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—Original Article—

Early production of offspring by *in vitro* fertilization using first-wave spermatozoa from prepubertal male mice

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Abstract. Mature male mice (aged 10–12 weeks or older) are conventionally used for *in vitro* fertilization (IVF) in order to achieve high fertilization rates (e.g., > 70%). Here, we sought to determine the earliest age at which male mice (C57BL/6J strain) can be used efficiently for producing offspring via IVF. Because we noted that the addition of reduced glutathione (GSH) to the IVF medium significantly increased the fertilizing ability of spermatozoa from prepubertal males, we used this IVF protocol for all experiments. Spermatozoa first reached the caudal region of the epididymides at day 35; however, they were unable to fertilize oocytes. Caudal epididymal spermatozoa first became competent for oocyte fertilization at day 37, albeit at a low rate (2.9%). A high fertilization rate (72.0%) was obtained at day 40, and 52.4% of the embryos thus obtained developed into offspring after embryo transfer. Moreover, we found that corpus epididymal spermatozoa in prepubertal mice could fertilize oocytes; however, the fertilization rates were always < 50%, regardless of the age of the males. Caput epididymal spermatozoa failed to fertilize oocytes irrespective of the age of the males. Therefore, we propose that caudal epididymal spermatozoa from male mice aged 40 days can be efficiently used for IVF, to obtain offspring in the shortest attainable time. This protocol will reduce the turnover time required for the generation of mice by ~1 month compared with that of the conventional IVF protocol.

Key words: Congenic strain, Epididymis, *In vitro* fertilization, Mouse, Sperm maturation

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The first wave of spermatogenesis in mice occurs after birth and takes approximately 35 days in a testis [1–3]. It is regulated by complex interactions between circulating hormones [4, 5]. After spermatogenesis, the spermatozoa acquire motility and fertilizing capacity during their passage through the caput and corpus regions of the epididymis [6, 7], and functionally matured spermatozoa are stored in the caudal region [8]. Without this process of maturation of spermatozoa in the epididymis, no mammal can produce offspring by natural breeding [9, 10]. Specifically, the maturation process involves the expression of several proteins and genes [11–16], nuclear [17, 18] and membrane [19, 20] alterations, morphological maturation events [8, 21], motility changes [22, 23], and physiological changes that are necessary for the processes occurring in the female reproductive tract, such as the acrosome reaction [24–28].

In vitro fertilization (IVF) is a popular reproductive technique that is extensively performed not only in large repository mouse facilities, such as ours [29–32], but also in a large number of laboratories using mice [33–35]. For conventional IVF, mature males aged over 10–12 weeks are usually used because of the consistently high fertilizing ability of their spermatozoa; the CARD (Center for Animal Resources and Development, Kumamoto University) protocol for IVF provides similar recommendations (http://card.medic.kumamoto-u.ac.jp/card/english/sigen/manual/mouseivf.html). Therefore, males in the prepubertal age period are not used for conventional IVF. Consequently, at least 13 weeks (including the pregnancy period) are required for obtaining a new generation of mice. If the technique of microinjecting mouse oocytes is available in laboratories, spermatids or nonmotile spermatozoa in the testes may be used for the production of offspring. This enables the use of the first-wave round spermatids from males at 17 days at the youngest, even though they are scarce among the testicular cell population [36]. Ogonuki *et al*. [37] established the high-speed congenic strategy using round spermatids from immature males aged 22–25 days, in which a new generation of mice can be obtained within 50 days.

In this study, we sought to determine the youngest age at which males can be used for IVF, to obtain the next generation in the shortest attainable time without using the microinjection technique. We found that males as young as 40 days can be used for IVF, with practical fertilization rates.

Materials and Methods

Animals

Mature male and female mice of the C57BL/6J strain were pur-

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chased from CLEA Japan, Inc. and used in experiments at 10–20 weeks of age. Prepubertal C57BL/6J males (5–6 weeks of age) were produced by the transfer of embryos generated by IVF using these mature males and females. ICR females (CLEA Japan) were used as recipients for embryo transfer at 10–20 weeks of age. All animals were housed under controlled lighting conditions (daily light period, 0700 to 2100 h). The animal experiments described here were approved by the Animal Experimentation Committee of the RIKEN Tsukuba Institute and were performed in accordance with the committee's guiding principles.

