Evaluation of Pre-maturity of Mouse Oocytes Ovulated from Prepubertal Females using an In Vitro Fertilization Technique

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Abstract: The cytogenetic normality and developmental competence of mouse oocytes derived from prepubertal females were investigated to determine ooplasmic maturity after in vitro fertilization (IVF) and to examine the ability of the resultant blastocysts to develop to term. To estimate the effect of body weight and age on ovulation, prepubertal female (BALB/c × C57BL/6J) F_1 mice, 20–30 days of age, were classified into two groups according to body weight as follows: a light group (L) of 8.5–12 g and a heavy (H) group of 13–16 g. The IVF blastocysts were fixed as chromosome samples, and some of the blastocysts were transferred to pseudopregnant recipients. The implantation rates and number of fetuses were subsequently evaluated at 20 days post-coitus. The average number of ovulated oocytes differed significantly, with 46.2, 18.1, and 37.1 in the H, L, and control groups, respectively. All fertilization rates were high, and there were no significant differences. However, 16.8% of zygotes from the L group were arrested at the 1-cell stage, with mostly male premature condensed chromosomes (PCC). The rate of development to the blastocyst stage was significantly low in the L group (36.6%). The rates of implantation and development to newborns were significantly lower in the H group than in the pubertal mice.

Key words: Chromosomal abnormality, Embryo transfer, In vitro fertilization, Premature chromosome condensation, Prepubertal mouse

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Introduction

In vitro production of embryos from prepubertal experimental animals has been used to reduce the generation interval [1, 2], to provide an economic and efficient source of viable oocytes and embryos [3], and to offer models of immature oocytes for humans [4]. Prepubertal mice can be induced to ovulate a greater number of oocytes than pubertal mice by means of superovulation treatments with equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) [5]. Thus, they have often been used as oocyte donors. However, it has been reported that triploidy is frequently observed at first cleavage in *in vitro* fertilization (IVF) of oocytes ovulated from prepubertal mice [5, 6]. Moreover, the frequency of hyperploid embryos is higher in prepubertal mice [5, 7]. Many physiological [8] and cytogenetic problems [4] have been reported in oocytes ovulated from prepubertal mice; however, little is known about the developmental competence of embryos after IVF and the cytogenetic normality of embryos at stages subsequent to first cleavage division.

Recently, iatrogenic infertility has been noticed in pediatric cancer patients treated with chemical therapy and/or radiotherapy [9]. There are many methods of rescuing patients from iatrogenic infertility, such as cryopreservation of oocytes [10] and/or ovaries [11] before cancer treatment. However, matured human oocytes have not been obtainable by *in vitro* culture of follicles isolated from cortical sections of cryopreserved ovaries until now [11]. We recommend harvesting oocytes from patients and sequential cryopreservation

of the oocytes before cancer treatment, as these oocytes will be usable for assisted reproductive technologies (ART) in the future. However, it is necessary to confirm the safety of the oocytes taken from prepubertal individuals for use with ART. It is especially important to verify their genetic safety experimentally. It was the aim of the present study to evaluate the cytogenetic normality, developmental competence to the blastocyst stage, and implantation ability of the resultant embryos derived from IVF of oocytes from prepubertal mice as a model experiment for the use of oocytes from pediatric cancer patients.

Materials and Methods

Mice

Prepubertal 20 to 30-day old (BALB/c × C57BL/6J) F_1 female mice and 3 to 4-month old adult mice were used in this study. To estimate the effect of body weight and age on ovulation, prepubertal mice were classified into two groups according to their body weight. These included a light group (L) of 8.5–12 g and a heavy group (H) of 13–16 g in weight. When mice were born, the number of pups in the litter was adjusted to 5 to reduce differences in the growth of individuals [12]. Rockefeller (RF) mice, which have 38 chromosomes containing one pair of homological Robertsonian translocation chromosomes (38 tr 5/15), were used as sperm donors. RF male mice have normal fertility, although females produce a slightly small litter size as compared with other strains of mice.

