Bypassing Spermiogenesis for Several Generations Does Not Have Detrimental Consequences on the Fertility and Neurobehavior of Offspring: A Study Using the Mouse

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Purpose: *This study was conducted to determine whether the omission of spermiogenesis and all prefertilization events for five generations in mice affects the fertility or behavior of offspring.*
Methods: *Fifth-generation hybrid (C57BL/6 × DBA/2) mice were produced using round spermatid injection (ROSI). Control groups consisted of mice born after natural mating with and without sham operation. The growth, fertility, and behavior of offspring were compared. Behavior tests conducted assessed elementary reasoning (Krushinsky test), emotionality (Mouse Defense Test Battery), and spatial learning and memory (Morris water maze).*
Results: *There were no significant differences in the growth and fertility of fifth-generation ROSI mice compared to natural fertilization mice. We also found no evidence of significant learning or behavioral deficits of the fifth-generation ROSI mice.*
Conclusions: *In this study, we found no evidence that bypassing the natural biological processes involved in spermiogenesis produces adverse effects on the growth, fertility, or behavior of mouse offspring.*

KEY WORDS: round spermatid injection; spermatid; mouse; behavior.

INTRODUCTION

The direct injection of sperm into oocytes, commonly referred to as intracytoplasmic sperm injection (ICSI), has become the method of choice to overcome human infertility due to severe male infertility factors (1–4). Even "immature" spermatozoa within the testis have been used for ICSI (5–7). Obviously, ICSI bypasses many natural biological processes such as sperm maturation in the epididymis, interactions within the female genital tract, sperm capacitation and interaction with oocyte vestments, and sperm membrane fusion with the oocyte. With the widespread use of this technology, its potential adverse outcomes need to be ascertained (8). Thus far in humans, ICSI has not yielded evidence of increased obstetric risk and the frequency of major malformations among ICSI offspring is comparable to that of the general population (9,10).

A more drastic method of assisted fertilization is round spermatid injection (ROSI) or round spermatid nucleus injection (ROSNI), which involve the injection of prespermatozoal cells, round spermatids, into oocytes. This bypasses spermiogenesis, in addition to those biological processes previously mentioned. In the mouse, ROSI resulted in the birth of offspring that appeared to be normal at birth and developed into normal, fertile adults (11,12). However, ROSI may cause detrimental changes that are not detectable after just one generation. The question we consider in this study is whether omission of spermiogenesis and all prefertilization events for several generations accumulate defects and affect the development, fertility, behavior, or learning ability of offspring.

There are no human studies reporting the effects of injecting immature round spermatids beyond the birth of apparently normal babies (13–16). As the average single generation in the human is approximately 20 years, this study utilized mice with a much shorter single generation (~3 months) to determine whether repeated ROSIs have serious consequences on the fertility and behavior of offspring.

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Fifth-generation mice reproduced exclusively by ROSI were compared with two groups of control mice. The first control group consisted of mice born after natural mating and consequently natural in vivo fertilization. The second control group was derived from natural mating, but with a sham operation on the mothers' oviducts, to permit evaluation of differences due to effects of the embryo transfer surgery on developing offspring.

MATERIALS AND METHODS

Animals

Hybrid B6D2F1 mice (C57BL/6 female × DBA/2 male) were purchased from the National Cancer Institute. These hybrid mice have been used in all previous experiments involving microsurgical operation of oocytes in this laboratory (12,17–20). Control and fifth-generation ROSI animals were derived using the procedure described below. All animals were individually housed in polycarbonate cages and were maintained in air-conditioned rooms. Until 3 months of age, mice were maintained under a 14-hr light/10-hr dark cycle, with light onset at 0500 hr. From 3 months on, they were kept on a 12-hr light/12-hr dark cycle, with light onset at 0600 hr. The animals were maintained in accordance with the guidelines of the Laboratory Animal Service at the University of Hawaii and those prepared by the Committee on Care and Use of Laboratory animals of the Institute of Laboratory Resources National Research Council [DHEW publication No. (NIH) 80-23, revised in 1985]. The protocol of animal handling and treatment for this study was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii. Animals were about 4 months old at the beginning of the experiments, which took place over a span of 8 weeks.