Motility and morphological analyses of sperm

Epididymal spermatozoa from each region (caput, corpus, and cauda) (Fig. 1A) were collected and preincubated in human tubal fluid (HTF) medium [38] for 1 h at 37°C under 5% CO_2 in humidified air. The overall sperm motility, progressive motility, average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN), and straightness (STR) were assessed by computer-assisted sperm analysis (CASA) using a Hamilton Thorne IVOS computerized semen analyzer (Hamilton Thorne, Beverly, MA). Motile spermatozoa were defined as those with any movement of the sperm head. Spermatozoa with progressive motility were defined as those with a forward, linear-direction movement at a speed > 50.0 μm/sec. All the parameters were measured in > 200 spermatozoa (except in prepubertal males) in at least three different fields. The morphological normality of the spermatozoa was examined under an inverted microscope.

IVF

IVF was performed using epididymal spermatozoa as described previously, with slight modifications [39, 40]. In brief, superovulation was induced in female C57BL/6J mice using an intraperitoneal injection of 7.5 IU of equine chorionic gonadotropin (eCG; Peamex, Sankyo, Tokyo, Japan), followed by 7.5 IU of human chorionic gonadotropin (hCG; "Gonatropin"; ASKA Pharmaceutical, Tokyo, Japan) with a 48–50 h interval. At 16–17 h after hCG injection, cumulus-enclosed oocytes were collected from the ampulla region of the oviducts and preincubated for 1–1.5 h in 80 μl droplets of HTF medium supplemented with or without 1.25 mM reduced glutathione (GSH) [41, 42]. Each droplet contained oocytes collected from one oviduct or oviducts of one female. Sperm masses collected from each region of the epididymis were suspended in 100 μl of sperm preincubation medium (HTF containing 0.4 mM methyl-β-cyclodextrin [43, 44] and 0.1 mg/ml of polyvinyl alcohol instead of bovine serum albumin) and incubated at 37°C under 5% CO_2 in humidified air for 45–60 min. At the time of insemination, the preincubated spermatozoa were transferred into the droplets containing oocytes at a concentration of 200–400 spermatozoa/μl. After 3–4 h of co-incubation, oocytes were freed from spermatozoa and cumulus cells using a fine glass pipette and transferred into 10 μl droplets of CZB medium [45] containing 5.6 mM glucose, 0.1 mg/ml of polyvinyl alcohol, and 3.0 mg/ml of bovine serum albumin. For embryo transfer experiments, a portion of these oocytes was cultured at 37°C under 5% CO_2 in humidified air for approximately 24 h. For the confirmation of sperm penetration, the remaining oocytes were fixed with 2.5% glutaraldehyde and stained with 1% aceto-orcein, in order to visualize the pronuclei [46]. Oocytes with a male and a female pronucleus and a sperm tail in the ooplasm were considered successfully fertilized.

