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Establishment of superovulation procedure in Japanese field vole, *Microtus montebelli*

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ABSTRACT

Japanese field vole (*Microtus montebelli*) is a wild-derived rodent and have unique characteristic. Thus, these species have been expected as model animal. This study was performed to develop novel superovulation procedure for Japanese field vole. First, when 30 IU pregnant mare's serum gonadotropin (PMSG) and 30 IU human chorionic gonadotropin (hCG) were administrated 48 hours apart, females showed higher response to hCG compared with three concentrations of PMSG. Second, to effectively induce ovulation on females after vaginal opening, they were mated with vasectomized male instead of hCG administration. Average number of ovulated oocytes using PMSG mating (13.9 ± 1.9 oocytes) was higher than PMSG–hCG (control; 6.9 ± 2.3 oocytes) or PMSG–hCG mating (6.8 ± 0.8 oocytes). Finally, we attempted superovulation using GnRH agonist (GnRHa). With this treatment, we speculated that GnRHa might induce endogenous luteinizing hormone releasing to cause ovulation. Such superovulation was performed with 30 IU PMSG and different concentration of 20% polyvinylpyrrolidone–GnRHa (15, 30, 45, and 60 μg/kg). As results, average number of ovulated oocytes was highest with 30 μg/kg GnRHa (14.5 ± 4.1 oocytes). The numbers of ovulated oocytes of other concentrations were 5.0 ± 1.4 (15 μg/kg), 12.8 ± 2.7 (45 μg/kg), and 8.8 ± 3.7 oocytes (60 μg/kg). Nuclear status of most collected oocytes was the second meiotic division (range, 94.3%–100%). These superovulation procedures will be useful for development of *in vitro* culture systems and assisted reproductive technologies for not only Japanese field vole but also other voles.

1. Introduction

The *Microtus* is distributed over the world widely from frigid zone to temperate zone, and they inhabit underground of the lands that are worthless for agriculture and residential development by human. In addition, 10 of 64 species in this genus have been classified into the endangered category in Red List [1], and preservation of these species has been required from viewpoint of biologic diversity protection.

The characteristic of vole including Japanese field vole, *Microtus montebelli*, is a herbivorous animal with multiple stomachs [2] and some of them possess a mating system similar to human [3]. Thus, this has been expected as models for large herbivory and mating system. Recently, Manoli et al. [4] have been succeeded in establishment of induced pluripotent stem cell in prairie vole (*Microtus ochrogaster*) and then attempted development of transgenic prairie voles. Therefore, development of assisted reproductive technologies is necessary for future use of voles.

Little is known about reproductive activity on vole. Goto et al. [5,6] reported that Japanese field vole exhibits a copulatory ovulation and that its vaginal smear does not

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reveal pattern of a regular estrous cycle, whereas, *in vitro* fertilization with modified Krebs–Ringer–bicarbonate medium supplemented with 1 mM hypotaurine was carried out [7] and production of term offspring from *in vitro* fertilized oocytes [8] was successful. Keebaugh et al. [9] reported about superovulation of prairie voles, showed the age was important for superovulation, and concluded that use of young females was most efficient. For example, females aged 6 to 11 weeks ovulated more oocytes (14.0 ± 1.4 oocytes) compared with females aged 12 to 20 weeks (4.0 ± 1.6 oocytes), although females aged 4 to 5 weeks did not produced oocytes.

A stable supply of large numbers of high-quality oocytes with high competence for fertilization and embryo development will be important for not only basic reproductive studies but also applied researches in vole. Therefore, control of collecting oocytes as many as possible and the timing is extremely essential.

Treatment of superovulation can obtain many synchronized oocytes and/or embryos from experimental animals, but success of these methods is dependent on each species. Protocols on the basis of administration of gonadotropic hormones have been standardized in species, such as mouse [10], rat [11], rabbit [12], goat [13], pig [14], and cow [15]. Superovulation with a combination of pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) has been widely used to induce superovulation in many rodent species including rat [16,17], Syrian hamster [18], Siberian hamster [19], Vesper mouse [20], and Spiny mouse [21]. In contrast, a set of these hormones might not be proper to induce superovulation in some species, such as Chinese hamster [22], Steppe mouse, and Algerian mouse [23]. Therefore, the sensitivity of each species to superovulation treatment varies, and then superovulation procedure must be optimized according to species specificity. From these reasons, present study was performed to establish the novel superovulation procedure on Japanese field vole for reliable supply of oocytes throughout all ages.

