Normal Progeny Produced by the Parents Derived from Mouse Eggs Fertilized *In Vitro*

Kenkichi KASAI, Masahito SUGIMOTO and Yutaka TOYODA

School of Veterinary and Animal Sciences, Kitasato
University, Towada-shi 034

(Received June 20, 1979)

Abstract Superovulated eggs from F₁ (C3H/He×C57BL/6J-at/at) female mice fertilized *in vitro* by epididymal sperm of JCL:ICR strain were cultured for 72 hr, transferred to the uteri of pseudopregnant recipients and the reproductive capacity of the young born from the recipients was examined by full-sib mating. Fertilization rate was 80.9% (418/517) as judged from the second polar body extrusion at 6 hr after insemination. The developmental rate of these fertilized eggs to the morula and early blastocyst was 97% (393/405) after 72 hr in culture under 5% CO₂ in air. Transferred to the uteri of Day 3 or Day 4 pseudopregnant recipients were 245 of these embryos, resulting 29 male and 23 female normal newborn young. Of these young, 7 females were randomly selected and mated polygamously within the same litter at 2 to 3 months of age. All females became pregnant and delivered 37 males and 41 females. No gross anomalies were found among these offspring. Average body weight at 3 weeks of age was 12.2±2.7 g for males and 12.3±1.7 g for females. From these findings, it is suggested that normal progeny can be produced by parents derived from superovulated eggs fertilized *in vitro*.

Jap. J. Zootech. Sci., 50 (12): 885-890, 1979

The fertilization process has been accomplished *in vitro* in many species of mammals¹⁾. Criteria for normal fertilization, however, have not in most cases included the production of viable young following transfer of the embryos to suitable recipient females. We may conclude at this point that the development of eggs fertilized *in vitro* into viable young when embryos were transferred to pseudopregnant animals was confirmed only in rabbits²⁻⁷⁾, rats⁸⁾, mice⁹⁻¹⁴⁾ and most recently human¹⁵⁾.

None of the reports, however, have so far clarified the reproductive potentiality of the young derived from eggs fertilized *in vitro*.

The purpose of this paper is to investigate the reproductive capacity of female and male mice developed from eggs fertilized *in vitro*.

Materials and Methods

1) *In vitro* fertilization and culture of fertilized eggs

Virgin 2 to 6 months old F₁ (C3H/He×C57BL/6J-at/at) females were superovulated by injection of 5 IU PMSG (Peamez, Sankyo Zoki), followed 48 hr later by 5 IU hCG (Puberogen, Sankyo Zoki). Methods and medium for fertilization and subsequent culture *in vitro* used were as described by KASAI *et al.*¹⁰⁾. The females were killed 15-16 hr after the injection of hCG and their oviducts were removed and put into the

culture dish (35×10 mm, Falcon Plastic) containing 0.4 ml of medium under paraffin oil (Art 7160, Merck) maintained at a temperature of 37°C under 5% CO₂ in air. The wall of the ampullar portion of the oviduct was dissected with a needle and the eggs surrounded by cumulus cells were introduced into the medium. Sperm was obtained from the cauda epididymidis of 4 months old JCL:ICR male mice and suspended into 0.4 ml of the medium under paraffin oil. After preincubation for 1 hr at 37°C under 5% CO₂ in air, a small volume of sperm suspension was added to the medium containing eggs with a calibrated glass pipett according to TOYODA *et al.*¹⁹⁾. The final sperm concentration was adjusted to 150 cells/ μ l in each experiment. Following incubation for 6 hr, eggs which extruded second polar body were picked up from the medium under the dissecting microscope, washed twice and cultured in 0.2 ml drop-lets of medium under oil for 66 hr (total culture period: 72 hr). The fertilization rate was calculated by determining the proportion of eggs with second polar body of the total number of eggs examined. The preimplantation development rate was calculated by determining the proportion of cultured embryos which reached the morula and early blastocyst stage after 72 hr in culture. After cultivation, embryos to be transplanted were removed from culture dishes, washed once and then transferred to other dishes freshly prepared. These dishes were kept at 37°C under 5% CO₂ in air until transfer.

