Development of early-stage embryos of the Japanese field vole, *Microtus montebelli*, *in vivo* and *in vitro*

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Although ovulation could be easily induced in the Japanese field vole by administering pregnant mares' serum gonadotrophin and hCG, the number of embryos obtained varied from 1 to 47 (mean, 9.6). One-cell embryos were small (57.8–63.3 μm in diameter; mean, 61.0 μm) compared with those in other mammals. Development of the preimplantation vole embryos *in vivo* was similar to that of mouse embryos. The first cleavage occurred between 24 and 26 h after mating. The second cleavage was between 46 and 52 h after mating, and subsequent cleavages occurred at about 12 h intervals. Blastocysts were clearly observed in the uterus 4 days after mating. Vole embryos could be cultured *in vitro* from the late two-cell to the blastocyst stage in M16 medium. However, development of one-cell and early two-cell embryos *in vitro* was limited, and few cleaved beyond the four-cell stage. Eliminating sodium pyruvate from M16 medium significantly improved the development of early two-cell embryos into blastocysts ($P < 0.05$). The Japanese field vole may be a useful experimental animal for reproductive biology, comparable with the mouse.

Introduction

The Japanese field vole, *Microtus montebelli*, is a herbivorous rodent known to be a potential animal model for herbivorous domestic animals (Kudo and Oki, 1984; Imai and Ogimoto, 1988; Obara and Goto, 1988). Although the field vole has been reported to have a digestive tract characteristic of this group of animals, little is known about its reproduction. Goto and Hashizume (1978) and Goto *et al.* (1977) reported that the Japanese field vole exhibits a copulatory ovulation and that its vaginal smear does not indicate a regular oestrous pattern. In a previous study (Wakayama *et al.*, 1993), we demonstrated that vole spermatozoa can penetrate the zona pellucida of mice and hamster eggs. Knowledge of embryonic development *in vivo* plus the ability to support cleavage *in vitro* permits the study of the physiology of early embryos in a defined environment, and also facilitates the determination of storage and transport requirements for embryo transfer and manipulation. In the present study, we examined the preimplantation development of early-stage Japanese field vole embryos *in vivo* and *in vitro*.

Materials and Methods

Animals

The Japanese field voles used in this study had been maintained by outbred mating at the National Institute of Animal Health, Tsukuba for more than 15 years. The voles

were housed at 25°C under a controlled photoperiod of 14 h light:10 h dark. The animals were fed commercial mouse diets (CMF, Oriental East Co., Ltd, Tokyo), a herbivorous diet (ZF, Oriental East Co., Ltd, Tokyo), and cubed hay. Food and water were given *ad libitum*.

Induction of ovulation and recovery of embryos

Sexually mature female voles ($n = 74$) were injected i.p. with 75 iu pregnant mares' serum gonadotrophin (PMSG) (Adachi *et al.*, 1993) at 13.00 h, followed by 30 iu hCG at 06.00 or 13.00 h 2 days later. Each animal was placed with a fertile male for 1 h, during which mating occurred. Mated females were then separated from the males, and embryos were recovered by flushing the reproductive tracts 12–92 h after mating. If unfertilized or one-cell eggs were found to contain cumulus cells, they were treated with 0.1% (w/v) hyaluronidase to remove the cumulus cells. M16 medium (Whittingham, 1971) was used to flush the tracts and wash the embryos. The diameters of fertilized one-cell embryos and unfertilized oocytes (i.e. vitellus alone) were measured within 22 h after mating using the calibrated eyepiece micrometer of a microscope.

Culture conditions

About 20 embryos were cultured in 3 ml fresh M16 medium in plastic Petri dishes (diameter 35 mm) at 37°C, under 5% CO₂ in air. Their developmental stage was examined every 24 h. Some early two-cell embryos (recovered within 26 h after

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Fig. 1. Comparative sizes of one-cell embryos in Japanese field voles and mice. Although the vole embryos are smaller (61.0 μm in diameter) than mouse embryos, the zona pellucida is thicker than in mice. Scale bar represents 20 μm .

mating) were cultured for the first 24 h in M16 medium supplemented with 100 $\mu\text{mol EDTA l}^{-1}$ (M16-EDTA) or in M16 without sodium pyruvate (M16-PF). The developmental rates of embryos in the different media were compared statistically using the chi-squared test.

Results

Ovulation

Ovulation occurred in voles after administration of PMSG and hCG, but the number of ovulated eggs was lower than that in the mouse and hamster. Of 74 females given both PMSG and hCG, 62 (83.8%) mated, and no embryos were found in eight mated females. A total of 519 embryos was obtained from the remaining 54 females (a mean of 9.6 ± 5.3 embryos per female). The number of embryos in each female varied from 1 to 47. The diameter of one-cell embryos (vitellus alone) ranged from 57.8 to 63.3 μm (a mean of $61.0 \pm 1.3 \mu\text{m}$; $n = 10$; number of embryos = 58; Fig. 1).

Development in vivo

Eggs were ovulated within 12 h and remained at the one-cell stage until 22 h after mating (Table 1). The first cleavage

occurred between 24 and 26 h. Four-cell embryos were first counted after 46 h and constituted 46.7% of all embryos obtained between 46 and 52 h. These results indicate that the second cleavage takes place between 46 and 52 h after mating. Thereafter, cleavages occurred about every 12 h. Blastocysts were found in the uterus after 94 h.