2. Materials and methods

All chemicals and reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless stated otherwise.

2.1. Ethics statement

The designed animal experimental protocol was approved by the Animal Experimental Committee of Nippon Veterinary and Life Science University (approval number: 27K-31). All procedures were complied with guideline for Proper Conduct of Animal Experimental by Science Council of Japan. All animals were humanely treated throughout the course of experiments, and maximum care was taken to minimize pain of experimental animals.

2.2. Animals

Original Japanese filed voles were derived from wild and have been maintained for >30 years by outbred mating. They were housed under 14 L:10 D at 20 ± 2 °C and fed a pellet for herbivores (ZC; Oriental East Co., Ltd., Tokyo, Japan) and cubed hay. Food and water were given *ad libitum*.

2.3. Vasectomy

Male voles were anesthetized by a mixture of medetomidine (Dorbene vet; Kyoritsu Seiyaku Corporation, Tokyo, Japan) at a dose of 0.23 mg/kg, midazolam (Midazolam Injection 10 mg [SANDOZ]; Sandoz, Yamagata, Japan) at a dose of 3 mg/kg, and butorphanol (Vetorphale; Meiji Seika Pharma Co., Ltd., Tokyo, Japan) at a dose of 3.75 mg/kg. When the paw withdrawal reflex was absent, voles were shaved at midventral incision sites. All voles were placed on a heating pad, and rectal body temperature was maintained at 36 °C to 38 °C. The skin was disinfected with 70% ethanol. Surgical consisted of a 1 cm midverntral vertical skin and abdominal wall. Vas deferens was identified and approximately 0.5 cm of vas removed by cauterizing using electrosurgical unit (South Pointe Surgical Supply, Inc., FL, USA). The wound was closed with no. 3 silk thread (Shirakawa Co., Ltd., Tokyo, Japan) for abdominal wall and clip (Reflex 9 mm Wound Clip; Cell Point Scientific, Inc., MD, USA) for skin. Vasectomized voles were administrated atipamezole (Atipame; Kyoritsu Seiyaku Corporation) at a dose of 0.23 mg/kg as antagonist of medetomidine. After 10 days, vasectomized voles were mated with fertile females and proved sterility.

2.4. Media

Medium used for collecting oocytes was HEPES buffered Chatot-Ziomek-Bavister (H-CZB) medium composed of 81.62 mM NaCl, 4.83 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$-$7H_2O$, 5.00 mM $NaHCO_3$, 1.70 mM $CaCl_2$-$2H_2O$, 0.10 mM Na_2-EDTA-$2H_2O$, 1.00 mM L-glutamine, 28.00 mM sodium lactate, 0.27 mM Na-pyruvate, 5.55 mM glucose, 20.00 mM HEPES-Na, 1 mg/mL polyvinyl alcohol, and 50 μg/mL gentamicin as described by Kimura and Yanagimachi [24].

For dilution of each hormone, PMSG (Serotrophin 1000; Aska Pharmaceutical Co., Ltd., Tokyo, Japan), hCG (Gonadotropin 1000; Aska Pharmaceutical Co., Ltd.), and 0.9% saline used. GnRH agonist (GnRHa) (Buserelin acetate; 40 μg/10 mL; Kyoritsu Seiyaku Corporation) and polyvinylpyrrolidone (PVP) (PVP K-30; molecular weight 40,000) was used; 20% of PVP–GnRHa solution was prepared by adding 2 g of PVP in 10 mL of GnRHa liquid.

For fixation of oocytes, 2.5% glutaraldehyde and 4% paraformaldehyde (Nacalai Tesque, Inc., Kyoto, Japan) in PBS–PVA were used.