Round Spermatid Injection (ROSI)

Injection of round spermatid nuclei into mature oocytes, transplantation of preimplantation embryos to foster mothers, and rearing of offspring were carried out as described previously (12) except that oocyte manipulation was carried out at room temperature (22–26°C) rather than at 16–17°C, and spermatid-injected oocytes were activated by Sr^{2+} (21) rather than an electric shock. Round spermatids were the smallest nucleated cells among various spermatogenic cells isolated from the testes of mature males. When viewed under an interference-contrast or phase-contrast microscope, mouse round spermatids were easily recognized by their small size (about 10 μm in diameter) and centrally located distinct chromatin mass (12). The ages of males and females that provided spermatids and oocytes for ROSI were 3–10 and 2–3 months, respectively. The steps for obtaining the fifth-generation of ROSI offspring are shown in Fig. 1.

All of the offspring resulting from the first ROSI (ROSI-1) were raised until puberty, then two were randomly selected to collect round spermatids for the second ROSI (ROSI-2), and so on.

Growth and Fertility of the ROSI Fifth Generation

Eight male and eight female newborn ROSI F_5 animals were randomly selected and weighed at 1-week intervals beginning on Day 10 after birth. The same was done for natural F_2 animals. When matured, these mice were mated with B6D2F_1 males or females and the litter sizes of their first and second pregnancies were determined.

Behavior Tests

For behavioral tests, we randomly selected 11 males and 12 females of the fifth-generation ROSI animals (ROSI F_5), 7 males and 9 females of the F_2 generation by natural mating animals (natural F_2), and 10 males and 11 females of the F_2 generation by natural mating with sham operation on mothers' oviducts to control for possible effects of the embryo transfer surgery (sham-operated F_2).

Krushinsky Test. The general capacity for elementary reasoning of ROSI F_5 mice was tested by the Krushinsky test. This test evaluates the ability of an animal to find food that has been presented and then removed from the animal's visual field.

The testing apparatus was modeled after that used by Poletaeva *et al.* (22). The box (24 × 21 × 15 cm) was made from Plexiglas, opaque black for the front wall and opaque white for the remaining three sides. Sweetened condensed milk (Springfield brand, Los Angeles, CA) was used as the food stimulus. Activity was recorded by a videocamera mounted above the apparatus.

The procedure used was adapted from those used by Dulioust *et al.* (23). Each subject was deprived of food and water for at least 18 hr prior to testing. The

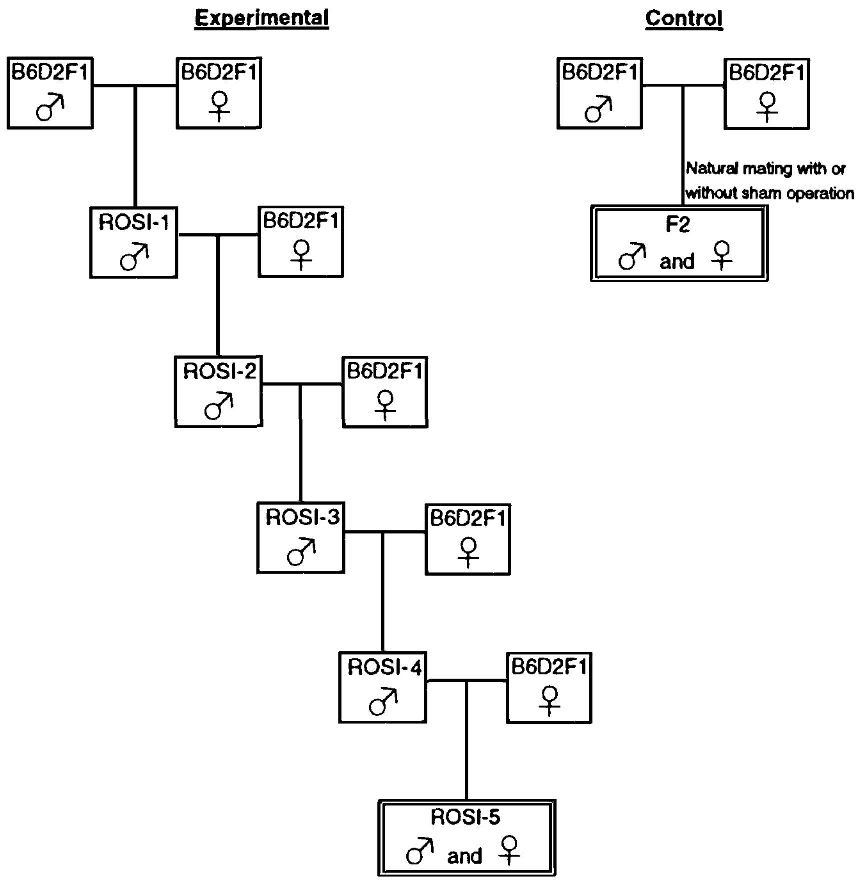

Fig. 1. Procedures for the production of experimental fifth-generation ROSI mice and control natural F_2-generation mice.

weights of all subjects were monitored to ensure that significant weight loss did not occur.

