In vitro fertilization and embryo development of Japanese field voles (*Microtus montebelli*)

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Optimal conditions for *in vitro* fertilization of Japanese field voles (*Microtus montebelli*) were analysed. The medium used was a modified Krebs–Ringer bicarbonate devised for *in vitro* fertilization in rats. Ovulated eggs and epididymal spermatozoa were co-incubated *in vitro* at 37°C under 5% CO₂ in air for 6 h, and the eggs were fixed with 2.5% (w/v) glutaraldehyde, stained with 0.25% (v/v) acetolacmoid and examined for evidence of fertilization at the pronuclear stage. Although the fertilization rate with spermatozoa preincubated at $1\text{--}2 \times 10^8$ cells ml⁻¹ for 2 h was very low (1–13%), it was significantly increased (43–51%, $P < 0.05$) when spermatozoa were preincubated at a lower concentration ($1\text{--}2 \times 10^7$ cells ml⁻¹). Furthermore, the fertilization rate was significantly higher with 1 mmol hypotaurine l⁻¹ (74.0%) than without hypotaurine (44.4%, $P < 0.05$). Fertilization rates of spermatozoa preincubated at $1\text{--}2 \times 10^7$ cells ml⁻¹ for 0.5 or 2 h were similar (69.0% and 73.6%), but a longer preincubation (10 h) resulted in a significantly lower fertilization rate (56.8%, $P < 0.01$). Vole spermatozoa preincubated for 2 h penetrated the zona pellucida 2 h after insemination, and the sperm heads became decondensed 3 h after insemination. At 6 h after insemination, male and female pronuclei were found in most penetrated eggs. When the eggs were left in the fertilization medium without washing and cultured for 96 h after insemination, they developed to two-cell (82.6%), four-cell (60.9%), eight-cell (23.2%) and morula/blastocyst (8.7%) stages in modified Krebs–Ringer bicarbonate supplemented with 1 mmol hypotaurine l⁻¹.

Introduction

In vitro fertilization (IVF) techniques contribute to improvement in domestic animal breeding, alleviation of infertility, and conservation of endangered species. Furthermore, IVF permits visual observation of direct interactions between spermatozoa and eggs. Thus, the IVF technique is valuable from many perspectives.

Although the Japanese field vole (*Microtus montebelli*), a herbivorous rodent, is considered a potential model for herbivorous domestic animals (Kudo and Oki, 1984; Imai and Ogimoto, 1988), its reproductive characteristics have not been thoroughly investigated. Goto *et al.* (1977) and Goto and Hashizume (1978) reported that voles exhibit a copulatory ovulation and that vaginal smears show no regular pattern. Wakayama *et al.* (1993, 1994) demonstrated that one- and early two-cell vole embryos cannot be cultured to the blastocyst stage, but early two-cell embryos in pyruvate-free M16 or late two-cell embryos in M16 can. They also reported that vole spermatozoa can penetrate the zona pellucida of mouse and hamster eggs *in vitro*. The present study examined media

composition, sperm concentration and capacitation time, and the time required to complete fertilization, to clarify optimal conditions for vole IVF. The development of IVF eggs was also evaluated.

Materials and Methods

Animals

Japanese field voles were obtained from the National Institute of Animal Health (Japan), where this species had been maintained for over 20 years by outbred mating. The voles were housed under controlled conditions of 14 h light:10 h dark at 25°C. They were fed a commercial mouse chow, a herbivore food, (CMF and ZF, respectively, Oriental East Co., Tokyo) and cubed hay. Food and water were available *ad libitum*.

