarb J, Woods L, Abdul-Karim FW, Hunt P.** The meiotic competence of in-vitro matured human oocytes is influenced by donor age: evidence that folliculogenesis is compromised in the reproductively aged ovary. *Hum Reprod* 1998; 13: 154–160.
38. **Liu L, Keefe DL.** Ageing-associated aberration in meiosis of oocytes from senescence-accelerated mice. *Hum Reprod* 2002; 17: 2678–2685.
39. **Obata Y, Kono T, Hatada I.** Gene silencing: maturation of mouse fetal germ cells in vitro. *Nature* 2002; 418: 497.
40. **Ogura A, Wakayama T, Suzuki O, Shin TY, Matsuda J, Kobayashi Y.** Chromosomes of mouse primary spermatocytes undergo meiotic divisions after incorporation into homologous immature oocytes. *Zygote* 1997; 5: 177–182.
41. **Ogura A, Suzuki O, Tanemura K, Mochida K, Kobayashi Y, Matsuda J.** Development of normal mice from metaphase I oocytes fertilized with primary spermatocytes. *Proc Natl Acad Sci U S A* 1998; 95: 5611–5615.
42. **Wakayama T, Yanagimachi R.** Fertilisability and developmental ability of mouse oocytes with reduced amounts of cytoplasm. *Zygote* 1998; 6: 341–346.