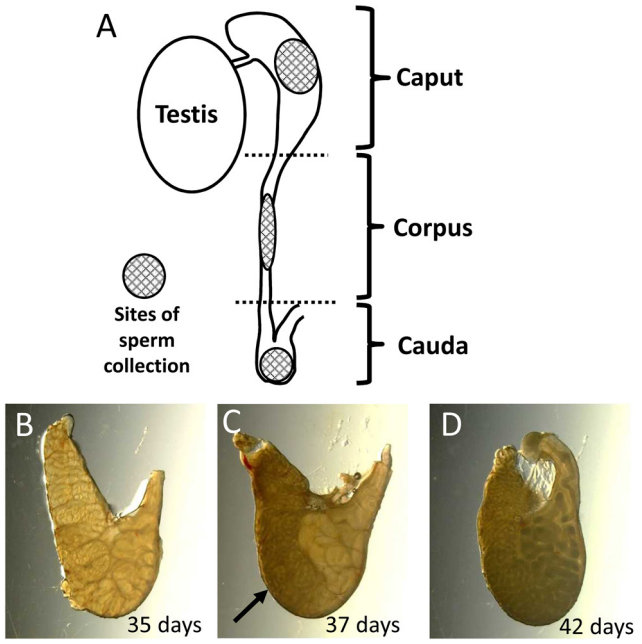

Fig. 1. Schematic representation of the epididymal regions and the appearance of spermatozoa in epididymides in prepubertal males. (A) Spermatozoa were collected from three regions of the epididymis. The cross-hatched circles show the sites of sperm collection. (B) Absence of spermatozoa (empty) in the cauda epididymis at 35 days of age. (C) Presence of spermatozoa in the upper half (arrow) of the cauda epididymis at 37 days. (D) Presence of spermatozoa in the entire cauda epididymis at 42 days. B–D, horizontally flipped from the originals.

Embryo transfer

Two-cell embryos produced by IVF were transferred into the oviducts of day 1 pseudopregnant females of the ICR strain. In the evening of days 18 and 19, each female was injected subcutaneously with 2 mg of progesterone, to avoid spontaneous delivery. In the morning of day 20, the recipient female mice were examined for the number of implantation sites and live offspring by Caesarian section.

Statistical analysis

The relationship between the presence of spermatozoa at the cauda epididymis and the age or body weight of males was analyzed using Spearman's correlation coefficient by the rank test. The rates of motile spermatozoa and spermatozoa with abnormal morphology and the values of the kinetic parameters of spermatozoa were evaluated statistically by one-way analysis of variance (ANOVA). The parameters that were calculated as percentages were subjected to arcsine transformation before statistical analysis and significance was set at $P < 0.05$. Other statistical analyses were described in the text, as appropriate.

Results

The appearance of spermatozoa at cauda epididymides in prepubertal male mice

We determined the proportion of prepubertal males that had spermatozoa in their cauda epididymides. The epididymides from males at 35, 37, and 42 days contained different amounts of spermatozoa, as clearly observed by transmitted light microscopy (Fig. 1B–D). Spermatozoa were observed in the caudal region in 50% and 70% of males aged 35 and 36 days, respectively (Table 1). However, none (35 days) or only 40% (36 days) of the males had progressively motile spermatozoa. The majority (≥ 90%) of males aged 37 days or older had spermatozoa in the caudal region, most of which were progressively motile. These data indicate that spermatozoa acquire motility about 1 day after their arrival at the cauda epididymis. The plotting of the body weight of individual males in the presence or absence of spermatozoa at cauda epididymides revealed that the timing of the arrival of spermatozoa at the caudal region depended on age (P = 0.001), but not on body weight (P = 0.204) (Fig. 2).

Motility and morphological analyses of spermatozoa retrieved from different regions of the epididymides of adult mice

To assess the functional and morphological maturation of spermatozoa in the epididymides, we collected spermatozoa from the caput, corpus, and caudal regions of the epididymides of prepubertal (37 and 40 days of age) and adult mice (120–180 days of age) (Fig. 1A) and analyzed their motility and morphology. Our CASA analysis indicated that spermatozoa of adult males gradually acquired motility as they descended the epididymis, from the caput to the cauda (Table 2). In prepubertal males, the proportion of motile spermatozoa in the caput region was very low (9–11%), with significant differences from those of corpus or caudal epididymal spermatozoa. Three velocity parameters (VSL, VAP, and VCL) and ALH increased predominantly at the transition from the caput to the corpus, regardless of the age of males. Additional three parameters (BCF, LIN, and STR) showed no or very little region-specific differences.

