THE ROLE OF CALCIUM IONS IN FERTILIZATION OF MOUSE AND RAT EGGS *IN VITRO*

H. MIYAMOTO AND T. ISHIBASHI

*Department of Animal Science, College of Agriculture,
Kyoto University, Kyoto, Japan*

(Received 14th April 1975)

In attempts to define suitable conditions for the in-vitro fertilization of mammalian eggs, it has been shown that the osmolality of the medium affects the fertilization rate of mouse and hamster eggs (Miyamoto & Chang, 1973b), and the pH values of the medium affect the fertilization rate of hamster eggs (Bavister, 1969; Miyamoto *et al.*, 1974), mouse eggs (Iwamatsu & Chang, 1971; Miyamoto *et al.*, 1974) and rat eggs (Miyamoto *et al.*, 1974). Iwamatsu & Chang (1971) showed that calcium ions affect the capacitation of spermatozoa and fertilization of mouse eggs *in vitro* in the presence of bovine follicular fluid. It has recently been shown that guinea-pig spermatozoa fail to fertilize eggs in calcium-free media (Yanagimachi & Usui, 1974). The achievement of fertilization *in vitro* by epididymal spermatozoa of mice (Toyoda *et al.*, 1971) and rats (Toyoda & Chang, 1974) has made it possible to define suitable conditions for in-vitro fertilization of these eggs in a defined physico-chemical environment. The present study was designed to determine if calcium ions have a particular role to play in the fertilization *in vitro* of mouse and rat eggs in a chemically defined medium.

Mature female DDN mice and immature female Wistar rats were induced to superovulate and their eggs were recovered according to the procedures reported elsewhere (Miyamoto & Chang, 1973a; Toyoda & Chang, 1974). Eggs in the cumulus clot were washed three times in 1 ml of a test medium in order to remove the oviducal fluid. The medium used in the present experiment was essentially the same as that used by Toyoda & Chang (1974), except that the concentration of CaCl_2 was varied. In some cases, the cumulus egg clot was placed in a watch glass containing 0.5 ml of 0.003% hyaluronidase in a test medium. After the removal of the cumulus cells, the hyaluronidase-treated eggs were washed three times.

A mouse sperm suspension was prepared by mincing the cauda epididymidis of a mature male DDN mouse in a watch glass containing 2 ml test medium. A rat sperm suspension was prepared by placing spermatozoa obtained from the cauda epididymidis of a mature male Wistar rat in 0.5 ml test medium covered with paraffin oil. About 0.02 ml of fresh sperm suspension was added to 0.5 ml test medium containing eggs under paraffin oil in a plastic dish. In some cases, mouse spermatozoa were preincubated for 2 hr in a medium containing 1.71 mM- CaCl_2 to induce capacitation before mixing with eggs (Toyoda *et al.*, 1971) and then 0.01 ml capacitated sperm suspension was placed in 0.5 ml medium

with (1.71 mM) and without calcium. The suspension of the gametes was incubated at 37°C in an atmosphere of 5% CO₂ in air. After incubation for 5–7 hr, the eggs were taken out, mounted on a slide and examined. They were then fixed with neutral formalin and stained with lacmoid for the assessment of fertilization (Miyamoto & Chang, 1973a).

Table 1. Effect of the concentration of calcium ions on the fertilization of mouse and rat eggs *in vitro*

Calcium concentration in the medium (mM)	No. of exps	% of motile sperm. at end of incubation	No. of eggs examined	No. of eggs penetrated (%)	No. of eggs undergoing fertilization (%)
Mouse					
0	6	5–20	116	0 (0)	0 (0)
0	4	5–20	77*	0 (0)	0 (0)
0.34	6	20–50	104	14 (13)	4 (4)
0.86	6	30–60	125	60 (48)	51 (41)
1.71	6	40–70	95	79 (83)	72 (76)
1.71	4	40–70	85*	61 (72)	52 (61)
5.13	6	5–30	126	15 (12)	11 (9)
8.55	6	5–10	102	0 (0)	0 (0)
Rat					
0	6	5–10	84	0 (0)	0 (0)
0	4	5–10	68*	0 (0)	0 (0)
0.34	4	5–40	81	6 (7)	5 (6)
1.71	6	30–60	73	40 (55)	34 (47)
1.71	4	30–70	79*	32 (41)	27 (34)
5.13	4	10–30	65	6 (9)	3 (5)
8.55	4	0–5	82	0 (0)	0 (0)

* Cumulus cells were removed before insemination.

The results of the experiments are shown in Table 1. When mouse or rat spermatozoa were incubated with eggs in calcium-free medium, the proportion of motile spermatozoa was decreased at the end of incubation. The proportion of motile spermatozoa at the end of incubation was high in the presence of calcium. No mouse eggs were penetrated *in vitro* in the absence of calcium ions whether the cumulus cells were removed or not, and thus calcium ions appear to be necessary for the fertilization of mouse eggs *in vitro* in a chemically defined medium. Capacitation of spermatozoa and penetration of mouse eggs *in vitro* occurred in media of 0.34 to 5.13 mM calcium. The proportions of penetrated mouse eggs and those of eggs undergoing fertilization were significantly higher ($P < 0.01$) when the gametes were incubated in media of 1.71 mM than in media of 0.34 to 0.86 mM or of 5.13 to 8.55 mM calcium. It seems that the most suitable calcium concentration for fertilization *in vitro* of mouse eggs under the present experimental conditions was 1.71 mM, as in the case of Krebs–Ringer bicarbonate solution. Higher (5.13 and 8.55 mM) or lower (0.34 and 0.86 mM) concentrations of calcium may have an inhibitory effect on sperm penetration. These results confirm the statement by Iwamatsu & Chang (1971) that the concentration of calcium ions affects the capacitation of mouse spermatozoa and the penetration of mouse eggs *in vitro* in the presence of bovine follicular fluid. When epididymal spermatozoa were incubated with eggs in calcium-free