In vitro fertilization

RF male mice were euthanized by cervical dislocation, and spermatozoa from the bilateral caudal epididymides were squeezed into drops of TYH medium [13] containing 4 mg/ml bovine serum albumin (BSA) and 2 mM caffeine. The suspension of spermatozoa was incubated at 37°C in 5% CO_2 and 95% air for 2 h until transfer to drops of insemination medium. The suspension of spermatozoa was added to the insemination media at a concentration of 2,000 sperm/μl, which was ten times the conventional sperm concentration used in our previous reports [14]. A high concentration of sperm was employed to compare the ability to block polyspermic penetration between oocytes from prepubertal and adult mice. Females were injected intraperitoneally with 5 IU of eCG followed 48 h later by 5 IU of hCG to induce superovulation. Oocytes were obtained from the ampullae of oviducts 16 h after hCG injection. Oocytes were introduced into insemination media and cultured for 6 h in a CO_2 incubator. After 6-h incubation, they were classified into morphologically normal oocytes, fertilized oocytes with a second polar body or abnormal oocytes, oocytes with a small amount of cytoplasm, fragmented cytoplasm, or without a second polar body. Correlation between the number of morphologically normal oocytes and body weight was analyzed. Zygotes were transferred into Whitten 514 medium [15] containing 3 mg/ml BSA and were incubated to the blastocyst stage for 96 h.

Chromosome preparations

Chromosome preparations were made by the method of Yoshizawa *et al.* [16]. Preparations for some zygotes that did not cleave to the 2-cell stage were made 31 h after insemination without mitotic inhibitors, such as colcemid or vinblastine sulfate. Embryos that developed to the blastocyst stage were treated with 30 ng/ml vinblastine sulfate in Whitten 514 medium for 10 h, placed in a hypotonic solution of 1% sodium citrate for 15 min, slightly fixed with a small volume of a mixture of methanol 3 and acetic acid 1, and fixed individually on glass slides with the same fixative as the chromosome samples. The procedure for production of chromosome preparations was performed under heavy moisture from supersonic humidifiers. Each sample was air-dried for at least one week and C-banded by a previously described method [16]. The number of chromosomes was checked according to a previously described method [14].

Embryo transfer

Some of the blastocysts were surgically transferred into the uteri of day 4 pseudopregnant ICR foster mice. Each uterus was pierced with a 25 G needle to create a small hole, and blastocysts in a minimal volume of medium were injected into the uteri through the small hole with a fine glass pipette. Caesarean section was performed to deliver pups at 20 days post-coitus. However, in the case of the L group, embryo transfer could not be accomplished because of low productivity to the blastocyst stage. The livers of newborns from the blastocysts of prepubertal mice were fixed as chromosome preparations for diagnosis of their karyotypes.

Statistics

Statistical evaluation was performed using the chi-square test for the fertilization rate, incidences of chromosomal abnormalities, and successful rates of embryo transfer in each group. Data concerning the

Table 1. The numbers of oocytes ovulated, morphologically normal, and fertilized *in vitro*

	Pubertal mice	Prepubertal mice	
		L group*	H group*
No. of mice used	25	18	35
No. of mice ovulated	25	15	35
No. of ovulated oocytes (mean ± SD)	928 (37.1 ± 12.9[a])	325 (18.1 ± 12.5[b])	1,617 (46.2 ± 19.2[c])
No. of morphologically normal oocytes (%) (mean ± SD)	622 (67.0[a]) (24.9 ± 12.4[a])	324 (99.7[b]) (18.0 ± 14.6[a])	1,599 (98.9[b]) (45.7 ± 20.3[b])
No. of fertilized oocytes (%)	591 (95.0)	304 (93.8)	1,528 (95.6)

*The body weights of the mice were 8.5–12 g in the L group and 13–16 g in the H group. [a–c]: Values with different superscripts in the same row are significantly different (P<0.05).