Table 1. Presence of spermatozoa and spermatozoa with progressive motility in the cauda epididymides of prepubertal males

Age of males (days)	No. (%) of males with	
	Sperm	Sperm with progressive motility
35	4/8 (50)	0/8 (0)
36	7/10 (70)	4/10 (40)
37	9/10 (90)	8/10 (80)
38	9/10 (90)	8/10 (80)
39	9/10 (90)	9/10 (90)
40	10/10 (100)	10/10 (100)
42	9/9 (100)	9/9 (100)

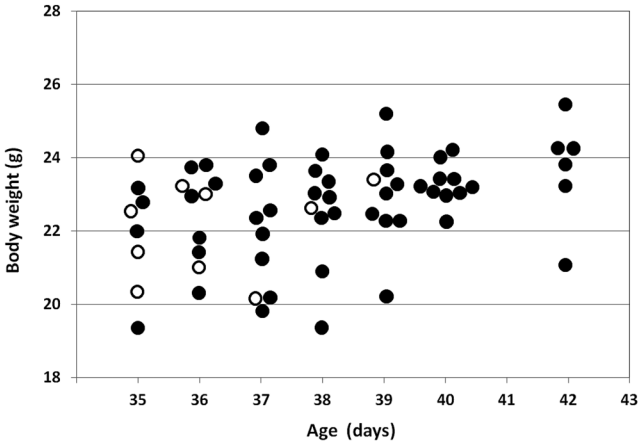

Fig. 2. Relationships between the presence/absence of spermatozoa at the cauda epididymis and body weight or age in prepubertal male mice. The black circles indicate mice with caudal spermatozoa and the white circles indicate those without caudal spermatozoa. The time at which the spermatozoa reached the caudal region depended on age (P = 0.001), but not on body weight (P = 0.204) (Spearman's correlation coefficient by the rank test).

Table 2. Kinetic parameters of spermatozoa collected from each region of the epididymis

Age	Region of the epididymis	Percentage of spermatozoa with		Velocity (μm/sec)			ALH (μm)	BCF (Hz)	LIN (VSL/VCL, %)	STR (VSL/VAP, %)
		motility	progressive motility	VSL	VAP	VCL				
37 days	Caput	11 ± 3.2 [a]	6 ± 1.7 [a]	73 ± 6 [a]	132 ± 13 [a]	257 ± 20 [a]	16 ± 1.1 [a]	36 ± 1.4	34 ± 2.4	60 ± 4.0
	Corpus	44 ± 7.7 [b]	27 ± 4.4 [b]	127 ± 11 [b]	219 ± 19 [b]	458 ± 30 [b]	27 ± 2.2 [b]	34 ± 1.0	30 ± 0.7	58 ± 0.9
	Cauda	36 ± 11.2	25 ± 9.6	140 ± 24 [b]	219 ± 27 [b]	448 ± 52 [b]	24 ± 2.0 [b]	36 ± 2.9	32 ± 2.6	61 ± 2.8
40 days	Caput	9 ± 2.4 [a]	5 ± 1.8 [a]	70 ± 7 [a]	141 ± 15 [a]	268 ± 25 [a]	16 ± 1.9 [a]	36 ± 2.9	31 ± 1.7	57 ± 2.5
	Corpus	44 ± 8.1 [b]	25 ± 4.8 [b]	132 ± 8 [b]	236 ± 7 [b]	484 ± 14 [b]	29 ± 0.4 [b]	37 ± 0.6	28 ± 1.5	56 ± 2.9
	Cauda	49 ± 3.5 [b]	31 ± 2.5 [b]	151 ± 8 [b]	251 ± 9 [b]	524 ± 22 [b]	28 ± 1.2 [b]	36 ± 1.5	30 ± 1.3	60 ± 2.1
Adult	Caput	31 ± 8.1 [a]	14 ± 4.3 [a]	60 ± 5 [a]	115 ± 12 [a]	218 ± 21 [a]	16 ± 0.8 [a]	37 ± 3.0	30 ± 0.7 [a]	56 ± 1.7
	Corpus	47 ± 8.1	23 ± 8.1	87 ± 8 [b]	161 ± 10 [b]	369 ± 21 [b]	23 ± 1.3 [b]	39 ± 1.2	26 ± 1.8 [b]	55 ± 1.9
	Cauda	62 ± 5.6 [b]	31 ± 4.3 [b]	91 ± 7 [b]	159 ± 11 [b]	343 ± 26 [b]	23 ± 1.2 [b]	37 ± 1.0	28 ± 1.0	58 ± 1.4

VSL, straight-line velocity; VAP, average path velocity; VCL, curvilinear velocity; ALH, amplitude of the lateral head movement; BCF, beat cross frequency; LIN, linearity; STR, straightness. The data of each parameter were analyzed by one-way ANOVA, followed by a multiple comparison test. [a,b] $P < 0.05$ within the same column (Scheffe's F test). Mean ± SEM, n = 5–7 males.