2) *Transfer of embryos to pseudopregnant mice*

Pseudopregnancy was induced by mating JCL:ICR females to sterility tested vasectomized JCL:ICR males in the night following the day of proestrous vaginal smear. Females which displayed continuous diestrous vaginal smear were used for the recipients on Day 3 or Day 4 pseudopregnancy (Day 1 is the day on which the vaginal plug was noticed). After anesthetizing the recipient with 60 mg sodium pentobarbital per kg body weight and exposing the oviducal end of the uterus through dorsal incision, a short glass rod was passed through the mesometrium in order to hold the uterin horn in place. The cranial end of the uterus was punctured with a 25-gauge needle and through the resulting hole, embryos suspended in a small amount of medium (0.5 μ l) were then introduced. This was achieved by using a fine glass pipett attached to a mouth piece. Six to 12 embryos were transplanted per recipient.

The remaining embryos were transferred into the 0.2 ml of culture medium containing colcemid (0.08 μ g/ml) under paraffin oil, cultured further for a few hours and then examined for their cell numbers by TARKOWSKI's method¹⁰⁾.

3) *Observation of progeny*

The recipients were examined daily for their vaginal smears. They were housed individually after a placental sign had been observed and allowed to deliver their young. Sex of the young was determined on the day following delivery. All of them developed normally with the exception of one died during nursing. When they reached 2 to 3 months of age, 7 females from 3 litters born from Day 3 recipients were randomly selected and mated polygamously within the same litter and observed for

conception and delivery. All young were left with their mothers. Their growth was observed during the nursing period for 3 weeks. Body weight was measured individually at 3 weeks of age.

Results and Discussion

1) *In vitro* fertilization and development in culture

The percentage of fertilization judged from the observation of second polar body extrusion at 6 hr after insemination was 80.9% (Table 1). Almost all of the eggs judged to be undergoing fertilization developed to the 2-cell stage within 24 hr after insemination (99.8%) and most of them (97.5%) proceeded to the 4-8-cell stage during 48 hr of culture. At the end of the culture period, 72 hr after insemination, 97.0% (393/405) of eggs developed to the morula and early blastocyst stage. The developmental rate to 4-8-cell stage was similar to our previous results¹⁰⁾. The rate of development to morula and early blastocyst, however, was much higher than earlier test results¹⁰⁾. It may have been caused by technical improvements. The developmental rate to the morula and early blastocyst was similar to the results of HOPPE and PITTS¹²⁾, in spite of our simple culture condition using a gas phase of 5% CO₂ in air, instead of 5% CO₂, 5% O₂, 90% N₂. Although the effectiveness of reduced oxygen partial pressure was substantiated for the embryonic development of several mammalian species¹³⁻¹⁶⁾, the present result seems to show that the gametes of F₁ female mice fertilize better *in vitro* and that more fertilized eggs are capable of developing to the blastocyst stage adapting themselves to the environmental condition during culture than those from inbred or randombred strains.

Table 1. Fertilization and preimplantation development *in vitro*.

No. of animals used	No. of fertilized eggs/ No. of eggs examined (%)	No. of eggs cultured	No. and (%) of eggs developed to		
			2-cell (24 hr) ^{a)}	4-8-cell (48 hr) ^{a)}	morula & early blastocyst (72 hr) ^{a)}
23	418/517 (80.9%)	405	404 (99.8%)	395 (97.5%)	393 (97.0%)

a): Culture period.

2) *Development of embryos following transfer*

The embryos were composed of a mean cell number of 24.4 ± 6.73 (Mean $\pm$ S. D., $n=88$) at the end of culture period for 72-80 hr. As shown in Table 2, 9/10 (90%) recipients which had received the embryos on Day 3 became pregnant and 41 young (34.2%), 27 males and 14 females were born from 8 recipients. On the other hand, in the case of Day 4 transfer, 5/11 (45%) recipients became pregnant and 11 young (8.8%), 2 males and 9 females were obtained from 5 recipients. A total of 52 (21.1%) offspring, 29 males and 23 females were born from 245 embryos transferred to 21 recipients. The difference in conception rate was not significant between Day 3 and

Table 2. Results of transplantation of morula and early blastocyst at days 3 and 4 of pseudopregnant recipients.

	No. of recipients used	No. of embryos transferred	No. of pregnant animals	No. of animals which delivered live young	No. of live born	
					♂	♀
Day 3	10	120	9	8	27 ^{a)}	14 ^{a)}
Day 4	11	125	5	4	2	9

a): Seven females and males from the same litter were used for progeny test (see Table 3).