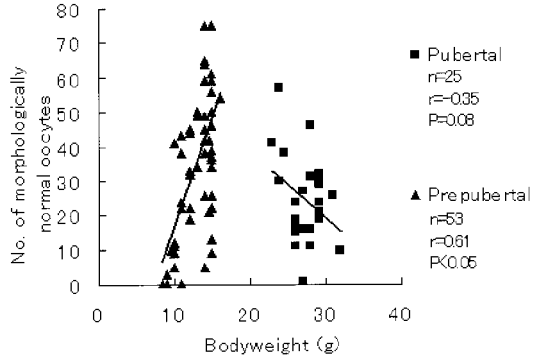

Fig. 1. Correlation between body weight and the number of morphologically normal oocytes in pubertal and prepubertal mice. Correlation was observed in prepubertal ($P=1.6 \times 10^{-6}$) but not pubertal mice (P=0.08).

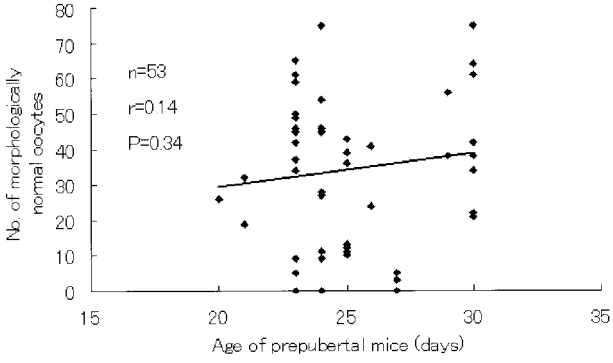

Fig. 2. Correlation between the age of prepubertal mice and the number of morphologically normal oocytes. There was no statistical correlation (P=0.34).

number of ovulated oocytes was subjected to one-way analysis of variance (ANOVA). P<0.05 was considered to be significant.

Results

Although all mice treated by administration of eCG and hCG in the pubertal and H groups ovulated, 3 of 18 mice in the L group did not ovulated (Table 1). The number of ovulated oocytes was significantly larger in the H group of prepubertal mice (46.2 oocytes/mouse) and significantly smaller in the L group of prepubertal mice (18.1) than in the pubertal mice (37.1) (P<0.05). The correlation coefficient between body weight and the number of ovulated oocytes was 0.61 in the prepubertal mice, and the P value was 1.6×10^{-6}, showing a significant correlation between body weight and the number of ovulated oocytes (Fig. 1). This correlation was not observed in the pubertal mice (r=–0.35, P=0.08). There was no significant correlation (r=0.14, P=0.34) between the age of the prepubertal mice and the number of ovulated oocytes (Fig. 2). The proportion of morphologically normal oocytes was significantly higher in the prepubertal mice (L, 99.7%; H, 98.9%) than in the pubertal mice (67.0%) (P<0.05). In IVF of morphologically normal oocytes, oocytes having a second polar body were assumed to be fertilized oocytes, and there were no statistical differences among the fertilization rates across all groups; all groups showed a high fertilization rate (93.8–95.6%).

In some experiments, especially in the L group, 51 of 304 zygotes (16.8%) were arrested at the 1-cell stage (Table 2). The rate of 1-cell arrested embryos in the L group was significantly higher than those of the H group (4.0%, 51/1528) and the pubertal (0.7%, 4/591) mice (P<0.01). We attempted to fix these arrested zygotes at

Table 2. Results of chromosomal analysis of 1-cell arrested zygotes after IVF

	Pubertal mice	Prepubertal mice	
		L group	H group
No. of oocytes fertilized	591	304	1,528
1-cell arrested zygotes (%)	4 (0.7[a])	51 (16.8[b])	51 (4.0[c])
Preparations	–	46	51
At the pronuclear stage		4	51
With PCC (%)		42 (91.3)	0
Analyzable embryos		33	
Diploid PCC		19 (57.6%)	
Haploid PCC		5 (15.2%)	
Triploid PCC		8 (24.2%)	
Tetraploid PCC		1 (3.0%)	

[a–c]: Values with different superscripts in the same row are significantly different (P<0.01).