Data collected included the total session duration and number of correct choices made. *Session duration* was defined as the amount of time elapsed for the subject to complete ten trials. *Percentage correct responses* was defined as the number of correct choices divided by the number of trials a subject completed. In analyzing data using total session duration we calculated the number of correct choices made per minute during the entire session (correct choice rate).

Mouse Defense Test Battery (MDTB). The MDTB measure a variety of defensive behaviors, including flight, freezing, defensive threat/attack, and risk assessment, to a natural predator of the mouse, a hand-held (anesthetized) rat. It has been used to evaluate changes in specific defensive behaviors with anxiolytic and panicolytic drugs (24,25). The MDTB was utilized to determine whether repeated ROSIs affect emotionality in mice.

Activity was recorded with videocameras mounted above the apparatus, and behaviors were evaluated from these videotape records by scorers who were not informed of the status of individual subjects.

The testing procedure is described in detail elsewhere (24–26) and included the following: pretest, rat avoidance test, chase/flight test, straight alley test, and forced contact test.

Morris Water Maze. The Morris water maze has been used extensively in evaluating spatial learning ability and long-term and short-term memory in rodents (27,28). In this test, a platform is hidden in opaque water, but distal visual cues are available for the animal to use for navigation. To find the platform efficiently, the animal must develop a spatial map of the platform's location using the distal visual cues provided. Memory is demonstrated by the decrease in latency to locate the hidden platform using extramaze cues in successive trials. A block of reversal trials in which the goal position is reversed, with the distal cues remaining in the same locations, is administered after the initial acquisition trials to further assess spatial learning ability. This test has been used extensively in studies to assess the effect of brain lesions (29), physiological differences in the brain (30), chemical stimuli (31), dietary changes (32), and age-related memory decline in mice and rats (27,28,33,34).

The hidden platform procedure utilized is described in detail by Upchurch and Wehner (31,35) except that trials were given in blocks of six on each of 3 consecutive days during the acquisition phase of the test.

Statistics

Data were analyzed by a one-way analysis of variance (ANOVA) or the nonparametric Kruskal–Wallis ANOVA for some infrequently occurring or non-parametrically distributed measures. Subsequent comparisons between animal types were carried out using Newman–Keuls procedures.

RESULTS

Growth and Fertility of Fifth-Generation ROSI Offspring

Figure 2 illustrates growth rates of ROSI F_5 offspring (eight males and eight females) and of the control F_2 group (eight males and eight females) over a period of 7 weeks after birth. There was no significant difference ($P > 0.2$) in the mean body weight of offspring at each week between these two groups of animals. When mature, males and females in each group were allowed to mate with B6D2F$_1$ females and males. The mean sizes of the first two litters were 9.1 ± 1.4 pups (ROSI F_5 group) and 9.2 ± 1.5 pups (natural F_2 group). The difference was not significant ($P > 0.5$). We did not find any physical malformations in growing and adult animals in both groups.

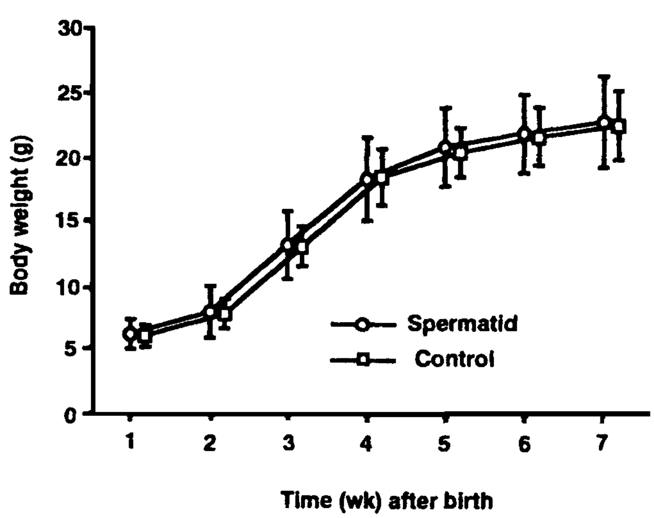

Fig. 2. Growth of ROSI F_5 and natural F_2 groups.

