In vitro Fertilization of Mouse and Hamster Eggs After Freezing and Thawing

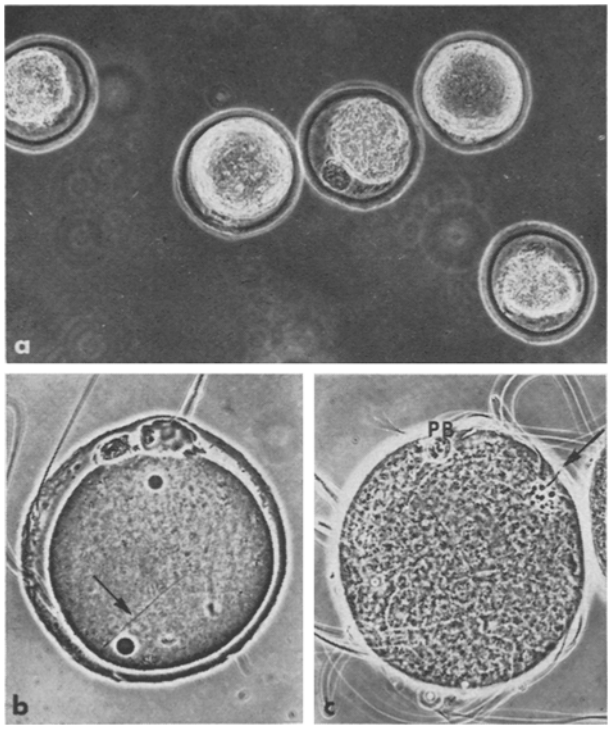
Y. Tsunoda, T. A. Parkening and M. C. Chang[1]

Worcester Foundation for Experimental Biology, Shrewsbury (Massachusetts 01545, USA), 1 September 1975.

Summary. Mouse eggs (12–14%) and hamster eggs (87-94%) appear normal upon thawing after having been stored for 30 min to 25 h at −70 °C and −196 °C. While 89–98% of the normal appearing hamster eggs are capable of fertilization in vitro, only 4–13% of the mouse eggs can be fertilized. The ability of fertilized eggs to develop in culture and their viability upon transfer to pseudopregnant recipients is under investigation.

Since the report by Whittingham[2] on the survival of mouse embryos after freezing and thawing, development of frozen embryos transferred to recipient mothers has been described for the mouse[3–5], rabbit[6,7] and cow[8]. Although fertilization in vivo of mouse eggs frozen at −10 °C has been reported[9], freezing of unfertilized eggs at much lower temperatures has not been examined. This paper describes the procedures and results of successful in vitro fertilization of thawed mouse and hamster eggs frozen to −70 °C and −196 °C.

Mature female CD-1 Swiss albino mice and golden hamsters were superovulated by an i.p. injection of 5-25 IU PMSG (donated by NIH) 44–48 h before an injection of 5–25 IU HCG. The females were killed 15 to 16 h after an injection of HCG. The ampullary portion of the oviducts was placed under paraffin oil in a Falcon plastic dish and the eggs in cumulus were dissected out and placed in a phosphate buffered solution (PBS) containing 0.05% hyaluronidase. After the dissolution and dispersal of cumulus and follicular cells the denuded eggs were washed 3 times in PBS and placed in 0.10 ml PBS in a plastic tube (10 × 75 mm). The tubes were then cooled to 0 °C at 0.5 °C min^{-1} and 0.05 ml dimethyl sulfoxide (DMSO) kept at 0 °C was added 3 times at intervals of 10 min (final concentration of DMSO was 1.0 to 2.0 *M*). After 15 min the samples were cooled to −4 to −5 °C and induced to freeze by placing a cooled hypodermic needle into the medium. After 5 min they were cooled to −70 to −79 °C at 0.33 °C min^{-1} by adding dry ice to 95% ethanol. The samples were either maintained in ethanol-dry ice or transferred directly to liquid nitrogen for 30 min to 25 h. For thawing, the tubes were placed in 60 ml ethanol in a 200 ml graduated cylinder or in a 15 × 150 mm pyrex test tube at −70 to −110 °C. The cylinder or pyrex tube was then removed to room temperature and allowed to reach 0 °C at a warming rate of approximately 4 °C min^{-1} or 12 °C min^{-1}. At 0 °C the tubes were removed to crushed ice and, as soon as the suspending medium had thawed, PBS, in the amount of 0.2, 0.2 and 0.4 ml was added at 1 min intervals. The egg suspension was placed in a watch glass and rinsed with a modified Krebs-Ringer bicarbonate solution containing bovine serum albumin for the mouse eggs[10], and Tyrode's solution containing 20% γ human serum (North American Biologicals, Inc.) for the hamster eggs[11]. Next, the washed eggs were introduced respectively to these media in which sperm were preincubated for 1–3 h for the mouse, or 4–5 h for the hamster. The preparations were then placed in an incubator at 37 °C saturated with 5% CO_2 in air for in vitro fertilization. 5 to 11 h after incubation the eggs were mounted in toto on a slide, stained with 0.25% lacmoid[12] and examined for evidence of fertilization. 'Penetrated eggs' denotes all the eggs that had perivitelline spermatozoa and those undergoing fertilization or fertilized. The eggs which had enlarged sperm head(s) or male pronucleus(ei) with fertilizing sperm tail(s) were considered as 'undergoing fertilization'.