Table 3. Rates of 2-cell embryos developed to the blastocyst stage

	Pubertal mice	Prepubertal mice	
		L group	H group
No. of 2-cell embryos	460	76	1,261
Blastocysts (%)	420 (91.3[a])	30 (39.5[b])	633 (50.2[c])
Degenerated embryos (%)	40 (8.7)	46 (60.5)	628 (49.8)

[a–c]: Values with different superscripts in the same row are significantly different (P<0.05).

31 h after insemination, and 46 of 51 zygotes were successfully produced as chromosome samples. Four (8.7%) of them showed two pronuclei, and the others (42 samples, 91.3%) showed premature condensed male chromosomes (PCC) and female chromosomes at the M-II stage (Fig. 3). PCC were observed only in 1-cell embryos from the L group; however, all embryos in the H group (51 embryos) stopped development at the pronuclear stage. Of the 42 embryos showing PCC, 33 were successfully analyzed. Of the 33 analyzable embryos in the L group, 19 (57.6%) showed normal diploidy, 5 (15.2%) were haploidy, 8 (24.2%) were triploidy and 1 (3.0%) was tetraploidy. The other samples could not be analyzed chromosomally because of overlap or lack of chromosomes.

In the *in vitro* fertilized embryos cultured for 96 h, the rate of development to the blastocyst stage was significantly lower in the L group (39.5%, 30/76) than the H group (50.2%, 633/1261), while the rate in the pubertal mice was much higher (91.3%, 420/460) (P<0.05, Table 3). Thus, body weight and puberty in the mouse could affect the number of ovulated oocytes and the developmental competence of *in vitro* fertilized oocytes.

The results of chromosomal analysis for the blastocysts of each group showed no statistical differences in the rate of mitosis (87.9–100%) and the rate of normal diploid blastocysts (86.3–91.5%) between the embryos from the pubertal and prepubertal mice (Table 4). Haploid blastocysts were derived from parthenogenesis showing 20 chromosomes without a translocation chromosome. All polyploid blastocysts possessed 2 or 3 translocation chromosomes originating from polyspermy (Fig. 4). Furthermore, mixoploidy (n/2n, Fig. 5; 2n/3n) was observed to a slight extent in each group. Haploid cells in mixoploids showed 19 chromosomes containing one translocation chromosome, and diploid cells in mixoploids showed signs of normal fertilization, i.e. 39 chromosomes containing one translocation chromosome. Thus, the embryos having chromosomal abnormalities were also capable of developing to the blastocyst stage.

After some of the blastocysts of the H and control groups were transferred to pseudopregnant ICR foster mice, their implantation rates and rates of development to term were checked at 20 days post-coitus by caesarean section. Consequently, as shown in Table 5, 38.3% (23/60) of all transferred blastocysts derived from pubertal mice were successfully implanted, and 33.3% (20/60) of the blastocysts developed to term. On the

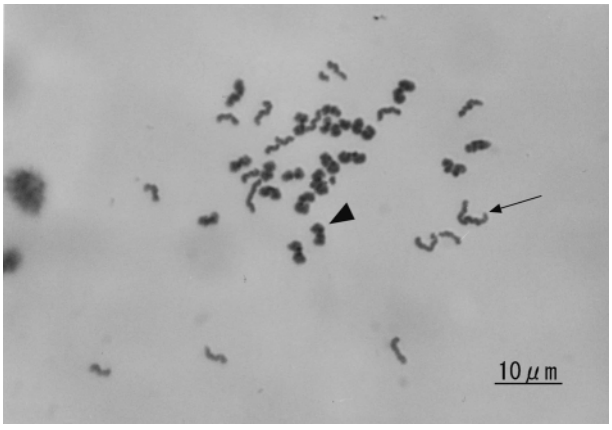

Fig. 3. Premature chromosome condensation (PCC) from a sperm and female chromosomes at the M-II stage in a 1-cell arrested embryo derived from a prepubertal mouse at 31 h after IVF. An arrow shows PCC of the translocation chromosome from a RF male mouse. Male chromosomes showing single chromatids as PCC are different from female chromosomes showing bivalent chromosomes at the M-II stage indicated by an arrowhead.