Microscopic observations revealed that epididymal spermatozoa always contained a certain population of spermatozoa with an abnormal head and/or tail (Fig. 3A–D). At 37 days, the proportion of spermatozoa with abnormal morphology was consistently high throughout the epididymides (Fig. 3E). At 40 days, the proportion of spermatozoa with an abnormal tail was significantly decreased in the caudal region (Fig. 3F). Interestingly, in adult males, the proportion of sperms with a morphologically abnormal head or tail decreased significantly as they passed from the caput to the caudal region (Fig. 3G). As a result, the rate of occurrence of normal-shaped spermatozoa in the caudal region increased with age (Fig. 3H). This is in accordance with the increase in the motility rates of caudal epididymal spermatozoa with an increase in the age of males, described above (Table 2).

Improvement of fertilization rates by the addition of GSH to the oocyte incubation medium

To devise the IVF protocol for prepubertal males, we examined the effect of GSH supplementation to the oocyte incubation medium on fertilization rates because treatment of oocytes with GSH facilitates sperm penetration through the zona pellucida [43]. Spermatozoa from adult (120–180 days) and prepubertal (40–42 days) male mice were used. After IVF using caudal epididymal spermatozoa, the fertilization rate in the absence of GSH was lower in the prepubertal group than it was in the adult group; however, it increased significantly when oocytes were treated with GSH (from 55% to 86%) (Fig. 4A). This was also the case for IVF using corpus epididymal spermatozoa; the fertilization rate in the prepubertal group increased from 6% to 37% in the presence of GSH (Fig. 4B). Therefore, we used GSH supplemented oocyte/fertilization medium for all IVF experiments performed in this study.

Fertilization and developmental ability of spermatozoa from prepubertal male mice

To identify the youngest age at which males can be used for IVF, we performed IVF using spermatozoa isolated from the caput, corpus, and cauda of epididymides from prepubertal mice of different ages (36–42 days) (Fig. 1B–D). The mass of spermatozoa was collected by puncturing the surface of the epididymis with a sharp needle and immediately transferred into a droplet for preincubation (Fig. 5A). At 4–5 h after insemination, oocytes were assessed for fertilization. Oocytes with two pronuclei and a sperm tail were considered to have been fertilized (Fig. 5B). No oocytes were fertilized with spermatozoa at 36 days, regardless of the region of the epididymis from where they were collected. We found that 37 days was the youngest age at which males were able to produce spermatozoa with fertilizing ability; however, the fertilization rates were very low at this time point (5.8% and 2.9% for corpus and caudal spermatozoa, respectively) (Table 3). The fertilization rates using corpus and caudal spermatozoa increased with age, reaching 47.0% and 77.9% respectively at 42 days. A high fertilization rate (72.0%) was obtained when using caudal epididymal spermatozoa at 40 days, whereas it was 22.1% for corpus epididymal spermatozoa. No fertilized oocytes were obtained when using caput epididymal spermatozoa, irrespective of the age of the male, including adulthood (120 days). In Fig. 6, we summarize the three age-related parameters of sperm maturation in the cauda

