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—Original Article—

In vitro maturation of immature rat oocytes under simple culture conditions and subsequent developmental ability

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Abstract. Rat oocytes can be produced artificially by superovulation. Because some strains show low sensitivity to superovulation treatment, *in vitro* maturation is an alternative method to produce numerous matured oocytes. Furthermore, establishment of an *in vitro* maturation system with simple culture conditions is cost effective and leads to easy handling of oocytes. This study examined developmental ability of rat germinal vesicle (GV) oocytes maturing *in vitro* under simple culture conditions. Significantly different numbers of ovulated oocytes reached the second metaphase of meiosis (MII) among Jcl:Wistar (17.0), F344/Stm (31.0), and BN/SsNSlc (2.2) rats in whom superovulation was induced by pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin. However, similar numbers of GV oocytes were obtained from ovaries of PMSG-injected Wistar (27.7), F344 (34.7), and BN (24.7) rats. These GV oocytes were cultured *in vitro* in HTF, αMEM, and a 1:1 HTF + αMEM or TYH + αMEM mixture. High proportions of Wistar and F344 oocytes that matured to MII in αMEM were parthenogenetically activated by strontium chloride treatment (78% and 74%, respectively). Additionally, 10% of matured oocytes of both strains developed into offspring after intracytoplasmic sperm injection and embryo transfer to foster mothers. Although BN oocytes cultured in αMEM could be parthenogenetically activated and developed into offspring, the success rate was lower than that for Wistar and F344 oocytes. This study demonstrated that numerous GV oocytes were produced in rat ovaries by PMSG injection. This simple *in vitro* maturation system of immature oocytes could be further developed to maintain valuable rat strains experiencing reproductive difficulties.

Key words: Germinal vesicle oocytes, Intracytoplasmic sperm injection, Second metaphase of meiosis

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Embryos are generally produced using oocytes collected from the oviducts of superovulation-induced female laboratory animals. In rats, intraperitoneal injection of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) has been used to induce superovulation [1]. It was also reported that the effect of superovulation by PMSG and hCG injection was not dependent on the estrous cycle and age of rats [2, 3]. However, although it is possible to induce superovulation in most rat strains using this method, some strains show negative responses [4].

Numerous variously sized follicles, including oocytes, are contained in the mammalian ovary [5, 6]. Some of these oocytes mature and are then ovulated. In the strains that show negative responses to superovulation, it is important to optimize conditions for the injection of PMSG and hCG to achieve efficient superovulation. The *in vitro* maturation (IVM) technique [7] can be used as alternative method for producing embryos from such strains. Using this technique, a large number of immature oocytes in antral follicles can mature and be used to produce embryos and offspring. Schroeder and Eppig [8] reported the first mouse offspring obtained from oocytes cultured *in vitro*. In rat, several IVM studies have been also reported [9–11]. It is thought that the addition of growth factors and hormones to the culture medium is required for maturation of mammalian oocytes. The establishment of an IVM technique that uses a simple culture medium without additional factors for supporting oocyte maturation is useful and cost effective. Furthermore, the easy handling of oocytes using a simple IVM technique contributes to storing valuable animal strains, which experience reproductive difficulties, as genetic resources in biobanking. It was reported that rat oocytes collected from ovaries could be matured in MEM medium [12, 13], and these matured oocytes developed into offspring after fertilization [14]. However, no detailed studies addressing the influence of strain and medium components on oocyte maturation, fertilization, and subsequent development have been reported.

This study evaluated the maturation to second metaphase of meiosis (MII) of germinal vesicle (GV) oocytes collected from the ovaries of Wistar, F344, and BN rats using various culture media. Furthermore, the subsequent ability of these IVM oocytes to develop into offspring was studied.

Materials and Methods

Animals

Jcl:Wistar and BN/SsNSlc rats were purchased from CLEA Japan (Tokyo, Japan) and Japan SLC (Shizuoka, Japan), respectively. F344/Stm rats were supplied by the National BioResource Project-Rat, Kyoto University (Kyoto, Japan). All animals were housed in plastic cages in a specific pathogen-free barrier facility that was air-conditioned (temperature = 24 ± 2°C, humidity = 50 ± 10%) and

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light-controlled (lights on from 0700 h to 1900 h). All animal care and procedures performed in this study conformed to the Guidelines for Animal Experiments of Kyoto University and were approved by the Animal Research Committee of Kyoto University.