2.5. Oocyte collection

At 10 hours after administration of hCG or GnRHa, females were sacrificed by cervical dislocation. The ampullae of oviducts were collected and placed on watch glass. Under a stereoscope, cumulus–oocyte-complexes were collected by flushing oviduct with 200 μL H-CZB medium or releasing from ampullae using a pair of needles.

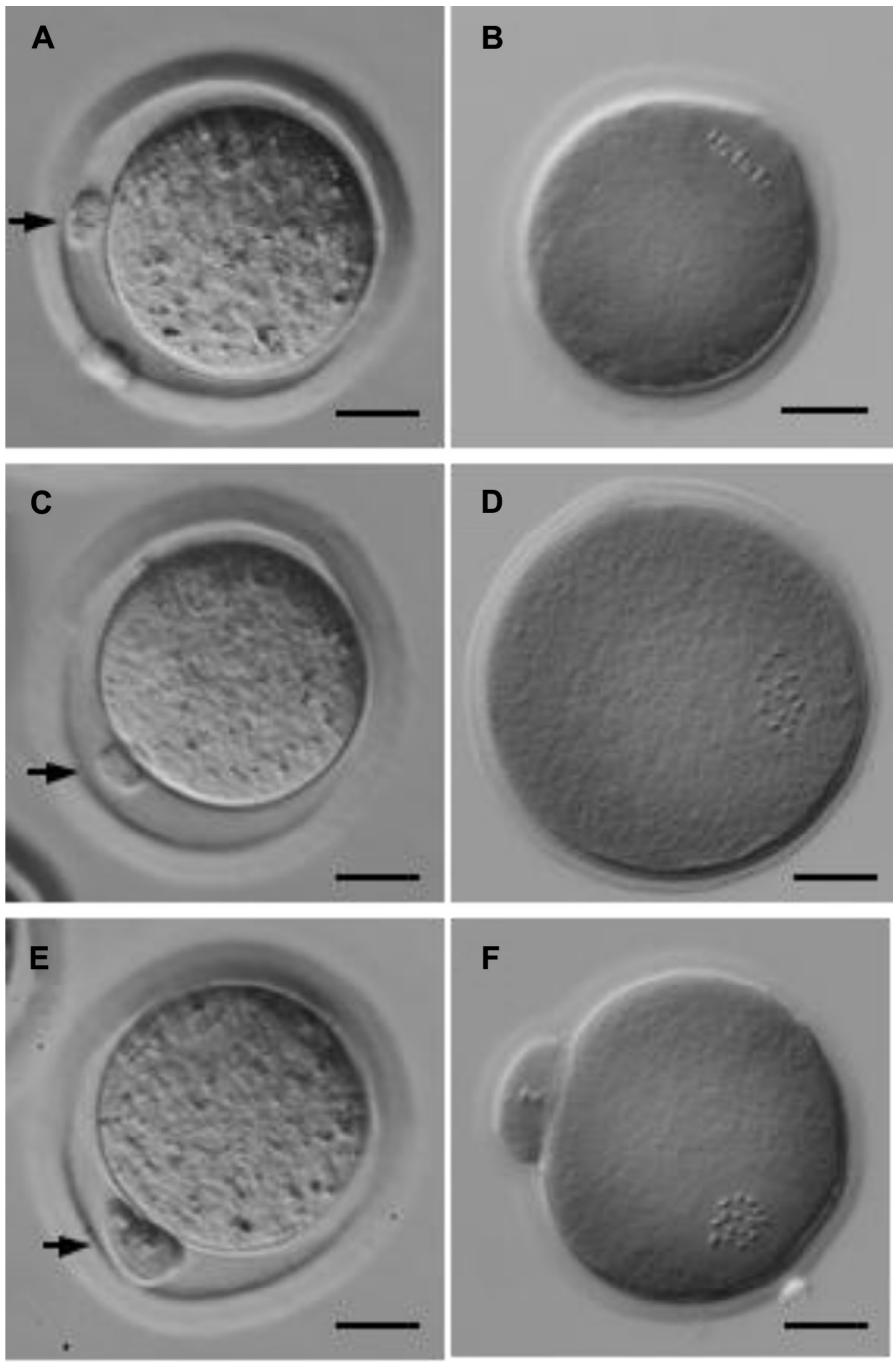

Fig. 1. Morphology of matured oocytes from Japanese- led vole with three superovulation procedures. Oocytes collected at 10 hours after hCG (A and B), mating (C and D), and 20% PVP-GnRHa (E and F) after PMSG administration. (A, C, and E) Morphologies of cumulus cell –free oocytes are shown. (B, D, and F) Nuclear statuses (M) were shown. Arrows indicate rst polar body. Scale bars represent 20 m. GnRHa, GnRH agonist; hCG, human chorionic gonadotropin; MII, metaphase stage II; PVP, polyvinylpyrrolidone; PMSG, pregnant mare 's serum gonadotropin.

2.6. Assessment of nuclear status

Cumulus–oocyte-complexes were treated for 3 minutes in H-CZB medium containing 0.01% hyaluronidase (Sigma – Aldrich, MO, USA) and then cumulus cells removed by pipetting. To prepare specimens, cumulus-free oocytes were whole mounted on slide grass, xed with 2.5% glutaraldehyde (rinsing), and 4% paraformaldehyde in PBS – PVA for 30 minutes at room temperature. After xing, specimens were washed with 100% ethanol, stained with

Table 1
Relationship on induced ovulation between days-old and concentrations of PMSG in Japanese field vole.

Days-old	Concentrations of PMSG[e,f,g]	Total no. of voles examined	No. of ovulated voles (%) [h,i]	No. of ovulated oocytes			Nuclear status (%)[j]	
				Total	Average[j,k]		M	Others[l]
19–29	0 IU	5	1 (20.0)	3	3		0 (0)	3 (100)
	7.5 IU	7	7 (100)	20	2.9	0.3[a]	15 (75.0)	5 (25.0)
	30 IU	6	6 (100)	135	22.5	2.6[b]	104 (77.0)	31 (23.0)
30–138	0 IU	13	6 (46.2)	15	2.5	0.6	10 (66.7)	5 (33.3)
	7.5 IU	7	6 (85.7)	29	4.8	1.8	21 (72.4)[c]	8 (27.6)[c]
	30 IU	11	8 (72.7)	55	6.9	2.3	26 (47.3)[d]	29 (52.7)[d]

Different superscript letters (a, b, c, and d) represent a significant difference ($P < 0.05$).