a) Mouse eggs frozen to −75 °C and maintained at this temperature for 30 min before thawing. While some eggs remained normal in appearance, others were in a state of deterioration. × 175. b) A mouse egg frozen to −75 °C was fertilized in vitro after thawing. The male and female pronuclei are visible together with the fertilizing sperm tail (arrow). × 240. c) A hamster egg frozen to −196 °C was fertilized in vitro after thawing. This egg was polyspermic but only 1 fertilizing sperm tail with its pronucleus is visible at this focal plane (arrow). The second polar body (PB) is present and should not be mistaken for a pronucleus. × 265.

[1] This work was supported by grants from the U.S. Public Health Service and the Ford Foundation. Sincere thanks are due to Prof. Y. Nishikawa of Kyoto University, Japan, for advice and to Mrs. Rose Bartke and Ms. D. M. Hunt for assistance.

[2] D. G. Whittingham, Nature, Lond. *233*, 125 (1971).
[3] D. G. Whittingham, S. P. Leibo and P. Mazur, Science *178*, 411 (1972).
[4] I. Wilmut, Life Sci. *11*, part II, 1071 (1972).
[5] R. R. Maurer and H. Bank, Cryobiology *10*, 509 (1973).
[6] M. Ferdows, C. L. Moore and A. E. Dracy, J. Dairy Sci. *41*, 739 (1958).
[7] H. Bank and R. R. Maurer, Expl Cell Res. *89*, 188 (1974).
[8] I. Wilmut and L. E. A. Rowson, Vet. Rec. *92*, 686 (1973).
[9] J. K. Sherman and T. P. Lin, Proc. Soc. exp. Biol. Med. *98*, 902 (1958).
[10] Y. Toyoda, M. Yokoyama and T. Hosi, Jap. J. Anim. Reprod. *16*, 147 (1971).
[11] R. Yanagimachi, Biol. Reprod. *3*, 147 (1970).
[12] M. C. Chang, J. exp. Zool. *121*, 351 (1952).

In vitro fertilization of frozen-thawed mouse and hamster eggs

Animal	Final temperature	No. of eggs frozen	No. of normal eggs after thawing (%)	No. of normal eggs inseminated	No. of eggs penetrated (%)	No. of eggs undergoing fertilization or fertilized (%)	No. of eggs penetrated by more than one sperm	
							Total (%)	Polyspermic (%)
Mouse	−70 to −79 °C	569	79 (14)	75	19 (25)	10 (13)	8 (42)	0
	−196 (liquid N_2)	227	27 (12)	25	1 (4)	1 (4)	0	0
Hamster	−70 to −79 °C	126	119 (94)	66	65 (98)	65 (98)	65 (100)	65 (100)
	−196 (liquid N_2)	188	164 (87)	88	79 (90)	78 (89)	79 (100)	78 (99)