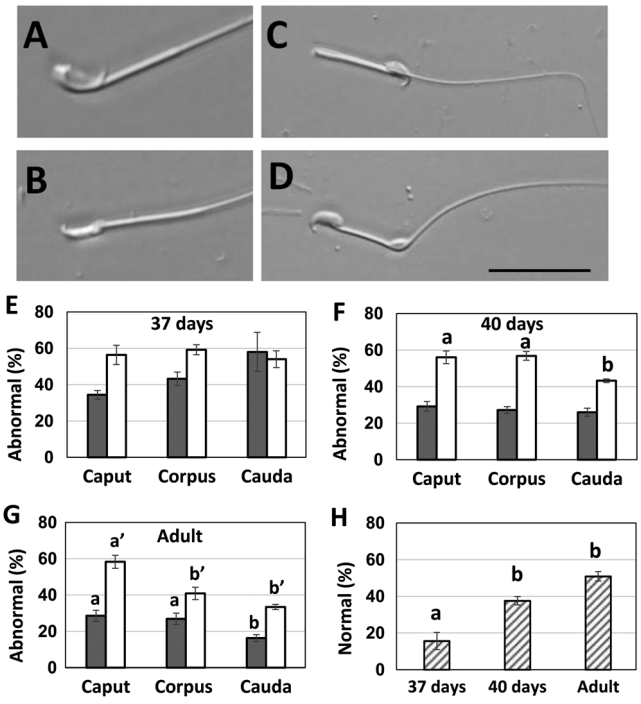

Fig. 3. Presence of morphologically abnormal spermatozoa at each region of the epididymis. (A, B) Spermatozoa with an abnormal head shape. (C, D) Spermatozoa with an abnormal tail (bending). (E to G) Percentages of spermatozoa with an abnormal head (gray bar) or tail (white bar) from the caput, corpus, and cauda epididymis at 37 days, 40 days, and adult age, respectively. (H) Percentage of morphologically normal spermatozoa in the cauda epididymides at different ages. Mean ± SEM, n = 5–7 males. Data were analyzed by one-way ANOVA, followed by a multiple comparison test. [a, b; a′, b′] $P < 0.05$ (Tukey-Kramer test). Bar = 25 μm.

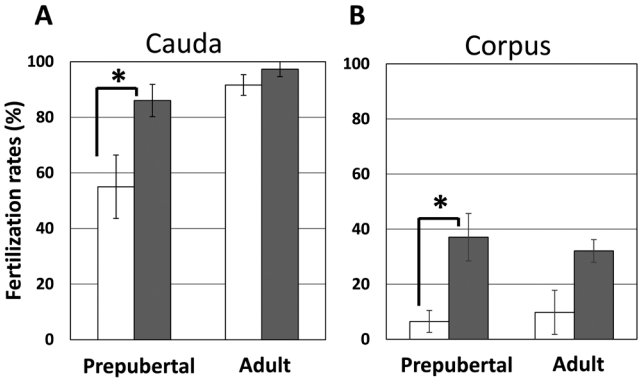

Fig. 4. Effect of GSH on fertilization rates after IVF using spermatozoa from prepubertal (40–42 days of age) and adult males. The spermatozoa were collected from the caudal (A) or corpus (B) region and were used for IVF. The asterisks indicate significant differences between the GSH (–) (white bar) and GSH (+) (gray bar) groups ($P < 0.05$, Wilcoxon rank-sum test; mean ± SEM, n = 3–5).

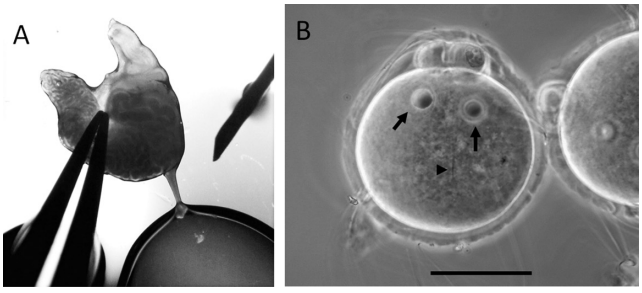

Fig. 5. Collection of spermatozoa for preincubation during IVF and visualization of a fertilized oocyte. (A) A mass of spermatozoa from an epididymis of a male (42 days) was collected via puncture using a fine needle and was introduced into the preincubation medium. (B) Fertilized oocyte with two pronuclei (arrows) and a sperm tail (arrowhead). Bar = 50 μm.

epididymides that were identified in this study. All three parameters, i.e., the appearance in the cauda, the acquisition of motility, and the acquisition of the fertilization ability, increased in that order with age, with a gap of 1 to 3 days between these events.