[e] Each PMSG dosage was 100 mL.
[f] Concentration of hCG was 30 IU and dosage was 100 mL.
[g] Group of 0 IU was control and 0.9% saline was administrated.
[h] The rate was calculated from total number of voles examined.
[i] Statistical analysis was performed between 7.5 and 30 IU PMSG.
[j] The rate was calculated from number of voles with ovulation.
[k] Data represent the mean SEM.
[l] Others were included fragmentation, degeneration and regression.

0.25% acetolacmoid (Lacmoido Resorcin Blue; Waldeck GmbH & Co. KG, Munster, Germany), rinsed off extra dye with acetoglycerol (glycerin:acetic acid:MilliQ ¼ 1:1:3), and enclosed with manicure. Oocytes containing first polar body were judged meiotic metaphase stage with differential interference contrast microscope.

2.7. Experimental designs

2.7.1. Experiment 1: superovulation induced by a combination of PMSG and hCG

Female Japanese field voles were divided into two groups before vaginal opening (19–29 days-old) and after vaginal opening (>30 days-old). Superovulation treatment was attempted with various concentrations of PMSG (0, 7.5, or 30 IU) and 30 IU hCG. Group of 0 IU PMSG was administrated 0.9% saline as a control. Ovarian and uterine weights were also measured after oocytes collection.

2.7.2. Experiment 2: superovulation induced by mating with vasectomized voles

Japanese field vole has been copulatory ovulation animal. Therefore, we tested whether mating with vasectomized male was effective for ovulation. It was found that administration of PMSG facilitated follicular growth because increase in ovarian weight was observed in experiment 1. Thus, females were subjected to two different treatments to induce superovulation: (1) females were intraperitoneally administrated with 30 IU of hCG and mated with vasectomized male after 30 IU of PMSG 48 hours later and (2) superovulation was induced with PMSG 48 hours later by mating with vasectomized males. Only females that confirmed copulation and plug were used for experiment. As a control of experiment, superovulation was induced with 30 IU of PMSG and hCG.