From the results presented in the Table, it is clear that after freezing and thawing only 12–14% of the unfertilized mouse eggs appeared to be intact and normal (Figure a) and only 4–13% of the apparently normal mouse eggs can be fertilized in vitro (Figure b). In contrast, 87–94% of the unfertilized hamster eggs appeared to be normal after freezing and thawing and 89–98% of these eggs can be fertilized (Figure c), but polyspermic fertilization was high since most eggs were penetrated by more than 5 spermatozoa. One might speculate whether the high proportion of hamster eggs fertilized after freezing and thawing is correlated with the survival of hamsters exposed to low temperatures[13].

Although the recovery rate of normal appearing hamster eggs was high after freezing, the high incidence of polyspermy suggested that only a small percentage might be capable of normal cleavage. No attempt to study further cleavage was made, since successful methods have not yet been developed for culturing hamster eggs beyond the 2-cell stage. Some normal appearing frozen-thawed mouse eggs fertilized in vitro have been successfully cultured beyond the 2-cell stage, but only one egg developed to a blastocyst. Studies are currently underway to improve techniques for freezing mouse eggs so that larger numbers of normal eggs can be fertilized and cultured to obtain blastocysts for transfer to pseudopregnant females. Only after achieving term fetuses or newborn will the potential of freezing unfertilized mammalian eggs be fully realized.

[13] A. U. SMITH, Proc. R. Soc. B147, 533 (1957).

Deciduoma Formation in Rats with Cornified Vagina

K. TAKEWAKI and Y. OHTA[1]

Endocrinology Laboratory, Department of Biology, Kawasaki Medical College, Kurashiki, Okayama-ken 701-01 (Japan),
26 August 1975.

Summary. In female rats given a single injection of 1.25 mg 5α-dihydrotestosterone during neonatal life, reactivity of the vaginal mucosa to hormones was permanently altered, so that the rats, when adult, could show vaginal cornification under the hormonic conditions supportive of development of deciduomata in the uterus.

It seems to be generally accepted that in female rats placed under hormone conditions which support development and maintenance of deciduomata, cornification of the vaginal epithelium cannot take place. While studying deciduoma formation in response to uterine trauma in adult rats treated neonatally with sex steroids, we found that, in those which had been ovariectomized and treated with 5α-dihydrotestosterone (DHT) during neonatal life, deciduoma formation in the uterus and cornification of the epithelium in the vagina could be induced simultaneously by administration of progesterone (P) plus oestradiol-17β (OD) combined with traumatization of the endometrium.

Eight T-strain rats ovariectomized on day 4 of postnatal life (the day of birth = day 1), under cold anaesthesia, received a single s.c. injection of 1.25 mg DHT (Sigma Chemical Co., St. Louis, Mo., USA) in 0.05 ml sesame oil on day 5 (OX-DHT-rats), while 8 other rats likewise ovariectomized on day 4 were not injected with DHT (OX-rats). From day 60 on, both groups of rats were given s.c. injections of 0.2 μg OD for 3 consecutive days, followed by combined injections of 2 mg P and 0.2 μg OD in 0.15 ml oil over a period of 7 days commencing 2 days after deprivation of OD. On the 4th day of P-OD injections, the antimesometrial wall of the right uterine horn of each animal was scratched longitudinally along its entire length with a hooked needle inserted into its lumen[2]. The contralateral uterine horn was left untouched.

In 4 of the 8 OX-DHT-rats, the vagina was patent on day 50. Vaginal smears remained leucocytic until day 60 when OD priming was started. Either on the day of, or following the last injection, all rats had the vaginal aperture giving smears dominated by cornified cells. On the first 1 or 2 days of the 7-day period of P-OD injections, vaginal smears were of the dioestrous type, but thereafter

[1] We wish to thank Prof. T. KIMOTO of Kawasaki Medical College for valuable advice and information.

[2] K. TAKEWAKI, Annotns. zool. japon. *42*, 126 (1969).