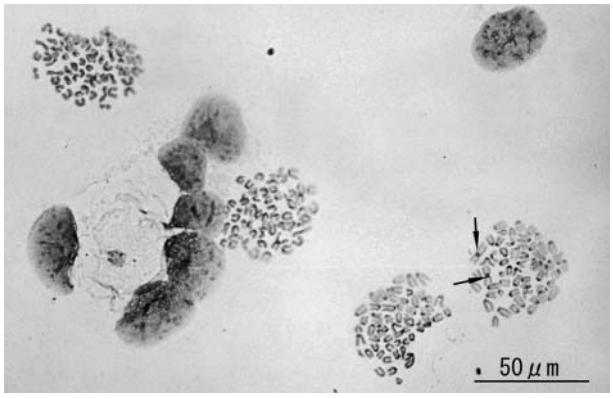

Fig. 4. Sample chromosome from a triploid blastocyst at 96 h after insemination. This embryo was derived from a prepubertal mouse oocyte fertilized *in vitro*. There are two translocated chromosomes in each metaphase (arrows). Therefore, this embryo originates from polyspermy.

Table 4. Results of chromosomal analysis of blastocysts

	Pubertal mice (%)	Prepubertal mice	
		L group (%)	H group (%)
No. of blastocysts prepared	146	30	349
In mitosis	135 (92.4)	30 (100)	307 (87.9)
Analyzable blastocysts	106 (78.5)	18 (60.0)	205 (66.8)
Diploidy	97 (91.5)	16 (88.9)	177 (86.3)
Abnormality	9 (8.5)	2 (11.1)	28 (13.7)
Haploidy	0	0	4 (1.9)
Triploidy	4 (3.8)	1 (5.0)	6 (2.9)
Tetraploidy	0	0	1 (0.5)
Mixoploidy (n/2n)	4 (3.8)	0	12 (5.9)
Mixoploidy (2n/3n)	1 (0.9)	1 (5.0)	3 (1.5)
Aneuploidy	0	0	2 (1.0)

other hand, 19.8% (36/182) of the blastocysts from the H group were successfully implanted, and 10.9% (20/182) developed to term. These were significantly lower than those of the pubertal mouse blastocysts ($P<0.05$). The chromosome preparations from livers of eleven newborns from prepubertal mice showed that all of them had the standard karyotype.

Discussion

The number of ovulated oocytes did not correlate with mouse age, and the response to gonadotropins depended on the body weight of the female mice in the present study. Català *et al.* [5] reported that prepubertal mice were highly sensitive to exogenous hormonal stimulation, but did not refer to the effect of body weight. In humans, there is evidence that the timing of puberty is associated with the rate of weight gain during early childhood in girls [17] and that increase in bodyweight during early childhood is relevant to activation of central gonadotrophin secretion [18]. These reports support the difference in the response to gonadotropins between prepubertal mice with 8.5–12 g and 13–16 g body weights; slight variation in body weight affected

