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

Atsuko Kageyama [a], Minako Tanaka [b], Mami Morita [b], Hitoshi Ushijima [a,b], Hiroshi Tomogane [b], Konosuke Okada [b,*]

[a] Graduate School of Veterinary Medicine and Life Science, Graduate School, Nippon Veterinary and Life Science University, Tokyo, Japan
[b] School of Animal Science, Faculty of Applied Life Science, Nippon Veterinary and Life Science University, Tokyo, Japan

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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

* Corresponding author. Tel.: +08 422 314 151 x5502; fax: +08 422 332 094.
E-mail address: okada@nvlu.ac.jp (K. Okada).

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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.