Behavior Tests

Krushinsky Test. ANOVA showed that there were no statistically significant differences in the correct choice rate between the experimental (ROSI F_5) and the two control (natural F_2 and sham-operated F_2) groups [$F(2,54) = 1.36$, $P < 0.26$]. There was a reliable difference between male and female subjects [$F(1,54) = 5.21$, $P < 0.05$], with females showing a higher correct choice rate than males. There were no significant interactions between type of animal and other variables. There was a significant improvement in the ratio across days [$F(5,270) = 77.70$, $P < 0.001$]. The increasing correct choice rate of each session (Fig. 3) was not a function of subjects making more correct choices, as the percentage of correct choices remained at chance level, at approximately 50%; rather, it was a result of the subjects taking a shorter amount of time to complete the session. This indicates that although the mice did not make more correct choices across sessions, they responded more quickly.

Figure 4 indicates the percentage of correct choices made. ANOVA revealed a statistically significant difference among the three types of animals [$F(2,49) = 3.68$, $P < 0.05$]. The mice in the ROSI F_5 group achieved a significantly higher percentage of correct responses compared to the two groups of natural fertilization mice. Post hoc analysis using Newman–Keuls test indicated a significant difference between ROSI F_5 mice compared to natural F_2 mice, with the ROSI F_5 mice achieving a higher percentage of correct responses [$F(1,33) = 6.77$, $P < 0.05$]. There were no reliable differences between male and female subjects [$F(1,49) = 2.87$, $P < 0.10$]. There were no significant interactions between type of animal and other variables.

Mouse Defense Test Battery. Table I summarizes data for pretest activity and avoidance and flight of the oncoming threat stimulus (rat). ANOVA indicated that these measures did not differ significantly among the three types of animals or for males compared to females.

Data for response to predator approach in a blocked alley are presented in Table II. ANOVA showed that there were no behavioral differences with the exception

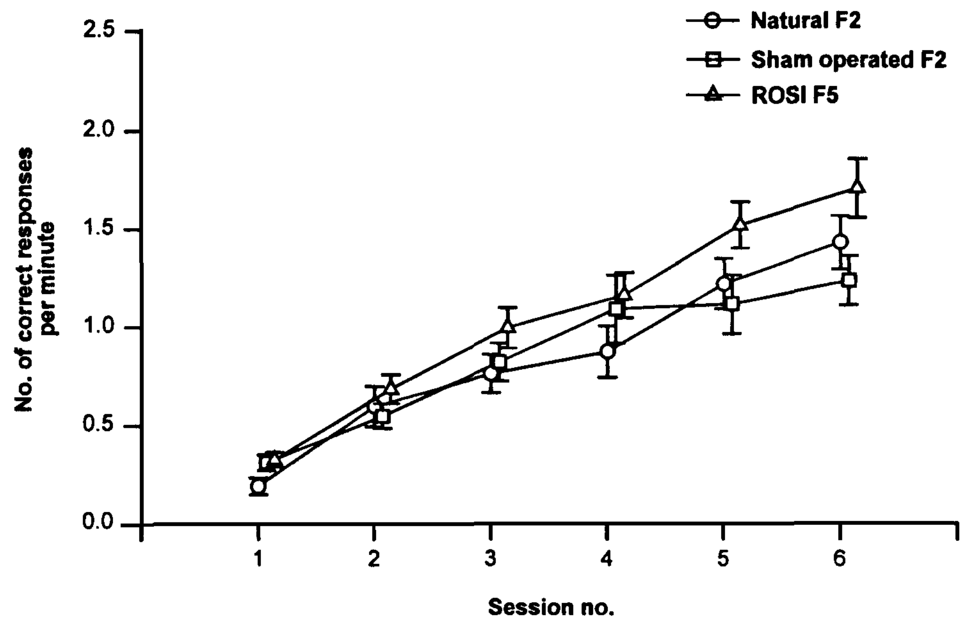

Fig. 3. Krushinsky test: ratio of the number of correct responses by session to the minutes of session duration (correct choice rate).

of the closest distance between subject and rat predator (a measure of risk assessment with the subject approaching the predator) [$F(2,54) = 6.26$, $P < 0.01$] and flight [$F(2,54) = 4.13$, $P < 0.05$]. Sham-operated F_2 animals had a significantly lower distance compared to ROSI F_5 animals (Newman–Keuls; $P < 0.01$). There was an interaction between type and sex for the minimum distance between subject and predator [$F(2,54) = 3.27$, $P < 0.05$], with sham-operated F_2 males exhibiting a significantly closer distance to the predator compared to any other group (Newman–Keuls; $P < 0.05$). With reference to the significant flight differences, post hoc Newman–Keuls tests indicated that sham-operated F_2 animals showed a significantly higher number of flight attempts compared to natural F_2 animals ($P < 0.05$) and ROSI F_5 animals ($P < 0.01$).

