

DEVELOPMENT OF HAMSTER EGGS FERTILIZED *IN VITRO* OR *IN VIVO*

D. G. WHITTINGHAM AND B. D. BAVISTER*

Physiological Laboratory, University of Cambridge, Cambridge CB2 3EG

(Received 18th December 1973)

Although many workers have fertilized hamster eggs *in vitro* since the technique was first described by Yanagimachi & Chang (1963), there is only one reported attempt to obtain further development of these eggs in culture (Yanagimachi & Chang, 1964). In this instance, development ceased at the two-cell stage. The present study examines the development in culture of hamster eggs fertilized *in vitro* and after transfer to recipient foster mothers.

Immature female hamsters (5 to 6 weeks old and 60 to 80 g in weight) were induced to superovulate with intraperitoneal injections of 25 i.u. PMSG and HCG given 48 to 56 hr apart. Following removal of the oviducts 15 to 17 hr after the injection of HCG, they were blotted on sterile filter paper to remove excess blood and immersed in liquid paraffin contained in a Petri dish (35 mm in diameter, Falcon Plastics). The eggs and surrounding cumulus cells were released by rupturing the ampullae and the oviducts were discarded. The culture medium used was a modified Tyrode's solution containing 112.40 mM-NaCl, 2.20 mM-KCl, 1.48 mM-CaCl₂, 0.41 mM-MgCl₂, 0.29 mM-NaH₂PO₄, 4.56 mM-glucose, 36.08 NaHCO₃, 0.10 mM-sodium pyruvate, 9 mg bovine serum albumin/ml, and 100 i.u. sodium penicillin/ml. This medium, designated Tyrode-B2, had an osmotic pressure of 285 to 290 mosmols, and a pH of 7.6 after equilibration with 5% CO₂ in air at 37°C. Approximately 2 to 3 vols of Tyrode-B2 were added to each vol. of eggs and cumulus, and the contents of the drop were gently mixed with a sterile needle. Epididymal sperm suspensions were prepared from the caudae of selected males (4 to 6 months of age and 90 to 100 g in weight). The epididymides were removed, immersed in paraffin oil contained in a Petri dish, and the epididymal contents were released by incising the distal portion of the duct at several points. Under the dissecting microscope, a small drop of the epididymal contents was picked up with watchmaker's forceps and inserted into the drop of medium containing eggs and cumulus. When the concentration of spermatozoa in the drop was judged to be sufficient, usually between 0.5 and 4.5 × 10⁵ spermatozoa/ml, the remainder of the drop of epididymal contents was withdrawn and discarded. After final mixing with a sterile needle, the eggs and spermatozoa were incubated at 37°C in an atmosphere of 5% CO₂ in air.

After 24 hr, a total of 257 out of 429 eggs (60%) had reached the two-cell

* Present address: Department of Anatomy, University of Hawaii Medical School, 1960 East-West Road, Honolulu, Hawaii 96822, U.S.A.

stage (Table 1) but no further development occurred when the culture period was extended, confirming the earlier findings of Yanagimachi & Chang (1964). Only two out of sixty-eight ova (3%) cleaved in control experiments in which ova were incubated without spermatozoa; one egg had two equal blastomeres, each with an apparently normal nucleus, and the other egg had divided into two unequal blastomeres with no nuclei. Many of the two-cell ova from the inseminated groups were examined and all had a sperm tail in the vitellus. For comparison, one-cell eggs fertilized *in vivo*, obtained between 10.00 and 11.00 hours on the day after coitus from naturally mated hamsters, were cultured in a modified Tyrode's medium (T6: Whittingham, 1971a), previously used to culture eight-cell hamster ova to the blastocyst stage (D. G. Whittingham, unpublished results). Forty-one out of fifty-seven eggs (72%) cleaved within 24 hr (Table 1) but no further development occurred *in vitro*.

Table 1. Development after 24 hr in culture of hamster eggs fertilized *in vitro* or *in vivo*

<i>Fertilization</i>	<i>Medium</i>	<i>No. of one-cell ova cultured</i>	<i>No. of two-cell ova after 24 hr in culture</i>	<i>%</i>
<i>In vitro</i>	Tyrode-B2	429	257	60
Control (no sperm. added)	Tyrode-B2	68	2*	3
<i>In vivo</i>	Tyrode-T6	57	41	72

* One two-cell ovum with equal blastomeres and one two-cell ovum with unequal blastomeres.

Since development of one-cell hamster eggs fertilized *in vitro* or *in vivo* was limited to one cleavage division *in vitro*, the culture conditions were assumed to be adverse for testing the normality of the eggs fertilized *in vitro*. In a second series of experiments, hamster eggs fertilized either *in vitro* or *in vivo* were transferred to the oviducts of pseudopregnant females on the day after coitus with vasectomized males. The data are summarized in Table 2. Initially, a total of 147 two-cell ova which had been fertilized *in vitro* were transferred, after washing with Tyrode-B2 medium, to the oviducts of eight animals (eight to nine ova/oviduct); all the ova recovered 48 hr later (ninety-four) had degenerated at the two-cell stage. Thirty-six two-cell ova flushed from donor oviducts with T6 medium were transferred to the oviducts of three animals (six ova/oviduct). None of the sixteen eggs recovered 48 hr later had developed to the blastocyst stage, but seven had proceeded to the four- to eight-cell stage; the remainder were still at the two-cell stage and six out of nine were degenerate when recovered. By contrast, when forty-six two-cell ova flushed from donor oviducts with PB1 medium (a modified Dulbecco's phosphate-buffered medium with a stable pH of 7.2: Whittingham, 1971b) were transferred to the oviducts of five animals (four to six eggs/oviduct), seventeen out of twenty-nine ova (59%) recovered 48 hr later had developed to the blastocyst stage.