Collection and IVM of oocytes

Female Wistar, F344, and BN rats aged 8 to 12 weeks were induced to superovulate by intraperitoneal injection of PMSG (ASKA Pharmaceutical, Tokyo, Japan) at doses of 150, 150, and 300 IU/kg, respectively, at 1600–1700 h, followed by intraperitoneal injection of hCG (ASKA Pharmaceutical) at doses of 75, 75, and 300 IU/kg, respectively, 48 h later [15, 16]. The doses of PMSG and hCG injected to each strain were optimized by our preliminary experiments. Cumulus-oocyte complexes were collected from the oviducts at 16–18 h after hCG injection. Oocytes were freed from cumulus cells by treatment with 0.1% hyaluronidase in PB1 medium [17] for 5 min. Oocytes that reached MII were then recorded as matured.

Female Wistar, F344, and BN rats aged 8 to 13 weeks were induced to superovulate by intraperitoneal injection of PMSG at doses of 150, 150, and 300 IU/kg, respectively, at 1600–1700 h. Forty-eight hours after PMSG injection, ovaries were removed from the females and washed in PB1 medium. Antral follicles of the ovarian cortex were cut with a steel needle, and oocyte-granulosa cell complexes (OGCs) were collected in PB1 medium. After washing twice in PB1 medium, 10–20 OGCs were cultured *in vitro* in 50 µl drops of HTF, αMEM (Thermo Fisher Scientific, Waltham, MA, USA), a 1:1 mixture of HTF and αMEM (HTF + αMEM), or a 1:1 mixture of TYH and αMEM (TYH + αMEM) [18]. All media were supplemented with 3 mg/ml bovine serum albumin. Oocytes were cultured for 16 h in 5% CO_2 and humidified air at 37.5°C. The *in vivo* matured MII oocytes that were collected from oviducts of females at estrous stage without superovulation were used as control.

Parthenogenetic activation of oocytes after IVM

After culturing *in vitro*, artificial parthenogenetic activation was carried out with oocytes that reached MII. Oocytes were physically denuded with a glass capillary pipette. Oocytes were then cultured in HTF supplemented with 10 mM strontium chloride ($SrCl_2$; Sigma-Aldrich, St. Louis, MO, USA) for 21 h in 5% CO_2 and humidified air at 37.5°C [19]. After culturing, the oocytes that contained a distinct pronucleus were counted.

IVM oocyte fertilization

The developmental ability of oocytes that matured *in vitro* in various culture media was estimated by fertilization with sperm using intracytoplasmic sperm injection (ICSI) [16, 20]. Sperm that were cryopreserved in Tris-EDTA (TE) buffer (10 mM Tris and 1 mM ethylenediaminetetraacetic acid; Thermo Fisher Scientific/Ambion, Waltham, MA, USA) were used in this study [21]. Sperm were thawed and then sonicated for 5 sec using an ultrasonic cell disrupter (VP-050, TAITEC, Saitama, Japan) to separate the sperm head from the tail. A small volume (1–2 µl) of the sperm suspension was thoroughly mixed with a 5 µl drop of HEPES-mR1ECM [20] supplemented with 12% (w/v) polyvinylpyrrolidone (PVP, MW = 360.000; ICN Pharmaceuticals, Costa Mesa, CA, USA). A single, normally shaped sperm head was hung on the tip of an injection pipette. The sperm head was then immediately injected into the oocytes in HEPES-mR1ECM. After sperm injection, the oocytes were cultured in modified Krebs-Ringer bicarbonate medium [22] in 5% CO_2 and humidified air at 37.5°C.

Embryo transfer

After 20 h of culturing, pronuclear- and 2-cell-stage embryos were transferred into the oviducts of female Jcl:Wistar rats that had been mated with vasectomized male rats of the same strain on the day before the transfer. Three or four females were used in each experiment. The numbers of implantation sites and offspring were assessed after 19 to 21 days of gestation.

Statistical analysis

The resulting data were analyzed using Tukey-Kramer method and chi-square test followed by multiple comparison using Ryan's method.

Results

The number of MII oocytes that ovulated by injection of PMSG and hCG are shown in Fig. 1A. The number of MII oocytes collected from Wistar and F344 rats were 17.0 and 31.0, respectively. The number of oocytes collected from BN rats (2.2) was significantly lower than that of F344 rats. However, similar numbers of OGCs were obtained from PMSG-injected Wistar (27.7), F344 (34.7), and BN (24.7) rats (Fig. 1B).