Table 2
Effect of PMSG concentrations on ovarian and uterine weights.

Days-old	Concentrations of PMSG[c,d,e]	Total no. of voles examined	Weight (mg) of a pair of [f,g]			
			Ovarian		Uterine	
19–29	0 IU	5	9.7	0.8	30	4.9
	7.5 IU	7	8.1	1.8[a]	61.9	9.1
	30 IU	6	22	1.9[b]	62.3	4.5
30–138	0 IU	13	8.9	0.7	57.7	10.1
	7.5 IU	7	19.9	2.2	98.9	16.9
	30 IU	11	21.8	3.7	76.5	12.2

Different superscript letters (a and b) represent a significant difference ($P < 0.05$).
[c] Each PMSG dosage was 100 mL.
[d] Concentration of hCG was 30 IU and dosage was 100 mL.
[e] Group of 0 IU was control and 0.9% saline was administrated.
[f] Data represent the mean SEM.
[g] Statistical analysis was performed between 7.5 and 30 IU PMSG.

2.7.3. Experiment 3: superovulation induced by GnRHa

GnRH is a simple polypeptide composed of 10 amino acids and advances the synthesis and secretion of endogenous follicle-stimulating hormone (FSH) and luteinizing hormone (LH). It is known that GnRH has similar structure of polypeptide among other animals. We estimated that GnRH facilitated endogenous LH release and then induced superovulation. In this experiment, PVP–GnRHa was administrated into cervix subcutaneous 48 hours later of PMSG injection. Concentration of PVP–GnRHa was tested with 15, 30, 45, and 60 mg/kg.

2.8. Statistical analysis

In experiment 1, comparison of number of ovulated oocytes and weight of ovarian and uterine between 7.5 and 30 IU PMSG were analyzed by Student's t test. The rate of ovulated voles and nuclear status were confirmed by the chi-square test. In experiments 2 and 3, number of ovulated oocytes was analyzed by 1-way ANOVA. The Tukey–Kramer procedure was used for multiple comparisons. The rate of ovulated voles and nuclear status were confirmed by the chi-square test. All analyses were conducted with Statcel 3 (OMS Ltd., Saitama, Japan). Difference was considered significant when P value was less than 0.05.

Table 3
No. of collected oocytes and nuclear status after superovulation using hCG mating or mating.

Procedure of collected oocytes[c]	Days-old	No. of voles examined	No. of ovulated voles[g]	No. of ovulated oocytes			Nuclear status (%)	
				Total	Average[h,i]		M	Others[j]
Control (hCG)[d]	30–138	11	8 (72.7)	55	6.9	2.3	26 (47.3)[a]	29 (52.7)[a]
hCG mating[e]	65–406	6	6 (100.0)	41	6.8	0.8	40 (97.6)[b]	1 (2.4)[b]
Mating[f]	68–301	14	14 (93.3)	195	13.9	1.9	190 (97.4)[b]	5 (2.6)[b]

Different superscript letters (a and b) represent a signi cant difference (P < 0.05).
[c] Concentration of PMSG was 30 IU and dosage was 100 mL.
[d] Control; 30 IU hCG was administrated.
[e] Voles were copulated with vasectomized males immediately after administrated of 30 IU hCG.
[f] Voles were copulated with vasectomized males at 48 hours after 30 IU of PMSG administration.
[g] The rate was calculated from total no. of voles examined.
[h] The rate was calculated from no. of voles with ovulation.
[i] Data represent the mean SEM.
[j] Others were included fragmentation, degeneration and regression.