Development in vitro

The development of one-cell and early two-cell embryos (collected within 26 h after mating) *in vitro* was limited and few cleaved beyond the four-cell stage (only 14% developed to blastocysts) (Table 2). However, the late two-cell embryos collected 45 h after mating developed readily to blastocysts (64%) in M16 medium. A higher proportion of four-cell and eight-cell embryos also developed to blastocysts (89% and 91%, respectively). Although many blastocysts expanded, none hatched in M16 medium.

In an attempt to overcome the block to development at the early two-cell stage, early two-cell embryos were cultured in M16 supplemented with EDTA (M16-EDTA) or in pyruvate-free M16 (M16-PF). Only 8–18% of the embryos cultured in M16 and M16 plus EDTA developed to blastocysts (Table 3). In M16-PF, development of the embryos to blastocysts was significantly increased to 42%.

Table 1. Development of Japanese field vole embryos *in vivo*

Time (h) after mating	Number of voles	Number of eggs	Developmental stage						
			One-cell	Two-cell	Four-cell	Eight-cell	Morula	Blastocyst	Abnormal
12	2	35	35						0
21–22	3	26	25	1					0
24–26	5	98	24	64					10
45–46	4	19		13	2				4
48–49	4	52		21	25				6
50–52	3	20		7	9				4
60–62	5	33			4	16			13
72–73	2	25				10	15		0
94	1	9					5	4	0

Table 2. Development of Japanese field vole embryos *in vitro*

Collection time after mating (h)	Stage of embryos	Number of embryos	Number of developing embryos (%)			
			≥ Two-cell	≥ Four-cell	≥ Eight-cell	≥ Blastocyst
12–22	One-cell	44	36 (82)	5 (11)	1 (2)	1 (2)
24–26	Two-cell (early)	44		17 (38)	7 (16)	6 (14)
45–52	Two-cell (late)	28		24 (82)	19 (64)	19 (64)
45–62	Four-cell	35			34 (97)	31 (89)
60–73	Eight-cell	22				20 (91)

Table 3. Culture of early two-cell embryos of the Japanese field vole *in vitro*

Medium	Number of voles	Number of embryos	Number of developing embryos (%)		
			≥ Four-cell	≥ Eight-cell	≥ Blastocyst
M16	4	28	17 (61) ^b	5 (18) ^a	5 (18) ^a
M16-EDTA	8	66	19 (29) ^a	5 (8) ^a	5 (8) ^a
M16-PF	8	65	47 (72) ^b	28 (43) ^b	27 (42) ^b

^{a,b}Values with different superscripts are significantly different ($P < 0.05$).

Data from four replicates were pooled.

PF: pyruvate-free.

Discussion

Ovulation may be induced in the Japanese field vole by administration of PMSG and hCG. Adachi *et al.* (1993) reported that the mean number of eggs obtained was highest (9.1 eggs, including abnormal eggs) when 30 iu hCG was injected 48 h after 75 iu PMSG. In the present study, the interval between the injections of the two hormones was 42 h yielding an average of 24.0 eggs per animal. The vole colony used in this study was genetically diversified (outbred), which might have caused a greater variability in the number of ovulations (1–47).

Blastocysts are found *in vivo* 4 days after hCG injection in mice, after 3.5 days in hamsters, and after 5 days in rats. Mystkowska (1975a) reported that the preimplantation devel-

opment of bank voles, *Clethrionomys glareolus*, resembles that of mice. The development of Japanese field vole embryos is similar to that of mice and bank voles.

Vole embryos can be cultured from the late two-cell to the blastocyst stage in M16, a medium devised for mouse embryos. Although the development of early two-cell embryos was limited in M16 or M16-EDTA, these embryos formed blastocysts at a rate significantly greater in M16-PF than in M16 or M16-EDTA ($P < 0.01$).

It is well known that EDTA can assist in overcoming the two-cell block in mouse embryos (Abramczuk *et al.*, 1977; Hoshi and Toyoda, 1985) and that the eight-cell block in hamsters may be overcome by chelating agents (Kameyama and Ishijima, 1991). However, supplementing with EDTA does

not overcome the two-cell block in vole embryos. Although sodium pyruvate is necessary for culture of mouse two-cell embryos (Brinster, 1965) and is consumed preferentially as an energy substrate during the early developmental stages of mouse (Leese and Barton, 1984; Gardner and Leese, 1986), rat (Brison and Leese, 1991) and human embryos (Hardy *et al.*, 1989), it is inhibitory to the development of hamster and pig embryos (Davis and Day, 1978; Davis, 1985; McKiernan *et al.*, 1991). These data suggest that there is species specificity in the type of energy source required by early preimplantation stage embryos.

The mean diameter of one-cell Japanese field vole embryos in this study was 61.0 μm . Austin (1957) reported that the eggs of the field vole, *Microtus agrestis*, were the smallest of all mammals that had been studied at the time, and Mystkowska (1975b) found that bank vole embryos were smaller and darker than mouse embryos. Niimura *et al.* (1987) found that Japanese field vole embryos possessed fewer lipid droplets in the cytoplasm than did mouse embryos. The Japanese field vole embryos were smaller than mouse embryos (which are 80 μm in diameter, Austin, 1961), although the features of both were similar. It is therefore possible to distinguish vole and mouse embryos by size.

The present study indicates that embryos of Japanese field voles can be cultured readily in medium used for mouse embryos and that development from one-cell embryos to the blastocyst *in vivo* is similar to that in mice rather than in rats and hamsters. The Japanese field vole may be a useful experimental animal for reproductive biology in addition to the mouse.

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