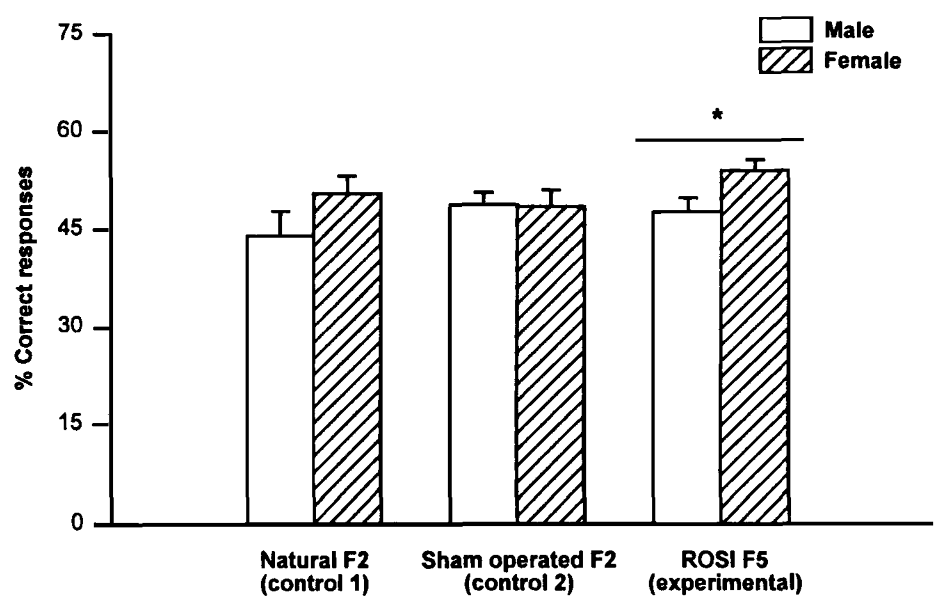

Fig. 4. Krushinsky test: percentage of correct responses.

Table I. MDTB: Pretest, Rat Avoidance, and Chase/Flight Tests (mean ± sem)

	Natural F_2		Sham-operated F_2		ROSI F_5	
	Male	Female	Male	Female	Male	Female
Pretest						
Line crossings	125.00 ± 17.52	141.00 ± 12.03	123.30 ± 16.46	134.91 ± 12.61	120.46 ± 9.19	106.25 ± 9.90
Wall rears	6.14 ± 1.50	7.11 ± 1.75	8.20 ± 1.29	9.09 ± 1.84	10.00 ± 2.01	6.75 ± 1.69
Wall climbing	6.14 ± 1.53	5.89 ± 1.26	4.80 ± 1.05	3.55 ± 0.90	4.73 ± 1.72	4.25 ± 0.99
Rat avoidance test						
Avoidance frequency	2.67 ± 0.33	2.83 ± 0.17	2.14 ± 0.40	2.00 ± 0.47	2.73 ± 0.51	2.25 ± 0.45
Avoidance distance (cm)	45.56 ± 17.88	23.89 ± 2.50	42.86 ± 6.28	55.05 ± 11.06	47.64 ± 9.02	42.50 ± 7.68
Escape frequency	3.57 ± 0.48	4.55 ± 0.48	4.33 ± 0.29	4.64 ± 0.15	4.55 ± 0.21	4.08 ± 0.38
Escape distance (cm)	61.10 ± 13.34	62.30 ± 5.21	61.74 ± 7.68	68.95 ± 7.70	57.45 ± 6.70	72.75 ± 12.56
Chase/flight test						
Average speed (m/sec)	0.90 ± 0.06	0.81 ± 0.09	0.73 ± 0.08	0.88 ± 0.08	0.93 ± 0.05	0.79 ± 0.03
Stops	4.29 ± 1.06	5.89 ± 0.96	5.80 ± 1.55	4.82 ± 0.92	3.55 ± 1.25	3.92 ± 0.67
Reversals	1.86 ± 0.80	1.11 ± 0.56	2.30 ± 0.50	1.82 ± 0.46	2.45 ± 0.67	1.50 ± 0.42
Orientations	0.43 ± 0.20	0.67 ± 0.33	0.80 ± 0.29	0.73 ± 0.24	0	0.75 ± 0.28
Jump escapes	2.00 ± 0.95	2.00 ± 1.41	1.40 ± 0.69	1.36 ± 0.51	1.73 ± 0.52	2.83 ± 1.19

During the forced contact phase (Table II), number of vocalizations showed a significant sex difference [ANOVA; $F(2,54) = 10.11$, $P < 0.01$], with females exhibiting higher frequencies than males in all groups. There were no other significant differences.