3. Results

3.1. Experiment 1: superovulation induced by a combination of PMSG and hCG

Females were treated with PMSG of various concentrations and 30 IU hCG, and then oocytes were recovered at 10 to 11 hours post-hCG (Fig. 1A). The results were listed in Table 1. In 19 to 29 days-old, average number of ovulated oocytes with 30 IU PMSG was signi cantly higher than 7.5 IU (22.5 2.6 and 2.9 0.3 oocytes, P < 0.05). Rate of M between 7.5 and 30 IU PMSG was of no signi cance (Fig. 1B). In 30 to 138 days-old, increase in average number of oocytes was concentration dependent of PMSG, but there was no signi cance between groups. Rate of M in 7.5 IU PMSG was signi cantly higher than 30 IU (72.4% and 47.3%, P < 0.05). With 30 IU PMSG, number of ovulated oocytes increased and females before vaginal opening ovulated more oocytes than after vaginal opening. The results of ovarian and uterine weight were listed in Table 2. With 30 IU PMSG in 19 to 29 days-old, ovarian weight was signi cantly higher than 7.5 IU (P < 0.05). In 30 to 138 days-old, ovarian weight was no signi cance between groups. Increase in ovarian weight was concentration dependent of PMSG in all ages and uterine weight was not signi cant among groups.

3.2. Experiment 2: superovulation induced by mating with vasectomized voles

To induce ovulation more effectively in over 30 days-old, females were treated with hCG mating or mating alone at 48 hours after PMSG (Fig. 1C). The results are listed in Table 3. Average number of ovulated oocytes using hCG mating was similar to control (6.8 0.8 and 6.9 2.3 oocytes, respectively). Mating alone tended to be higher average number of ovulated oocytes in all treatments, but there was no signi cance among groups. Rate of M in hCG mating and mating alone was signi cantly higher than control (97.6%, 97.4%, and 47.3%, P< 0.05; Fig. 1D).

3.3. Experiment 3: superovulation induced by GnRHa

Females were treated with 30 IU of PMSG and several concentrations of GnRHa, and then oocytes were recovered at 10 to 11 hours post-GnRHa administration (Fig. 1E). As a result, we found that ovulation occurred in all groups. The results are presented in Table 4. Average number of ovulated oocytes tended to be highest with 30 mg/kg of GnRHa (14.5 4.1 oocytes) and lowest with 15 mg/kg of GnRHa (5.0 1.4 oocytes). Morphology of most oocytes from all groups was normal (Fig. 1F). There was no signi cance among all groups with respect

Table 4
Effect of number of ovulated oocytes and nuclear status on different concentrations of 20% PVP –GnRHa.

Concentrations of GnRHa[a] (mg/kg)	Average dose of 20% PVP–GnRHa (mL)[b]	Days-old	Total no. of voles examined	No. of ovulated voles[c]	No. of ovulated oocytes			Nuclear status(%)	
					Total	Average[b,d]		M	Others[e]
15	120 4.1	50–79	4	4 (100.0)	20	5.0	1.4	20 (100)	0 (0.0)
30	230 17.8	66–302	4	4 (100.0)	58	14.5	4.1	56 (96.6)	2 (3.4)
45	358 19.7	50–67	4	4 (100.0)	51	12.8	2.7	50 (98.0)	1 (2.0)
60	460 38.9	60–226	4	4 (100.0)	35	8.8	3.7	33 (94.3)	2 (5.7)

[a] Concentration of PMSG was 30 IU and dosage was 100 mL.
[b] Data represent the mean SEM.
[c] The rate was calculated from total no. of voles examined.
[d] The rate was calculated from no. of voles with ovulation.
[e] Others were included fragmentation, degeneration and regression.

Table 5
Comparison of number of ovulated oocytes by different hormonal treatments on voles before opening vagina. [a]

Hormonal treatments [b]	Average dose of hormone (mL)	No. of voles examined	No. of ovulated voles [f]	No. of ovulated oocytes			Nuclear status (%)	
				Total	Average [e,g]		M	Others [h]
Control (hCG) [c]	100	5	3 (60.0)	63	21	10.6	59 (93.7)	4 (6.3)
20% PVP-GnRHa [d]	123 10.75	4	3 (75.0)	117	39.0	9.0	114 (97.4)	3 (2.6)

[a] Age was 19 to 20 days-old.
[b] Concentration of PMSG was 30 IU and dosage was 100 mL.
[c] Control; concentration of hCG was 30 IU, and dosage was 100 mL.
[d] Concentration of 20% PVP–GnRHa was 30 mg/kg.
[e] Data represent the mean SEM.
[f] The rate was calculated from total number of voles examined.
[g] The rate was calculated from number of voles with ovulation.
[h] Others were included fragmentation, degeneration and regression.

to average number of ovulated oocytes. When Japanese eld voles of 19 to 29 days-old were administrated PMSG and GnRHa, we obtained similar number of oocytes (Table 5).