In order to determine whether the two-cell eggs fertilized *in vitro* had been damaged by prolonged exposure to Tyrode-B2, eggs were transferred at the pronucleate stage of development. Seventy-eight pronucleate eggs which had

been fertilized *in vitro* were washed and transferred in PB1 medium but none of the fifty-seven eggs (this total included a proportion of native unfertilized eggs) recovered 48 hr later had cleaved. Finally, all three media were compared for recovery and transfer of pronucleate eggs which had been fertilized *in vivo*. All the eggs recovered 72 hr after manipulation in Tyrode-B2 and 48 hr after manipulation in T6 medium had degenerated at the one-cell stage (85/128 and 6/6 respectively). However, pronucleate eggs flushed and transferred in PB1 medium continued development; twelve out of fifteen (80%) had reached the four- to eight-cell stage when recovered 48 hr later. The comparison of T6 and PB1 was carried out in the same recipient; six pronucleate eggs flushed with PB1 were transferred to the right oviduct and six pronucleate eggs flushed with T6 were transferred to the left oviduct. At recovery 48 hr later, the six ova from the right oviduct were at the four- to eight-cell stage and the six ova from the left oviduct had degenerated.

Table 2. Development of hamster ova fertilized *in vitro* or *in vivo* following their transfer to pseudopregnant females

Fertilization	Developmental stage at transfer	Medium for manipulating ova during transfer	No. of ova transferred	Time in recipient oviduct (hr)	No. of ova recovered	No. of four- to eight-cell ova	No. of blastocysts
<i>In vitro</i>	Two-cell	Tyrode-B2	147	48	94*	0	0
<i>In vivo</i>	Two-cell	Tyrode-T6	36	48	16	7	0
		PB1	46	48	29	0	17
<i>In vitro</i>	One-cell (pronucleate)	PB1	78	48	57†	0	—
<i>In vivo</i>	One-cell (pronucleate)	Tyrode-B2	128	72	85*·†	0	0
		Tyrode-T6	6	48	6*·†	0	—
		PB1	15	48	24†	12	—

* All degenerate.

† Includes both native and transferred ova.

The present series of experiments indicate that Tyrode's bicarbonate-buffered medium has a detrimental effect upon further development of fertilized hamster eggs. Sato & Yanagimachi (1972) also found that one- and two-cell hamster eggs fertilized *in vivo* degenerated in recipient oviducts when recovered and transferred in a bicarbonate-buffered medium (TC 199). The finding that a phosphate-buffered medium could obviate this failure suggests that the deleterious effect on the early hamster embryos is the result of the uncontrolled pH of the bicarbonate-buffered medium when it is exposed to air. The failure of hamster eggs fertilized in modified Tyrode's solution to undergo more than one cleavage division may therefore be due to the high pH (7.6) which was found to be optimal for fertilization in this medium (Bavister, 1969). Human eggs fertilized *in vitro* in a similar modified Tyrode's solution do not appear to show this sensitivity since a proportion develop to the morula and blastocyst stages in culture (Edwards, Steptoe & Purdy, 1970; Steptoe, Edwards & Purdy, 1971). However, the optimum pH for development of mouse eggs fertilized in a

modified Krebs–Ringer bicarbonate medium (Whittingham, 1971a) is between pH 7.2 and 7.4 (D. G. Whittingham, unpublished observations).

In conclusion, we have shown that hamster eggs, whether fertilized *in vitro* or *in vivo*, are highly sensitive to culture conditions, especially pH. We believe that this finding may explain why hamster eggs that have been fertilized in Tyrode–B2 fail to develop beyond the two-cell stage, even though the process of fertilization appears morphologically normal. Evidently, the optimal conditions for cleavage *in vitro* differ significantly from those suitable for fertilization *in vitro*.

We thank Professor C. R. Austin for his valuable advice, and we are grateful to Mrs Jane Donaldson for her technical assistance. This investigation was supported by a grant from the Ford Foundation. One of us (D.G.W.) was the recipient of a Beit Memorial Fellowship, and B.D.B. received personal support from the World Health Organization.

REFERENCES

- BAVISTER, B. D. (1969) Environmental factors important for *in vitro* fertilization in the hamster. *J. Reprod. Fert.* **18**, 544, Abstr.
- EDWARDS, R. G., STEPTOE, P. C. & PURDY, J. M. (1970) Fertilization and cleavage *in vitro* of pre-ovulatory human oocytes. *Nature, Lond.* **227**, 1307.
- SATO, A. & YANAGIMACHI, R. (1972) Transplantation of preimplantation hamster embryos. *J. Reprod. Fert.* **30**, 329.
- STEPTOE, P. C., EDWARDS, R. G. & PURDY, J. M. (1971) Human blastocysts grown in culture. *Nature, Lond.* **229**, 132.
- WHITTINGHAM, D. G. (1971a) Culture of mouse ova. *J. Reprod. Fert.*, Suppl. **14**, 7.
- WHITTINGHAM, D. G. (1971b) Survival of mouse embryos after freezing and thawing. *Nature, Lond.* **233**, 125.
- YANAGIMACHI, R. & CHANG, M. C. (1963) Fertilization of hamster eggs *in vitro*. *Nature, Lond.* **200**, 281.
- YANAGIMACHI, R. & CHANG, M. C. (1964) *In vitro* fertilization of golden hamster ova. *J. exp. Zool.* **156**, 361.