Morris Water Maze. Evidence of response acquisition in the Morris water maze task is shown by the significant decrease in the latency to find the hidden platform over the 18 acquisition trials [$F(2,106) = 60.92$, $P < 0.0001$] (Fig. 5). There were no significant acquisition differences among the three types of animals [$F(2,53) = 0.38$, $P < 0.68$] or between males and females [$F(1,53) = 2.96$, $P < 0.09$]. Interactions between type of animal and other variables were also not significant.

Latencies to find the hidden platform for the retention trials are presented in Fig. 6. There were no signifi-

Table II. MDTB: Straight Alley and Forced Contact Tests (mean ± sem)

	Natural F_2		Sham-operated F_2		ROSI F_5	
	Male	Female	Male	Female	Male	Female
Straight alley						
Approach/withdrawal	1.00 ± 0.31	2.11 ± 0.63	1.80 ± 0.36	2.09 ± 0.64	1.73 ± 0.41	1.67 ± 0.51
Wall climb	2.26 ± 0.68	4.22 ± 1.10	3.20 ± 0.88	3.45 ± 0.79	3.36 ± 0.64	4.58 ± 0.78
Jump escape	1.14 ± 0.59	1.22 ± 0.55	1.40 ± 0.78	1.73 ± 0.71	2.91 ± 1.22	2.50 ± 0.75
Closest distance between animals (cm)	130.00 ± 20.59	108.89 ± 17.91	64.00 ± 15.65[b,c]	117.27 ± 12.66[b]	134.55 ± 10.12	142.08 ± 11.14
Flight	0	0	0.20 ± 0.13[a]	0.18 ± 0.12[a]	0	0
Contact	0.14 ± 0.14	0.22 ± 0.22	0.40 ± 0.31	0	0	0
Time out of first square (sec)	9.74 ± 3.13	16.00 ± 4.65	19.54 ± 4.61	12.66 ± 1.75	10.79 ± 2.36	12.3 ± 3.43
Immobility (sec)	4.29 ± 1.80	0.33 ± 0.33	3.20 ± 2.98	4.56 ± 2.18	1.64 ± 1.36	1.50 ± 1.25
Forced contact						
Vocalization	5.86 ± 1.24	9.11 ± 2.84[d]	4.90 ± 1.06	7.64 ± 1.39[d]	1.55 ± 0.55	7.25 ± 1.20[d]
Jump attack	1.00 ± 0.53	1.00 ± 0.47	1.80 ± 0.83	1.64 ± 0.68	2.45 ± 0.78	1.25 ± 0.37
Flight	1.71 ± 0.78	3.67 ± 1.11	2.60 ± 0.67	3.45 ± 0.81	2.91 ± 0.67	3.42 ± 0.70
Upright posture	2.86 ± 0.67	2.33 ± 0.75	2.20 ± 0.63	2.64 ± 0.72	1.09 ± 0.41	2.33 ± 0.58
Bite	0.57 ± 0.30	0.11 ± 0.10	0.20 ± 0.13	0.45 ± 0.21	0	0.25 ± 0.13
Jump escape	2.14 ± 0.94	2.67 ± 0.94	1.00 ± 0.47	1.73 ± 0.51	2.91 ± 0.65	2.67 ± 0.66

[a] Significantly different from the natural F_2 ($P < 0.05$) and ROSI F_5 groups ($P < 0.01$).
[b] Significantly different compared to the ROSI F_5 group ($P < 0.01$).
[c] Significantly different from males and females in the natural F_2 and ROSI F_5 groups and females in the sham-operated F_2 group.
[d] Significantly different from males ($P < 0.01$).