Table 1 shows the developmental ability of Wistar oocytes collected from ovaries of PMSG-injected females after culturing *in vitro* in various media. Five females were used in each experiment. The percentage of oocytes that were pronuclear upon activation with $SrCl_2$ was significantly higher after they were cultured in αMEM (78%) and TYH + αMEM (75%) compared with those that were cultured in HTF (34%) and HTF + αMEM (54%). There was no significant difference in the percentage of activated oocytes among the oocytes cultured in αMEM or TYH + αMEM and those that matured *in vivo* (91%).

The oocytes collected from ovaries of PMSG-injected female F344 rats were cultured *in vitro* in αMEM and TYH + αMEM (Table 2). Three females were used in each experiment. The percentage of activated oocytes was not significantly different among the oocytes that were cultured in αMEM (74%) or TYH + αMEM (78%) and those that matured *in vivo* (68%). Table 3 shows the developmental ability of BN oocytes collected from ovaries of PMSG-injected females after culturing *in vitro* in αMEM and TYH + αMEM. Three females were used in each experiment. The results also showed that there was no significant difference in the percentage of oocytes that were pronuclear among the oocytes cultured in αMEM (30%) or TYH + αMEM (23%) and those that matured *in vivo* (46%).

The oocytes from each strain that were cultured *in vitro* were fertilized with sperm from the same strain. The fertilized oocytes were then transferred to foster mothers. Offspring were obtained from fertilized Wistar oocytes that matured in all media types (Table 4). The percentage of fertilized oocytes matured in αMEM that developed into offspring (10%) was high. Additionally, there was no significant difference in the development into offspring of oocytes that matured in various media and of those that matured *in*

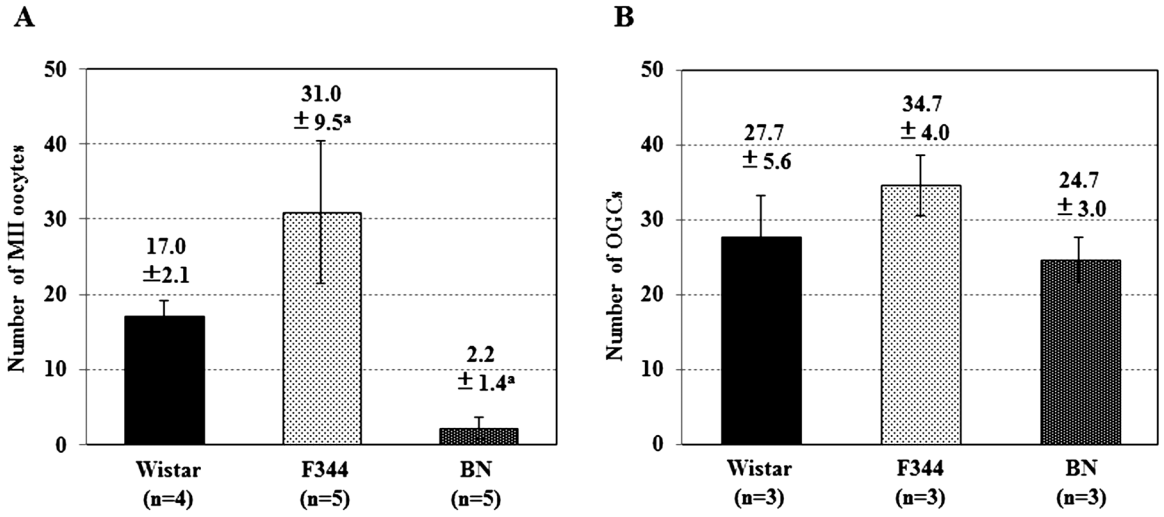

Fig. 1. (A) Number of MII oocytes collected from oviducts of females that were superovulated by injection of PMSG and hCG. (B) Number of OGCs collected from ovaries of females 48 h after injection of PMSG. All data shown represent the mean ± SE. [a] Significantly different at $P < 0.05$ (Tukey-Kramer method).