Day 4 recipients ($P > 0.05$), but the proportion of embryos that developed to newborn young was significantly higher in Day 3 recipients than in Day 4 recipients ($P < 0.01$).

The appearance of a placental sign and the duration of pregnancy were 11.0 ± 0.7 days and 19.9 ± 1.2 days in Day 3 recipients, and 11.0 ± 0.7 days and 21.5 ± 0.6 days in Day 4 recipients, respectively. The gestation period of Day 4 recipients seemed to be prolonged as compared to the period of Day 3 recipients, but the difference was not significant ($P > 0.05$). Average body weight of the young at 21 days after delivery was 15.0 ± 2.3 and 13.4 ± 1.7 g in Day 3 and Day 4 recipients, respectively.

3) Reproductive capacity of the young

Seven females, derived from Day 3 recipients and mated to males of the same litter, became pregnant and delivered normal newborn young. The results are summarized in Table 3. Thirty seven males and 41 females were obtained from 7 females. The average litter size was 11.1. There was no significant difference between the

Table 3. Average body weight of progeny produced by the parents derived from eggs fertilized *in vitro*.

Female no. used for progeny test ^{a)}	No. of young born		Total	Av. body weight (g) and (range) 21 days after delivery			
	Male	Female		No.	Male	No.	Female
K-2-1-1	5	5	10	5	16.08 ± 0.86^b (14.8 to 17.2)	5	14.76 ± 0.69 (14.0 to 15.8)
K-2-1-2	6	4	10	6	10.57 ± 2.08 (8.7 to 14.2)	4	10.68 ± 1.26 (9.5 to 12.3)
K-3-1-1	9	4	13	9	10.84 ± 0.92 (9.6 to 12.2)	4	11.48 ± 0.78 (10.9 to 12.6)
K-3-1-2	4	9	13	4	12.00 ± 0.26 (11.7 to 12.3)	9	11.96 ± 0.66 (11.1 to 13.3)
S-2-5-1	4	4	8	4	16.15 ± 1.92 (13.7 to 18.0)	4	15.43 ± 0.66 (14.6 to 16.2)
S-2-5-2	5	9	14	5	10.2 ± 2.42 (6.2 to 12.9)	9	11.42 ± 0.79 (10.1 to 12.3)
S-2-5-3	4	6	10	4	11.4 ± 0.74 (10.9 to 12.5)	6	11.95 ± 0.62 (11.3 to 12.8)

a): All females were mated to males of the same litter. b): Mean $\pm$ S.D.

number of female and male young ($P > 0.05$). The average body weight at 21 days after delivery was 12.2 ± 2.7 g in males and 12.3 ± 1.7 g in females. No gross abnormalities were found among these young.

In the control experiment, 2-cell embryos were collected from the oviducts of mated females, cultured for 48 hr and 24 embryos developed to morula and early blastocyst were transferred to the uterine horns of 2 pseudopregnant recipients. Nine males and 5 females were obtained from the recipients. These young were mated polygamously within the same litter at 2 to 3 months of age. The average litter size was 11.2 ($n=5$) and average body weight at 3 weeks of age was 12.4 ± 1.5 g in male ($n=28$) and 12.0 ± 1.5 g in females ($n=28$). There was no significant difference between the experimental and control groups as to litter size and body weight.

The present results show that a normal progeny can be produced by the parents derived from eggs fertilized *in vitro*. This research has just been started and further studies are being conducted.

The authors wish to express their gratitude to Dr. K. MURAKAMI of Sankyo Zoki Company for the generous gifts of PMSG and hCG, and to Mrs. Hildegard HOWELL, M.A. for her kindness in reading and revising the manuscript. This study was supported by a grant-in-aid for scientific research No. 248074 from the Ministry of Education, Science and Culture of Japan, and a grant aided by the Ministry of Health and Welfare of Japan for research on handicapped children.