Culture media

IVF medium was prepared according to the method of Toyoda and Chang (1974) with modifications as described

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Table 1. Effect of concentrations of Japanese field vole spermatozoa during preincubation and insemination on fertilization *in vitro*

Concentration of spermatozoa		Number of fertilized eggs					Unfertilized eggs	
Preincubation (cells ml ⁻¹)	IVF (cells ml ⁻¹)	Number of voles	Number of eggs	Total (%)	Monospermic eggs (%)	Polyspermic eggs (%)	Parthenogenetic eggs (%)	Abnormal eggs (%)
1–2 × 10 ⁸	1 × 10 ⁷	10	105	1 (1.0) ^a	1 (1.0)	0 (0)	0 (0)	1 (1.0)
	1 × 10 ⁶	10	93	12 (12.9) ^b	11 (11.8)	1 (1.1)	2 (2.2)	1 (1.1)
1–2 × 10 ⁷	1 × 10 ⁶	10	105	53 (50.5) ^c	67 (45.7)	5 (4.8)	1 (1.0)	3 (2.9)
	1 × 10 ⁵	10	90	39 (43.3) ^c	39 (43.3)	0 (0)	2 (2.2)	4 (4.4)

Data from five replicates are pooled.

Values with different superscripts are significantly different ($P < 0.05$).

below. All media were filter sterilized. For preincubation (0.6 ml) and insemination (0.2 ml) of spermatozoa, media were placed in the centre of a 35 mm Petri dish and immediately covered with mineral oil. Media were equilibrated under 5% CO₂ in air for at least 2 h at 37°C.

The optimal concentration of spermatozoa was determined in Expt 1 using mKRB medium for preincubation, fertilization and embryo culture. For Expt 2, which examined composition of the media, hypotaurine (Sigma Chemical Co., St Louis, MO) was dissolved in mKRB (without BSA) and the stock solution kept at 100 mmol l⁻¹ at -20°C. Medium supplemented with or without 1 mmol hypotaurine l⁻¹ was used for all three IVF phases. BSA (Sigma Chemical Co.) at 4 mg ml⁻¹ or 10 mg ml⁻¹, with or without hypotaurine (1 mmol l⁻¹), was used for all IVF phases during investigation of preincubation of spermatozoa (Expt 3) and timing of *in vitro* fertilization (Expt 4). The preimplantation development of eggs that remained in the fertilization medium (mKRB with 10 mg BSA ml⁻¹ and 1 mmol hypotaurine l⁻¹) or that were washed and cultured in either mKRB or pyruvate-free mKRB with 1 mmol hypotaurine l⁻¹ was evaluated in Expt 5.

Egg collection

Immature female voles (4–6 weeks of age), 20–30 g body mass, were injected with 30 iu pregnant mares' serum gonadotrophin (PMSG) and 30 iu hCG at a 44 h interval and killed 14–15 h after hCG injection. Oviducts were removed, and the ampullae placed in a Petri dish with mineral oil. Eggs with follicular cells were dissected from the ampullae and deposited in 0.2 ml fertilization medium under oil.

Collection, concentration and preincubation of spermatozoa

Nineteen sexually mature (3 months of age) male voles were killed by cervical dislocation. The epididymides were excised and washed in mKRB to remove any traces of blood. After cutting the epididymal ducts with a pair of sharp iridectomy scissors, one to three drops of the dense spermatozoa mass were placed in 0.6 ml medium. The concentration of spermatozoa was determined with a haemocytometer.

For Expt 1, sperm suspensions of 1–2 × 10⁷ or 1–2 × 10⁸ cells ml⁻¹ were preincubated for 2 h under 5% CO₂ in air at 37°C. After preincubation, each suspension was added to

different Petri dishes that contained eggs in IVF medium, so that the final concentration for insemination was 1 × 10⁵ or 1 × 10⁶ (1–2 × 10⁷ cells ml⁻¹ at preincubation) and 1 × 10⁶ or 1 × 10⁷ cells ml⁻¹ (1–2 × 10⁸ cells ml⁻¹ at preincubation). Expts 2–5 were conducted with 1–2 × 10⁷ cells ml⁻¹ for a 2 h preincubation and 1 × 10⁶ cells ml⁻¹ for insemination. In Expt 3, vole spermatozoa were preincubated for 0.5, 2 or 10 h.