Table 1. Developmental ability of Wistar oocytes matured *in vitro* in various culture media

Culture media	No. of oocytes collected from ovaries	No. (%) of oocytes matured to MII	No. of MII oocytes activated	No. (%) of oocytes after activation		
				Pronuclear stage	MII stage (non-activated)	Degenerated and fragmented
In vivo	-	-	32	29 (91) [c]	3 (9)	0 (0)
HTF	109	100 (92) [a]	100	34 (34) [d]	50 (50)	16 (16)
αMEM	137	110 (80) [b]	110	86 (78) [e]	17 (15)	7 (6)
HTF + αMEM	183	169 (92) [a]	169	92 (54) [f]	63 (37)	14 (8)
TYH + αMEM	168	142 (85)	142	107 (75) [g]	27 (19)	8 (6)

The percentages of oocytes that matured to MII and those that formed a pronucleus after activation were statistically analyzed. $P < 0.05$: a *vs*. b; c *vs*. d and f; d *vs*. e, f, and g; e *vs*. f; f *vs*. g (chi-square test followed by multiple comparison using Ryan's method).

Table 2. Developmental ability of F344 oocytes matured *in vitro* in various culture media

Culture media	No. of oocytes collected from ovaries	No. (%) of oocytes matured to MII	No. of MII oocytes activated	No. (%) of oocytes after activation		
				Pronuclear stage	MII stage (non-activated)	Degenerated and fragmented
In vivo	-	-	47	32 (68)	1 (2)	14 (30)
αMEM	97	69 (71)	69	51 (74)	9 (13)	9 (13)
TYH + αMEM	102	64 (63)	64	50 (78)	6 (9)	8 (13)

Table 3. Developmental ability of BN oocytes matured *in vitro* in various culture media

Culture media	No. of oocytes collected from ovaries	No. (%) of oocytes matured to MII	No. of MII oocytes activated	No. (%) of oocytes after activation		
				Pronuclear stage	MII stage (non-activated)	Degenerated and fragmented
In vivo	-	-	28	13 (46)	2 (7)	13 (46)
αMEM	128	92 (72)	92	28 (30)	63 (68)	1 (1)
TYH + αMEM	141	94 (67)	94	22 (23)	69 (73)	3 (3)

Table 4. Development into offspring of Wistar IVM oocytes after fertilization with sperm

Culture media	No. of fertilized oocytes transferred	No. (%) of embryos implanted	No. (%) of embryos that developed into offspring
In vivo	54	16 (30)	5 (9)
HTF	73	12 (16)	1 (1)
αMEM	72	20 (28) [a]	7 (10)
HTF + αMEM	80	21 (26)	4 (5)
TYH + αMEM	116	13 (11) [a]	4 (3)

[a] Significantly different at $P < 0.05$ (chi-square test followed by multiple comparison using Ryan's method).

Table 5. Development into offspring of F344 IVM oocytes after fertilization with sperm

Culture media	No. of fertilized oocytes transferred	No. (%) of embryos implanted	No. (%) of embryos that developed into offspring
In vivo	52	20 (38)	12 (23) [a]
αMEM	96	21 (22)	10 (10)
TYH + αMEM	67	15 (22)	4 (6) [a]

[a] Significantly different at $P < 0.05$ (chi-square test followed by multiple comparison using Ryan's method).

Table 6. Development into offspring of BN IVM oocytes after fertilization with sperm

Culture media	No. of fertilized oocytes transferred	No. (%) of embryos implanted	No. (%) of embryos that developed into offspring
In vivo	ND	ND	ND
αMEM	110	14 (13)	2 (2)
TYH + αMEM	61	10 (16)	1 (2)

ND: No data. The number of oocytes that ovulated after superovulation was too low for fertilization.

vivo (9%). Offspring were also obtained from fertilized F344 oocytes after maturing in different media (Table 5). There was no significant difference in the development into offspring of fertilized oocytes after maturing in αMEM (10%) and of those that matured *in vivo* (23%). However, the development into offspring of fertilized oocytes after maturing in TYH + αMEM (6%) was significantly lower than those that matured *in vivo* or in αMEM. Furthermore, as shown in Table 6, only 2% of BN oocytes matured in αMEM and TYH + αMEM developed into offspring.