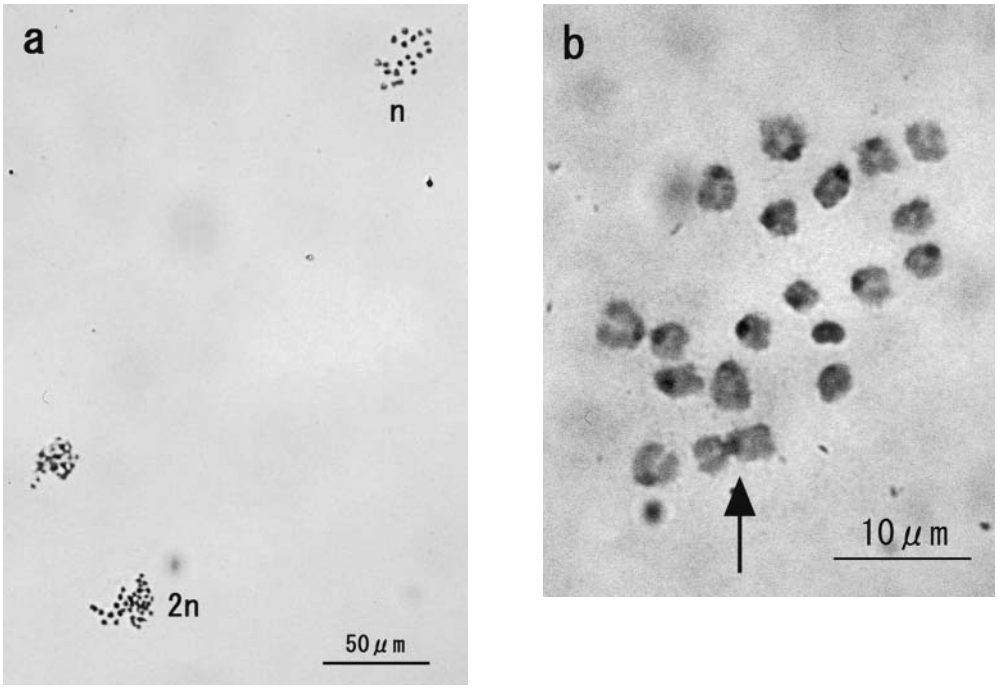

Fig. 5. Chromosomal mosaicism found in a blastocyst derived from an *in vitro*-fertilized mouse oocyte. Three metaphases, two diploid blastomeres (at the lower left), and one haploid blastomere (at the upper right) can be observed in this photo (a). High magnification of the haploid metaphase (b). An arrow shows the translocation chromosome, indicating that this haploid blastomere originated from an odd fertilized sperm.

Table 5. Rates of implantation and development to newborns after blastocyst transfer

	Pubertal mice	Prepubertal mice (H group)
No. of blastocysts transferred	60	182
Pregnant females (%)	4/4 (100)	10/12 (83.3)
Implanted blastocysts (%)	23 (38.3[a])	36 (19.8[b])
Newborns (%)	20 (33.3[a])	20 (10.9[b])

[a, b]: Values with different superscripts in the same row are significantly different ($P<0.05$).

the number of ovulated oocytes in the present study. Furthermore, a significantly low rate of morphologically normal oocytes was obtained in pubertal mice. We considered that this might result from pubertal mouse ovaries having more degenerating follicles than those of prepubertal mice. Thus, morphologically abnormal oocytes might have been ovulated from those degenerating follicles when stimulated with eCG and hCG.

Oocyte maturity as shown by the differences in the rates of 1-cell arrested oocytes between the L and H groups of prepubertal mice also depended on body weight. The rate of oocytes arrested at the 1-cell stage after IVF increased significantly in the L group as compared to the H group and control group of pubertal mice. Moreover, most of the 1-cell arrested oocytes in the L group showed female chromosomes at the M-II stage and male premature condensed chromosomes (PCC). PCC, a chromosomal abnormality that can be detected in immature oocytes fertilized *in vitro*, has often been observed in human oocytes after intracytoplasmic sperm injection [19–22]. These oocytes exhibiting PCC have extremely immature cytoplasm and can not be activated after sperm incorporation. Therefore, no male pronucleus can be formed due to the high level of maturation-promoting