Finally, we examined whether the embryos generated via IVF using prepubertal males could develop normally into offspring after embryo transfer. Indeed, following IVF using caudal epididymal spermatozoa at 40 days, live offspring were obtained at term with normal efficiency (52% of the embryos that were transferred). To our knowledge, this study reports the youngest male mice (40 days) to have been used till date to generate viable offspring after IVF (Table 4, Fig. 7A). Similarly, 50% of the embryos obtained from either corpus or caudal spermatozoa at 42 days developed into offspring (Table 4, Fig. 7B, C). These birth rates are comparable to those of our previous study (58%, 23/40), which used adult male mice of the same strain [40].

Discussion

Although it is known that, in adult mice, spermatozoa undergo functional maturation as they pass through the epididymides, the mechanism by which the first-wave of spermatozoa mature in the epididymides remains unexplored. In this study, we investigated the time course of the first wave of spermatozoa that descended the epididymides with respect to its acquisition of motility and fertilizing ability in prepubertal male mice. Our findings demonstrated that spermatozoa first appeared in the caudal region at 35 days of age, acquired motility at 36 days, and became competent to fertilize oocytes at 37 days of age. At 40 days, the fertilization rate increased to > 70%, and the fertilized oocytes developed into live offspring. Therefore, the first-wave of spermatozoa acquired functional competence between 35 and 40 days of age in prepubertal male mice, although the addition of GSH to the IVF medium was necessary for this process. GSH reduces the disulfide bonds (-S-S-) between cysteines that constitute the zona protein, thereby leading to easier penetration of spermatozoa through the zona [41, 44]. The increase in morphologically normal spermatozoa in the cauda epididymides at 40 days might have also contributed to the high fertilization rate observed (Fig. 3H). Importantly, these steps seemed to be dependent on age, and not body weight, which is in contrast with that observed for the sexual maturity of females, which shows dependency on body weight [47].

Our CASA analysis using spermatozoa from different regions of the epididymides provided important clues about how spermatozoa acquire motility in these regions. Spermatozoa with a high velocity appeared in the corpus region, indicating that caput spermatozoa cannot swim fast enough to penetrate the egg investments (cumulus cells and zona

Table 3. Age-related changes in the fertilization rate of epididymal spermatozoa from each region

Age of males (days)	Fertilization rates using spermatozoa from		
	Caput	Corpus	Cauda
36	0 (5)	0 (5)	0 (5)
37	0 (5)	5.8 ± 2.8 (7)	2.9 ± 1.4 (7)
38	0 (5)	18.5 ± 10.0 (7)	2.2 ± 2.2 (6)
39	0 (3)	11.2 ± 5.9 [a] (7)	48.9 ± 10.3 [b] (7)
40	0 (2)	22.1 ± 4.3 [a] (7)	72.0 ± 12.1 [b] (7)
42	0 (6)	47.0 ± 5.9 [a] (9)	77.9 ± 8.4 [b] (9)
120	0 (5)	27.4 ± 6.3 [a′] (8)	97.7 ± 0.9 [b′] (8)

Values are the mean ± SEM (%). The number in parentheses indicates the number of males used. [a,b] $P < 0.05$; [a′,b′] $P < 0.01$, Wilcoxon rank-sum test.

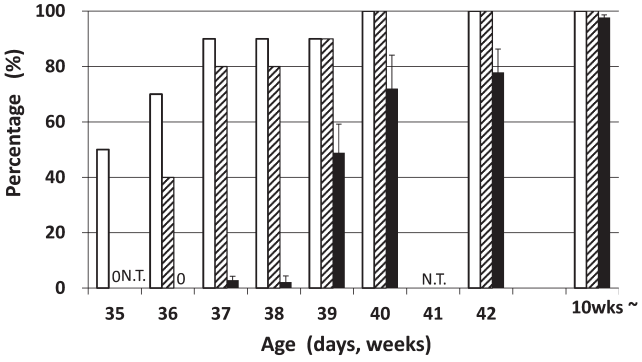

Fig. 6. Age-related changes in the percentages of the presence/absence of spermatozoa at the cauda (white bar), of spermatozoa with progressive motility (striped bar), and of fertilization by IVF (black bar) (n = 7–10, refer to Table 1). The black bar indicates the fertility rates of spermatozoa from the caudal region of the epididymis (mean ± SEM, n = 5–9, refer to Table 3). N.T., not tested.