Fertilization and development of IVF eggs

For Expts 1, 2 and 3, eggs and spermatozoa were co-cultured for 6 h under 5% CO₂ in air at 37°C, and then eggs were examined for penetration by spermatozoa and pronuclei formation. For Expt 4, eggs were examined 1, 2, 3, 4, 5 or 6 h after insemination. If eggs incubated for 1 and 2 h contained cumulus cells, these were removed by treatment with 0.1% (w/v) hyaluronidase. Whole mount egg preparations were examined under a phase-contrast microscope after they were fixed with 2.5% (w/v) glutaraldehyde for 2 h at room temperature and stained with 0.25% (v/v) acetolacmoid. Eggs that contained an enlarged sperm head, or one or more male pronuclei with sperm tails and second polar bodies, were judged to be fertilized.

Preimplantation development was examined (Expt 5) by washing some eggs 6 h after insemination and then placing them in 0.2 ml mKRB or pyruvate-free mKRB with 1 mmol hypotaurine l⁻¹. The remaining eggs were left in the fertilization medium without washing. All eggs were then cultured under 5% CO₂ in air at 37°C for 96 h after insemination. Development was observed under a dissecting or phase-contrast microscope.

Statistical analysis

Each experiment was carried out at least four times. The data were analysed by the chi-squared test.

Results

Effect of concentration of spermatozoa

As shown in Table 1, when spermatozoa were preincubated at a high concentration (1 × 10⁸ cells ml⁻¹) and then inseminated at concentrations of 1 × 10⁷ or 1 × 10⁶ cells ml⁻¹,

Table 2. Effect of hypotaurine and BSA in mKRB medium on fertilization of Japanese field vole eggs *in vitro*

Media		Number of fertilized eggs					Unfertilized eggs	
BSA (mg ml ⁻¹)	Hypotaurine (1 mmol l ⁻¹)	Number of voles	Number of eggs	Total (%)	Monospermic eggs (%)	Polyspermic eggs (%)	Parthenogenetic eggs (%)	Abnormal eggs (%)
4	—	10	75	28 (37.3) ^a	27 (36.0)	1 (1.3)	0 (0)	1 (1.3)
4	+	10	106	79 (74.5) ^b	79 (74.5)	0 (0)	1 (0.9)	5 (4.7)
10	—	10	105	52 (49.5) ^a	52 (49.5)	0 (0)	0 (0)	5 (4.8)
10	+	10	91	67 (73.6) ^b	64 (70.3)	3 (3.3)	3 (3.3)	2 (2.2)

Data from five replicates are pooled.

Values with different superscripts are significantly different ($P < 0.05$).

Table 3. Effect of preincubation time of Japanese field vole spermatozoa on fertilization *in vitro*

Preincubation time of spermatozoa (h)	Number of voles	Number of eggs	Number of fertilized eggs			Unfertilized eggs	
			Total (%)	Monospermic eggs (%)	Polyspermic eggs (%)	Parthenogenetic eggs (%)	Abnormal eggs (%)
0.5	27	268	185 (69.0) ^a	180 (67.2)	5 (1.9)	0 (0)	2 (0.7)
2	10	140	103 (73.6) ^a	101 (72.1)	2 (1.4)	2 (1.4)	8 (5.7)
10	10	185	105 (56.8) ^b	101 (54.6)	4 (2.2)	1 (0.5)	9 (4.9)

Data from five to nine replicates are pooled.

Values with different superscripts are significantly different ($P < 0.01$).

the fertilization rates were very low (1.0% and 12.9%, respectively). However, when the concentration was reduced to 1×10^7 cells ml⁻¹ at preincubation and to 1×10^6 or 1×10^5 cells ml⁻¹ at insemination, the fertilization rates were significantly higher at 50.5% and 43.3%, respectively ($P < 0.05$).

Effect of hypotaurine and BSA

Inseminating eggs in the presence of hypotaurine significantly increased the fertilization rate (74.5% in 4 mg BSA ml⁻¹ in mKRB and 73.6% in 10 mg BSA ml⁻¹ in mKRB) compared with that in the absence of hypotaurine (37.3 and 49.5%, respectively) (Table 2).