factors or cytoplasmic- chromosome- condensing factors [23]. The cause of this phenomenon in these immature oocytes seems to be asynchrony of nuclear maturation and cytoplasmic maturation. PCCs were observed when a cytoplasmically immature oocyte having a nucleus at the M-II stage was fertilized *in vitro* [4]. Therefore, it was indicated that some oocytes ovulated in prepubertal mice, especially mice with light body weights of 8.5–12 g, are cytoplasmically immature. Furthermore, it has been reported that some oocytes from prepubertal mice show immature meiotic stages [24]. Thus, it may be concluded that the prepubertal mouse ovaries of the L group may have had some immature follicles that could react to gonadotropins but not support normal oocyte maturation. Calafell *et al.* [6] recommended employment of a longer preincubation time or addition of hormones to the media for *in vitro* maturation of oocytes to solve the problem of immature oocytes showing PCC.

The fertilization rates of the oocytes from the prepubertal and pubertal mice were equivalent; therefore, this result showed that oocytes from prepubertal mice can be fertilized normally as reported by Santaló *et al.* [4]. The rates of development to the blastocyst stage of the zygotes derived from the oocytes in the L and H groups decreased significantly as compared with the rate in oocytes from the pubertal mice. This result corresponds with reported results in which mouse age [25], follicular size [26], oocyte volume [27], and cytoplasmic immaturity [28] were elucidated as the causes of low embryonic development of oocytes from prepubertal females. The body weight of prepubertal mice is also considered a cause of low embryonic development.

Incidences of chromosomal abnormalities were relatively higher in prepubertal groups than in pubertal mice. Our preliminary experiments demonstrated that significantly high chromosomal abnormalities, especially polyploidy, were shown at first cleavage in IVF of oocytes from prepubertal mice (unpublished data). In the present study, some mixoploids, i.e. mosaics, were found in all groups, although they were not very frequent except in the H group. Mixoploid blastocysts were also observed by Macas *et al.* [29]. However, they reported that the mosaic was only produced by subzonal sperm injection, which is likely to induce polyspermy, but was not induced by IVF. In the present study, it can also be presumed that the chromosomal mosaics resulted from polyspermy because polyspermy might have been induced by the high sperm concentration or the supplementation of caffeine into the insemination medium. Schatten *et al.* [30] reported the slight presence of dipolar spindle formation within polyspermic embryos at first cleavage as an evidential phenomenon showing that some of the polyspermic embryos are mixoploids, as was observed in the present study.

The present study revealed that some blastocysts from the prepubertal mice could develop to term regardless of the body weight and age of the female mice that were the oocyte donors; however, the implantation rates and rates of development to term of the blastocysts were significantly lower in the prepubertal mice than in the pubertal mice. Similar results, namely that oocytes from various prepubertal animals, including rodent species, pigs, sheep, and cattle, have limited competence to undergo normal embryogenesis and produce viable offspring, have been previously reported [27]. Duby *et al.* [2] reported that cytoplasmic deficiency compromised the embryonic development at pre- and post-implantation; hence, some embryos from prepubertal mice might also have cytoplasmic deficiencies that result in an increase in embryo loss after embryo transfer. However, our results demonstrated that newborns from the prepubertal mouse embryos had normal karyotypes and natural fertility. Thus, although chromosomal abnormality of blastocysts from the prepubertal mice was reflected in the rates of implantation and development to term, it did not affect on the normality of newborn babies.

In conclusion, the present study showed that the prepubertal mice yielded some cytoplasmic immature oocytes that were developmentally arrested at the 1-cell stage and that contained chromosomal abnormalities such as PCC after IVF. The rates of development to the blastocyst stage and to term for IVF of oocytes from the prepubertal mice were significantly low; however, the newborn babies had normal karyotypes and were fertile. The present study is the first report concerning the cytogenetic normality and development of embryos after the first cleavage stages in IVF of oocytes from prepubertal mice. Thus, the results of the present study will be helpful for obtaining cytogenetically safe prepubertal human embryos in the practical application of ART.

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