Tyrode's solution containing bovine follicular fluid, a low proportion (2.2%) of mouse eggs underwent fertilization (Iwamatsu & Chang, 1971). This may be due to calcium contained in mouse oviducal fluid and bovine follicular fluid (Lutwak-Mann, 1954).

When the mouse eggs in a medium with and without calcium were inseminated with capacitated spermatozoa which had been preincubated in a medium containing calcium, none of 75 eggs was penetrated in a calcium-free medium, and 58 (87%) of 67 eggs were penetrated and 52 (78%) were undergoing fertilization in a medium containing 1.71 mM calcium. This result shows that, even if mouse epididymal spermatozoa are capacitated by preincubation in a medium containing calcium ions before mixing with the eggs, they are unable to penetrate through the zona pellucida in a calcium-free medium. Thus calcium ions in a medium play an important role in the fertilization of mouse eggs *in vitro*.

As for mouse (present study) and guinea-pig (Yanagimachi & Usui, 1974) eggs, rat eggs were not penetrated *in vitro* in a calcium-free medium (Table 1). Capacitation of spermatozoa and penetration of rat eggs *in vitro* occurred in media of 0.34 to 5.13 mM calcium. The proportions of penetrated rat eggs and those of eggs undergoing fertilization were significantly higher ($P < 0.01$) when gametes were incubated in media of 1.71 mM than in media of 0.34, 5.17 or 8.55 mM calcium. As for mouse eggs, calcium ions in a medium are necessary for capacitation of spermatozoa and fertilization of rat eggs *in vitro* and a concentration of 1.71 mM was found to be the most suitable of these examined in the present study.

The absence of calcium is known to prevent development of eight-cell mouse embryos (Whitten, 1957), and two-cell mouse embryos cultured in calcium-deficient media had a decreased rate of cleavage (Wales, 1970). The incidence of activation, as well as of fertilization, of mouse and rat eggs in the absence of calcium was examined in the present study. The proportions of spontaneously activated eggs which had a pronucleus(ei) after incubation for 5–7 hr in media with (1.71 mM) and without calcium were 4% and 70%, respectively, in mouse eggs and 6% and 67% in rat eggs. Since calcium affects various enzymes important in energy metabolism (Wyatt, 1964), the action of calcium in the incubation medium may be on the general metabolic activity of the eggs.

REFERENCES

- BAVISTER, B.D. (1969) Environmental factors important for *in vitro* fertilization in the hamster. *J. Reprod. Fert.* **18**, 544–545.
- IWAMATSU, T. & CHANG, M.C. (1971) Factors involved in the fertilization of mouse eggs *in vitro*. *J. Reprod. Fert.* **26**, 197–208.
- LUTWAK-MANN, C. (1954) Note on the chemical composition of bovine follicular fluid. *J. agric. Sci., Camb.* **44**, 477–480.
- MIYAMOTO, H. & CHANG, M.C. (1973a) The importance of serum albumin and metabolic intermediates for capacitation of spermatozoa and fertilization of mouse eggs *in vitro*. *J. Reprod. Fert.* **32**, 193–205.
- MIYAMOTO, H. & CHANG, M.C. (1973b) Effect of osmolality on fertilization of mouse and golden hamster eggs *in vitro*. *J. Reprod. Fert.* **33**, 481–487.
- MIYAMOTO, H., TOYODA, Y. & CHANG, M.C. (1974) Effect of hydrogen-ion concentration on *in vitro* fertilization of mouse, golden hamster, and rat eggs. *Biol. Reprod.* **10**, 487–493.
- TOYODA, Y. & CHANG, M.C. (1974) Fertilization of rat eggs *in vitro* by epididymal spermatozoa and the development of the eggs following transfer. *J. Reprod. Fert.* **36**, 9–22.

- TOYODA, Y., YOKOYAMA, M. & HOSI, T. (1971) Studies on the fertilization of mouse eggs *in vitro*. II. Effects of *in vitro* pre-incubation of spermatozoa on time of sperm penetration of mouse eggs *in vitro*. *Jap. J. Anim. Reprod.* **16**, 152–157.
- WALES, R.G. (1970) Effects of ions on the development of the pre-implantation mouse embryo *in vitro*. *Aust. J. biol. Sci.* **23**, 421–429.
- WHITTEN, W.K. (1957) Culture of tubal mouse ova. *Nature, Lond.* **177**, 96.
- WYATT, H.V. (1964) Cations, enzymes and control of cell metabolism. *J. theor. Biol.* **6**, 441–470.
- YANAGIMACHI, R. & USUI, N. (1974) Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. *Expl Cell Res.* **89** 161–174.