4. Discussion

Our goal is production of high-quality vole oocytes that can be used for establishment of assisted reproductive technologies. The results of present study report that novel superovulation procedure induces superovulation in Japanese eld vole for collecting oocytes stably.

In experiment 1, we examined superovulation induced by PMSG–hCG on voles before and after vaginal opening. When 30 IU PMSG–hCG was administrated to voles, we obtained many oocytes in three groups. Moreover, ef - ciency of superovulation in vole varied with female age, and such superovulation was most ef cient in females before vaginal opening female. Notably, 19 to 29 days-old females produced a mean 36.5 3.5 oocytes, whereas more than 30 days-old females produced only a mean 6.9 2.3 oocytes. These results were consistent with reports that young mice could ovulate more oocytes than aged mice by PMSG–hCG. As this reason, it was considered that speci- city of hormone receptor was different between ages. FSH and LH, including PMSG and hCG, are heterodimer and are composed of the common a-subunit (CGa). CGa is shared by FSH, LH, and TSH, and hormone-speci c b subunits (FSHb, LHb, and CGb) have been possessed [25]. CGa is a maturity protein and shows high homology with more than 60% of complementary DNA among animal species [26]. Although FSHb shows very high homology [27], LHb was with low homology and different among species [28–30]. Thus, it was speculated that low hormonal response of vole after vaginal opening was because of difference of LH b. On the other hand, receptor of FSH and LH is increased by estradiol 17 b and combination of FSH to granulosa cell and LH to theca cell is necessary for production of estradiol 17 b [31]. Increase or decrease of FSH and LH are controlled by hypophysis in general. Hormone treatment to female before vaginal opening is important because ovary of young voles is not under adjustment from pituitary gland. Thus, we considered that young voles ovulated many oocytes. Consequently, combination of 30 IU PMSG and hCG was ef cient in 19 to 29 days-old voles.

In experiment 2, to induce ovulation more effectively in over 30 days-old, we examined superovulation induced by mating with vasectomized voles. Ovulation could be induced by mating stimulus. Because mating stimulus induced ovulation, we speculated that mating may facilitate endogenous LH release. In musk shrew, mating stimulus induced ovulation through the release of endogenous LH [32]. Then, it is thought that Japanese eld vole which is a copulatory ovulation animal may possess similar ovulation mechanism to musk shrew. However, average number of oocytes of PMSG–hCG mating was decreased. This result was considered that hCG obstructed facilitation of endogenous LH. In this result, we succeeded in superovulation induced by PMSG mating.

Recently, superovulation using GnRH has been developed [33–35]. GnRH is a high-rank hormone than hCG and difference of base sequence is less among animal species [36–38]. GnRH facilitated endogenous LH release and then high-quality oocytes could be obtained in experiment 3. In preliminary experiment, when GnRH was intraperitoneally administered, it was ineffective (data not shown). Thus, we used PVP that absorption of hormone became slower, and such effect was maintained as dilute solution of GnRHa. Effect of GnRH diluted with PVP was reported by cow [39] and sheep [40]. We conducted examination in expectation of these two effects. In regard to PVP, molecular weight and concentration of PVP were variously modi ed at preliminary experiment (data not shown). Because it was easiest to handle in K-30 at 20% and all administrated PVP was absorbed, we determined at these conditions. In present study, we revealed that GnRHa was also an effective hormone to induce superovulation because this procedure could obtain as similar number of oocytes as PMSG mating.

In conclusion, oocytes from Japanese eld vole could be stably obtained with PMSG and hCG, mating, or GnRHa. These procedures allow for the recovery of a large number of developmentally synchronized oocytes. Further research will be needed to evaluate the fertilizing and developmental ability of these oocytes.

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