References

- 1) ROGERS, B. J., Gamete Res., 1: 165-223. 1978.
- 2) CHANG, M. C., Nature, 184: 466-467. 1959.
- 3) BRACKETT, B. G. and G. OLIPHANT, Biol. Reprod., 12: 260-274. 1975.
- 4) BRACKETT, B. G., J. A. MILLS and G. G. JEITLES, Fertil. Steril., 23: 898-909. 1972.
- 5) FRASER, L. R. and P. V. DANDEKAR, J. Exp. Zool., 184: 303-312. 1973.
- 6) MILLS, J. A., G. G. JEITLES and B. G. BRACKETT, Fertil. Steril., 24: 602-608. 1973.
- 7) SEIDEL, G. E., R. A. BOWEN and M. T. KANE, Fertil. Steril., 27: 861-870. 1976.
- 8) TOYODA, Y. and M. C. CHANG, J. Reprod. Fert., 36: 9-22. 1974.
- 9) WHITTINGHAM, D. G., Nature, 220: 592-593. 1968.
- 10) CROSS, P. C. and R. L. BRINSTER, Biol. Reprod., 3: 298-307. 1970.
- 11) MIYAMOTO, H. and M. C. CHANG, J. Reprod. Fert., 30: 135-137. 1972.
- 12) MUKHERJEE, A. B., Nature, 237: 397-398. 1972.
- 13) HOPPE, P. C. and S. PITTS, Biol. Reprod., 8: 420-426. 1973.
- 14) MUKHERJEE, A. B. and M. M. COHEN, Nature, 228: 472-473. 1970.
- 15) STEPTOE, P. C. and R. G. EDWARDS, The Lancet, ii: 366: 1978.
- 16) KASAI, K., Y. MINATO and Y. TOYODA, Japan. J. Anim. Reprod., 24: 19-22. 1978.
- 17) TOYODA, Y., M. YOKOYAMA and T. HOSI, Japan. J. Anim. Reprod., 16: 147-151. 1971.
- 18) TARKOWSKI, A. K., Cytogenetics, 5: 394-400. 1966.
- 19) AUERBACH, S. and R. L. BRINSTER, Nature, 217: 465-466. 1968.
- 20) QUINN, P. and G. M. HARLOW, Theriogenology, 8: 161. 1977.
- 21) TERVIT, H. R., D. G. WHITTINGHAM and L. E. A. ROWSON, J. Reprod. Fert., 30: 493-497. 1972.
- 22) TERVIT, H. R. and L. E. A. ROWSON, J. Reprod. Fert., 28: 177-179. 1974.
- 23) WRIGHT, R. W., G. B. ANDERSON, P. T. CUPPS and M. DROST, Biol. Reprod., 14: 157-162. 1976.
- 24) WRIGHT, R. W., J. Anim. Sci., 44: 854-855. 1977.
- 25) DAVIS, D. L. and B. N. DAY, J. Anim. Sci., 46: 1043-1053. 1978.
- 26) QUINN, P. and G. M. HARLOW, J. Exp. Zool., 206: 73-80. 1978.

体外受精に由来するマウス間の交配による 正常産子について

笠井健吉・杉本正仁・豊田 裕

北里大学獣医畜産学部，十和田市 034

体外受精卵の移植によって得られた雌および雄マウスの繁殖能力について検討した。PMSG 及び hCG (各 5 iu) を投与して過排卵を誘起した F₁ (CSH/He×C57BL/6J-at/at) 成熟雌マウスを卵子提供雌とし，一方，精子提供雄としては，JCL:ICR 系成熟雄マウスを用いて体外受精を行った。授精後6時間に第2極体放出の有無で判定した受精率は，80.9% (418/517) であった。培養した受精卵 406 個のうち受精後 24 時間で 2 細胞，48 時間で 4-8 細胞及び 72 時間で桑実胚あるいは初期胚盤胞へ発生したものはそれぞれ，99.8%，97.5%，及び 97.0% であった。体外培養によって得られた桑実胚及び初期胚盤胞 245 個を 21 匹の偽妊娠雌マウスの子宮へ移植した

結果，雄 29 匹及び雌 23 匹の合計 52 匹の新生子が得られた。生後 3 週で離乳した後に，7 匹の雌とその同腹子の雄を選び 2-3 か月齢できようだい交配を行った。7 匹の雌マウスのすべてが妊娠し，雄 37 匹及び雌 41 匹の健康な新生子が得られた。外形異常は認められず，3 週齢での雄，雌の平均体重はそれぞれ， 12.2 ± 2.7 g 及び 12.3 ± 1.7 g (Mean $\pm$ S. D.) で対照区と差のない成績であった。本研究の結果から，体外受精卵の移植によって得られた子の成熟後の交配，妊娠，分娩及び哺乳に関する一連の繁殖能力は正常であることが明らかにされた。

日畜会報，50 (12)：885-890，1979