Effect of preincubation time

As shown in Table 3, when spermatozoa were preincubated for 0.5 or 2 h, the fertilization rate was significantly higher (69.0% and 73.6%, respectively) than for the 10 h preincubation (56.8%).

In all experiments, the fertilized eggs had one female pronucleus, one or more male pronuclei and penetrating sperm tails (Fig. 1b). The sperm tail was never incorporated inside the vitelline membrane of egg. The percentage of polyspermy was low (0–5%). Parthenogenetic eggs with only one pronucleus and without a sperm tail were also rare (0–3%).

Time of penetration and fertilization

Vole sperm penetration of the zona pellucida starts around 2 h (8.1%) and 75% are penetrated within 4 h after insemina-

tion (Table 4). Decondensation of sperm head(s) commences at about 3 h (16.9%; enlarged sperm head; Fig. 1a) and finishes within 6 h (86.8%; male and female pronuclei; Fig. 1b) after insemination.

Development *in vitro*

As shown in Table 5, most eggs cleaved to the two-cell stage (76.9–82.6%, Fig. 1c). Although eggs that were washed 6 h after insemination rarely developed to the four-cell stage in mKRB and pyruvate-free mKRB medium (9.6% and 4.6%, respectively), the non-washed eggs developed to at least the four-cell stage (60.9%, $P < 0.01$). In all experiments, a few (1.9–8.7%) eggs developed to the blastocyst stage (Fig. 1d) by 96 h after insemination.

Discussion

In the study reported here an IVF technique for Japanese field voles was established. IVF embryos developed to the blastocyst stage in the defined medium. Optimal concentration of spermatozoa and addition of hypotaurine to the medium made vole IVF highly repeatable.

Hypotaurine is found at high concentrations in many tissues, including the male and female reproductive tracts (Van der Horst and Brand, 1969; Van der Horst and Kuipfer, 1972), and in blood serum (Jacobsen and Smith, 1968) and preimplantation embryos (Schultz *et al.*, 1981). Mrsny *et al.* (1979) and Meizel

Fig. 1. (a) Enlarged sperm head (arrow) and early male pronucleus (arrowhead) together with corresponding sperm tail in vole eggs cultured for 4 h after insemination. The sperm tail is not incorporated inside the vitelline membrane of eggs. Scale bar represents 30 μm . (b) A pronuclear vole egg inseminated *in vitro* and fixed 6 h later. The egg has two pronuclei (PN), a sperm tail (arrowhead) and a second polar body (PB). Scale bar represents 20 μm . (c) Vole embryos at the two-cell stage cultured for 24 h after insemination. Scale bar represents 60 μm . (d) Vole embryos at the blastocyst stage cultured for 96 h after insemination. Scale bar represents 30 μm .

Table 4. *In vitro* fertilization by Japanese field vole spermatozoa preincubated for 2 h

Incubation time (h)	Number of eggs	Number (%) penetrated	Number fertilized			
			Total (%)	Stage I	Stage II	Polyspermy
1	69	0 (0)	0 (0)	0	0	0
2	74	6 (8.1)	1 (1.4)	1	0	0
3	77	34 (44.2)	13 (16.9)	13	0	0
4	84	63 (75.0)	60 (71.4)	22	38	2
5	84	73 (86.9)	71 (84.5)	10	61	2
6	91	79 (86.8)	79 (86.8)	3	76	5

Data from four replicates are pooled.
Stage I: eggs with enlarged sperm head in vitellus.
Stage II: eggs with male and female pronuclei.

et al. (1980) reported that taurine and hypotaurine may play roles in the maintenance and stimulation of sperm motility and in the stimulation of capacitation and acrosome reactions *in vivo*.