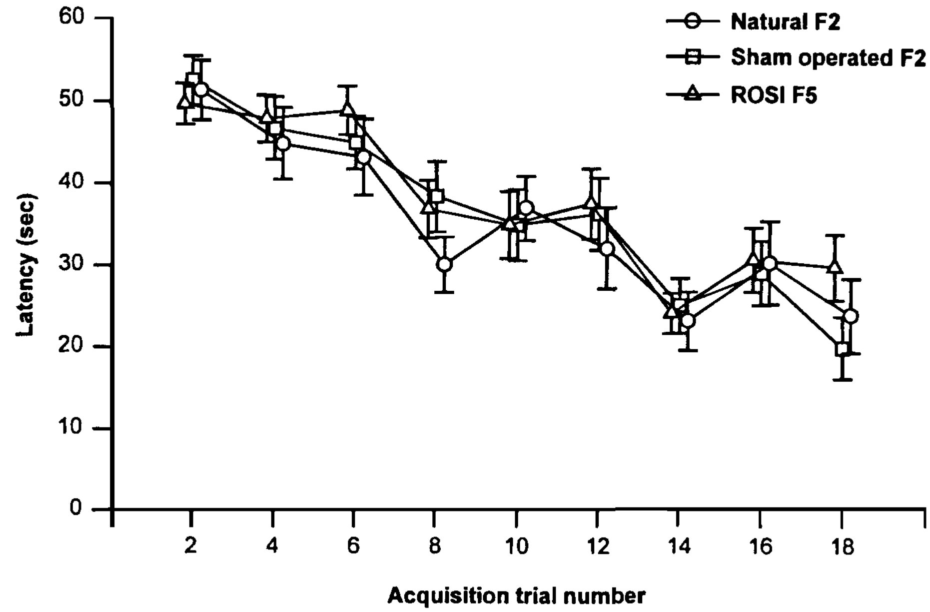

Fig. 5. Morris water maze: latency to find the hidden platform during acquisition trials.

cant group [$F(2,53) = 0.80$, $P < 0.46$] or gender [$F(1,53) = 0.30$, $P < 0.58$] differences.

Figure 7 shows the latencies to find the hidden platform in the reversal trials. There were no significant group differences [$F(2,53) = 0.62$, $P < 0.54$], but the difference between males and females was reliable [$F(1,53) = 18.55$, $P < 0.0001$], with males taking less time to find the hidden platform. The interaction between trials and sex was statistically significant [$F(5,265) = 2.31$, $P < 0.05$], with males significantly faster than females on the final trial (Newman–Keuls; $P < 0.05$).

DISCUSSION

There were no reliable or apparent differences between ROSI F_5 offspring and control F_2 groups in

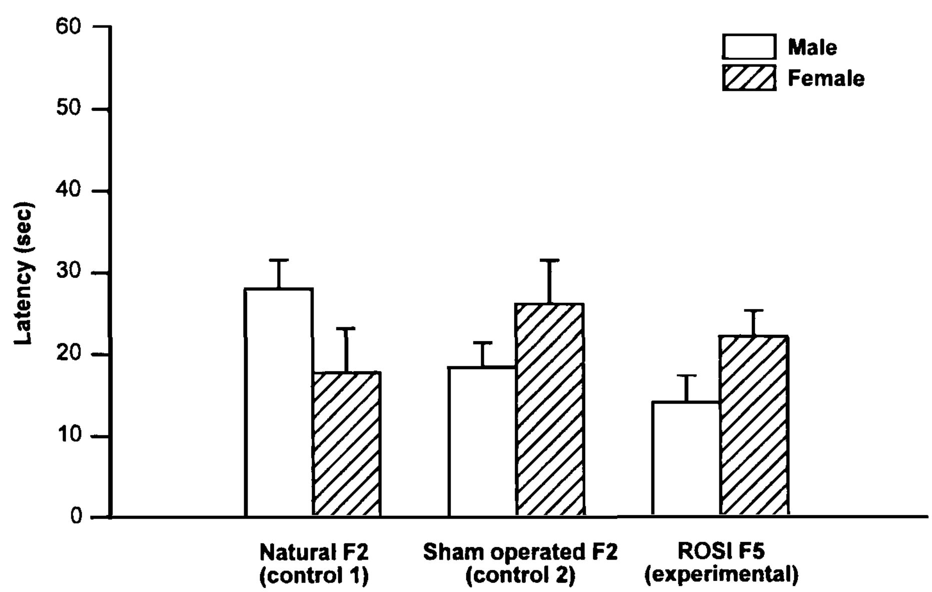

Fig. 6. Morris water maze: latency to find the hidden platform during retention trials.