Discussion

This study evaluated the developmental ability of immature rat oocytes under simple *in vitro* culture conditions. It has been reported that superovulation in Wistar and F344 rats can be induced by injecting PMSG and hCG [1, 15]. On the contrary, it is empirically known that the number of BN oocytes that ovulated by induction of superovulation is extremely low. Indeed, the number of MII oocytes that ovulated after injection of PMSG and hCG was significantly different between the F344 and BN rats (Fig. 1A). However, a similar number of OGCs was obtained from the ovaries after injection of PMSG among Wistar, F344, and BN rats (Fig. 1B). The results of this study indicate that a large number of follicles, including oocytes, were also present in BN rat ovaries [5, 6]. The development of these follicles was stimulated by injection of PMSG. In this study, the sensitivity of BN rats to hCG injection at 48 h after PMSG injection was extremely low. Although it has been reported that the effect of superovulation by PMSG and hCG injection was not dependent on the estrous cycle and age of rats [2, 3], a detailed study on such dependence to different factors is required in BN rats. Kon *et al*. [3] injected hCG to females at 55 h after PMSG injection by changing time interval of hCG injection. Synchronizing the hCG injection to the endogenous luteinizing hormone surge might improve the ovulation of oocytes [23, 24].

OGCs collected from ovaries of Wistar rats after injection of PMSG were cultured *in vitro* in HTF, αMEM, HTF + αMEM, and TYH + αMEM. A high proportion of oocytes matured to MII in all media. The percentage of oocytes that were parthenogenetically activated was high in αMEM and TYH + αMEM (Table 1), which suggests that αMEM and the addition of αMEM to other culture media strongly support the *in vitro* maturation of oocytes. Furthermore, the percentage of oocytes that were parthenogenetically activated after IVM showed no significant difference compared with the oocytes that matured *in vivo*. However, our result shows that the components of HTF were insufficient for IVM of immature rat oocytes. Similar results were also obtained in the F344 rats (Table 2). van de Sandt *et al*. studied the fertilization and subsequent development to offspring of mouse oocytes that matured in various culture media and found that the media used for IVM of mouse oocytes affect the subsequent development of the oocytes [25]. Our present study also showed

that the maturation of rat oocytes was affected by the components of culture medium.

The development into offspring of Wistar oocytes after culturing *in vitro* in αMEM was high and was not significantly different compared with that of the oocytes that matured *in vivo* (Table 4). Although the oocytes that were cultured *in vitro* in αMEM and TYH + αMEM were successfully parthenogenetically activated, the percentage of development into offspring of oocytes cultured in TYH + αMEM was lower than that of oocytes cultured in αMEM (Tables 1 and 4). Similar results were also obtained in the F344 rats (Tables 2 and 5). These results indicate that αMEM strongly promotes the maturation of rat oocytes. In the maturation of oocytes using TYH + αMEM, germinal vesicle breakdown, polar body formation, and parthenogenetic activation occurred normally (Tables 1 and 2). However, the development into offspring of the oocytes that underwent IVM after fertilization was not successful (Tables 4 and 5). This may be caused by the incomplete cytoplasmic maturation of the oocyte [26]. Miki *et al*. reported that mouse cumulus-free oocytes could mature *in vitro* in αMEM and TYH + αMEM and that the development of oocytes cultured in TYH + αMEM was slightly greater than those cultured in αMEM [18]. It is possible that various factors, such as differences in species and the interaction between granulosa and cumulus cells, caused this discrepancy in results. In this study, rat GV oocytes were cultured with the enclosing granulosa and cumulus cells. Cumulus cells are beneficial for maturation of oocyte cytoplasm, fertilization, and subsequent development [14]. It is thought that the addition of growth factors and hormones to the culture medium are required for maturation of mammalian oocyte. However, Vanderhyden and Armstrong [9] reported that the addition of follicle-stimulating hormone at low concentrations (1 to 10 ng/ml) to the maturation media decreased the fertility of rat oocytes. Although suitable culturing conditions for IVM may vary for each species, IVM of oocytes without the addition of growth factors and hormones is useful and cost effective. Furthermore, the easy handling of oocytes using a simple IVM technique contributes to storing valuable animal strains, which experience reproductive difficulties, as genetic resources in biobanking.

The results of this study showed that the development into offspring of *in vitro* matured BN oocytes was low (Table 6). It was thought that BN oocytes could not be fully matured in the culture media used in this study [26]. However, the number of oocytes collected from ovaries after injection of PMSG was significantly higher than the number of oocytes that ovulated by induction of superovulation (Fig. 1). BN rats are a valuable strain, and the sequence of over 90% of its genome was reported [27]. Further improvement of the IVM technique could lead to efficient production of BN rats and various other animals that experience reproductive difficulties.

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