Table 4. Development into offspring after the transfer of embryos obtained via IVF using the first-wave of spermatozoa

Age of males (days)	Region of collection of spermatozoa	No. (%) of embryos		
		Transferred	Implanted	Developed to offspring
40	Cauda	42	40 (95%)	22 (52%)
42	Corpus	24	21 (88%)	12 (50%)
42	Cauda	28	25 (89%)	14 (50%)

There was no significant difference among the three groups with respect to the rates of implantation and offspring generation (Fisher's exact test).

Fig. 7. Offspring born after IVF using spermatozoa from the caudal (A, C) or corpus (B) regions of the epididymides of prepubertal male mice at 40 (A) and 42 (B, C) days of age. These findings correspond to the results shown in Table 4.

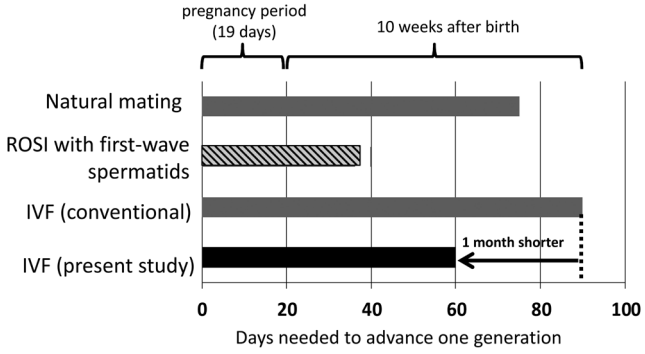

Fig. 8. Comparison of the time necessary for generation turnover by natural mating, round spermatid injection (ROSI), and IVF (conventional IVF and that described in the present study). The IVF procedure reported in the present study, which used prepubertal males, yielded generations that were approximately 1 month shorter than those obtained from the conventional IVF method. Although ROSI using first-wave spermatids, as reported by Miki *et al*. [36], results in the shortest generation turnover, it requires microinjection.

pellucida). Consistently, spermatozoa from the caput were unable to fertilize intact cumulus-enclosed oocytes but could fertilize them when the cumulus cells and the zona were removed [6], when sperm were microinseminated into the perivitelline space [48], or when sperm were preincubated with corpus or caudal epididymal epithelial cells [49]. It would be interesting to identify the factor(s) of the corpus that may facilitate the high-velocity movement of spermatozoa. In contrast, the proportion of motile spermatozoa gradually increased as they passed from the caput to the cauda. In addition to the motility parameters, other parameters, such as morphological normality, increased as spermatozoa descended the epididymides (Fig. 3G). Numerous epididymal factors are expected to be involved in the maturation processes of spermatozoa in the epididymides.

Intriguingly, the inability of caput spermatozoa to support the processes of fertilization or embryonic development has been reported. Caput spermatozoa injected into oocytes did not transform into pronuclei [50] or did not induce oocyte activation [28]. A more recent study reported that although, embryos were generated using caput spermatozoa by ICSI, they failed to develop to full term because of the lack of the small RNAs supplied by the epididymides [51]. Conversely, injection of caput spermatozoa into oocytes resulted in normal embryonic and fetal development [52, 53]. It remains unclear as to why discrepancies exist regarding the competence of caput epididymal spermatozoa.

There are several advantages in the use of spermatozoa from prepubertal males for the production of offspring. First, if mice carry a mutation that causes death or systemic weakness before puberty (e.g., early-onset diabetes), our IVF protocol using prepubertal males would help the propagation of the mutations to the next generation. Second, we may expect to generate a large number of mice quickly for experimentation purposes. Third, as our IVF protocol may shorten the generation turnover time, the establishment of congenic strains by backcrossing will be completed more quickly compared with conventional IVF (Fig. 8). Using this strategy, we established knockout mouse strains with the NOD/scid background in approximately 7 months, which is a process that requires more than 1 year when using conventional IVF (unpublished).

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