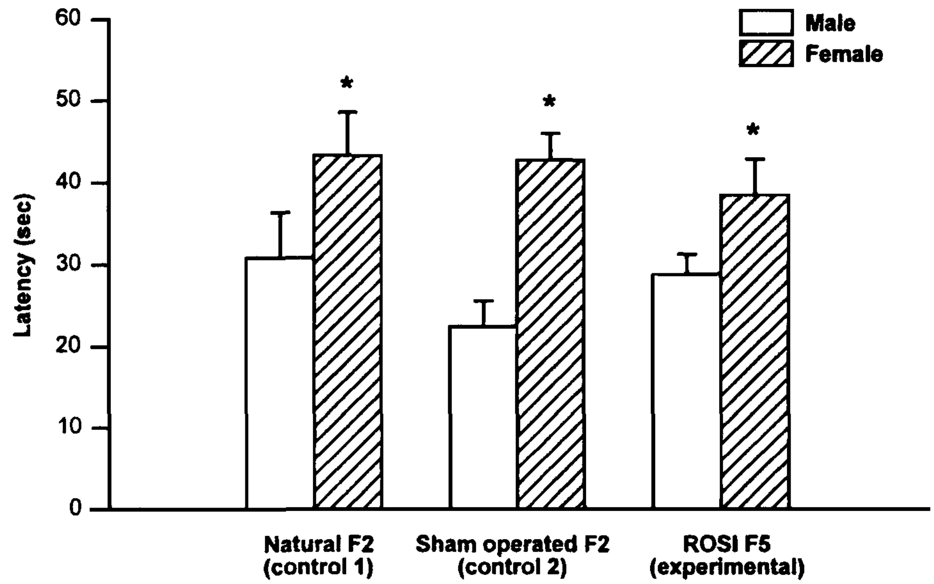

Fig. 7. Morris water maze: latency to find the hidden platform during reversal trials.

their growth rates and fertility. In addition, there was no evidence of learning deficiencies in any of the tests performed in this study.

There was no clear evidence of learning or performance deficit in the Krushinsky test. The increasing correct choice rate over successive sessions demonstrates reward conditioning by the mice. The mice learned that food would be available at one of the two possible choice positions in the apparatus. However, rather than making a choice based on what was observed in the central opening (i.e., direction of movement of the food cup from which milk was sipped), they moved to either of the choice positions with no evidence of discrimination. This is a behavior akin to that of animals in a straight alley test, in which learning is manifested by a decrease in the time it takes to traverse the alley to obtain food located at the end. However, the percentage of correct choices made remained approximately at the chance level of 50% for all mice throughout the Krushinsky test, indicating the lack of discrimination learning. Although there was no evidence of learning to make correct choices in the task, an increase in speed with time was indicated by a ratio improvement for all animal groups.

The MDTB and the Morris water maze are used more extensively in analyses of emotional response and spatial learning, respectively, in laboratory rodents. The results of the MDTB provided no evidence of differences in intensity of emotional responding among the three types of animals. Defensive behaviors measured were similar for all three groups, with the exception of reduced risk assessment and increased flight for the sham controls compared to the ROSI F_5 and natural F_2 controls, respectively. This pattern, an apparent shift in the relative prevalence of two defensive behaviors, is intriguing but does not suggest any deficiency in emotional responding for the ROSI F_5's.

The Morris water maze results provided no evidence that ROSI F_5 mice exhibit learning deficiencies. Short-term memory was measured by the decrease in latency to find the hidden platform over the initial 18 acquisition trials. The absence of significant differences between the groups on this task indicated that all groups were successful in demonstrating short-term memory of the position of the hidden platform and were able to use the information obtained in previous trials to improve their performance in successive trials. Long-term memory was assessed by use of a retention task and was satisfactorily completed by all three groups, with no significant variation among the groups. Spatial learning ability was also measured by employing a reversal task in which the mice were required to navigate using the same distal visual cues to a different hidden platform position. ROSI F_5 mice performed as well as or slightly (nonsignificantly) better than natural F_2 and sham-operated F_2 mice in all phases of the Morris water maze.

The complexity of the ROSI fertilization procedure and the goal of investigating the effects of ROSI in the fifth generation derived from this procedure limited the sample size in this study. There may be subtle differences that this study did not detect due to the limited sample size. However, the purpose of this experiment was to determine whether there were any gross abnormalities in growth, fertility, or behavior that could possibly be attributed to the omission of spermiogenesis and all prefertilization processes or the ROSI procedure.

Although the results of our experiments provide no evidence that the ROSI procedure itself yields cumulative, deleterious effects on the fertility and behavior of offspring, it is important to stress that normal, fertile animals were used in these experiments. As human ROSI is performed exclusively for infertile men with serious sperm production problems, these findings cannot be extrapolated directly to humans. Further studies must be carried out using animals with severe sperm production problems comparable to those seen in human patients. Human ROSI offspring need to be followed closely to ensure that assisting infertile patients does not yield serious problems in successive generations.

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