At a high concentration (1×10^8 cells ml^{-1}), the motility and survival rate of Japanese field vole spermatozoa increased (Wakayama *et al.*, 1993) but the fertilization rate decreased. Even at a low concentration of spermatozoa, a marked

Table 5. *In vitro* culture of Japanese field vole IVF eggs

Treatment of embryo	Number of embryos	Conceptus development (%)				
		≥ 2-Cell	≥ 4-Cell	≥ 8-Cell	≥ Morula	≥ Blastocyst
Non-washed	69	57 (82.6)	42 (60.9) ^a	16 (23.2) ^a	6 (8.7)	6 (8.7)
Washed	52	40 (76.9)	5 (9.6) ^b	3 (5.8) ^b	3 (5.8)	3 (5.8)
Washed and PF-mKRB	65	52 (80.0)	3 (4.6) ^b	1 (1.9) ^b	1 (1.9)	1 (1.9)

Data from four replicates are pooled.

Values with different superscripts are significantly different ($P < 0.05$).

PF-mKRB: pyruvate-free Krebs–Ringer bicarbonate.

improvement in fertilization rate was achieved by addition of hypotaurine to mKRB medium. Therefore, as in hamsters, hypotaurine may act to maintain and stimulate the motility and capacitation of spermatozoa in field voles.

In general, preincubated spermatozoa undergo capacitation in culture medium, although the time required for capacitation varies among species. For example, 1–2 h is required for mouse spermatozoa (Toyoda *et al.*, 1971), 2.5 h for hamsters (Yanagimachi, 1970), 5–7 h for rats (Niwa and Chang, 1974), and 8–12 h for guinea-pigs (Yanagimachi, 1972). In the study reported here, vole spermatozoa preincubated for 0.5 h penetrated vole eggs and male pronuclei were formed 6 h after insemination. However, vole spermatozoa preincubated for 2 h could penetrate the zona pellucida within 2 h after insemination and completely formed male pronuclei 6 h after insemination. It is suggested that vole epididymal spermatozoa require a very short period of preincubation for capacitation when the sperm concentration is 1×10^6 cells ml^{-1} .

Rabbits exhibit a copulatory ovulation and their ovulation occurs within 10 h after mating. Rabbit epididymal spermatozoa require 10 h for capacitation in a defined medium (Hosoi *et al.*, 1981; Niwa *et al.*, 1983). Although cats also exhibit induced ovulation and ovulate at 24–28 h after mating (Greulich, 1934), the capacitation time is short for spermatozoa in the ductus deferens and epididymis (Bowen, 1977; Niwa *et al.*, 1985). In Japanese field voles, ovulation occurs within 12 h after mating; however, the sperm capacitation time is as short as in cats. It is not clear why the required time for capacitation in voles is so short when compared with the period between mating and ovulation.

Japanese field vole embryos can be cultured from the late two-cell to blastocyst stage (64%) using M16 medium (Whittingham, 1971). Although most one- and early two-cell embryos did not develop to the blastocyst stage (2 and 14%, respectively), early two-cell embryos cultured in pyruvate-free M16 developed to blastocysts at a significantly higher rate (42%, $P < 0.05$, Wakayama *et al.*, 1994). In the present study, IVF vole eggs developed to the two-cell stage in mKRB medium with hypotaurine (76.9–82.6%), but eggs that were washed after insemination developed to the four-cell stage at very low rates in mKRB and pyruvate-free mKRB media (9.6 and 4.6%, respectively). However, when the IVF eggs were cultured with inseminating spermatozoa for 96 h without washing, the development rate to the four- and eight-cell stages was significantly higher (60.6% and 23.2%, respectively, $P < 0.01$), although the rate of development to the blastocyst

stage remained very low (8.7%). Because few differences are found in M16 and mKRB medium, vole embryos might be affected by washing. Although Barmett and Bavister (1992) and Reed *et al.* (1992) reported that hamster and pig embryos require hypotaurine for *in vitro* development, this does not appear to be the case in vole embryos.

Japanese field vole eggs can be fertilized and cultured *in vitro* as easily as those of rats and hamsters. Therefore, they may become a useful experimental model for reproductive and developmental biology.

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