FULL PAPER *Laboratory Animal Science*

Anesthetic effects of a combination of medetomidine, midazolam and butorphanol on the production of offspring in Japanese field vole, *Microtus montebelli*

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ABSTRACT. Pentobarbital sodium (Somnopentyl) can induce surgical anesthesia with a strong hypnotic effect that causes loss of consciousness. Animals have been known to die during experimental surgery under anesthesia with Somnopentyl, causing it to be declared inadequate as a general anesthetic for single treatment. An anesthetic combination of 0.3 mg/kg medetomidine, 4.0 mg/kg midazolam and 5.0 mg/kg butorphanol (M/M/B:0.3/4/5) was reported to induce anesthesia for a duration of around 40 min in ICR mice; similar anesthetic effects were reported in both male and female BALB/c and C57BL/6J strains of mice. However, the anesthetic effects of this combination in Japanese field vole, *Microtus montebelli,* remain to be evaluated. In the present study, we assessed the effects of Somnopentyl and different concentrations of anesthetic combination (M/M/B:0.3/4/5, 0.23/3/3.75 or 0.15/2/2.5) in Japanese field voles, by means of anesthetic scores. We also examined effect of these anesthetics on production of offspring. Death of the animals was observed only with Somnopentyl. The anesthetic score of Somnopentyl was lower than those of the other anesthetics, although there were no significant differences in duration, body weight and frequency of respiratory among the evaluated anesthetics. Abortion rate with Somnopentyl was significantly higher than that with the M/M/B:0.23/3/3.75 combination, although there was no significant difference in the number of offspring between two. In conclusion, results of this study provide basic information for achieving appropriate anesthetic concentrations in addition to indicating a new, safe and effective surgical anesthetic for Japanese field voles.

KEY WORDS: anesthetic combination, atipamezole, Japanese field vole, offspring, Somnopentyl

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Pentobarbital sodium (Somnopentyl) is an anesthetic agent authorized for use in animals. It is categorized as a prescription medicine. This anesthetic has been commonly used in animal experiments and scarcely has any analgesic effect, although it can induce surgical anesthesia with a strong hypnotic effect that causes loss of consciousness at extremely lethal doses. Somnopentyl rapidly acts on the central nervous system and can cause reduction in blood pressure, respiratory depression and respiratory arrest during conditions of deep anesthesia. It is known to often cause death of animals during experimental surgery; therefore, general anesthesia with a single administration of Somnopentyl is not recommended [4, 5]. In general, inhalation anesthetics are safe and reliable for the induction of anesthesia for longer durations; however, they require specialized devices for administration. In scenarios where many animals are simultaneously operated upon under anesthesia, the space required and cost of inhalation anesthetics are greater than those of injection anesthetics. Therefore, easy and effective injection anesthetics are required as alternatives to inhalation anesthetics. Kawai *et al.* reported a new injectable anesthetic agent with an effect in mice equivalent to that of the combination of ketamine and xylazine [11]. This combination anesthetic was composed of two tranquilizers—medetomidine and midazolam—along with butorphanol, a nonnarcotic analgesic; it exhibited an adequate anesthetic duration of approximately 40 min in ICR mice. Kirihara *et al.* reported similar anesthetic effects in both male and female mice of the BALB/c and C57BL/6J strains [14]. Combination anesthetics are used in capture of wild animals [1, 18, 25, 33] as well as in various experiments involving laboratory animals [8–10, 15, 16, 29].

Japanese field vole (*Microtus montebelli*) is a wild-derived rodent with unique characteristics. Voles, including *Microtus montebelli*, are herbivorous animals with multiple stomachs [17], and the members of some species exhibit mating habits similar to that of humans [19]. The chromosome number of voles varies among the different species [21]. Therefore, several model animals are expected to be produced from the members of this genus. Manoli *et al.* [20] were successful in establishing induced pluripotent stem cells (iPS cells) in prairie voles (*Microtus ochrogaster*), and development of transgenic prairie voles has also been attempted. Additionally, new assisted reproductive technologies have been developed for voles [6].

In addition, 10 of 64 species of this genus have been classified under the endangered category in the International Union for Conservation of Nature (IUCN) Red List [28], and

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preservation of these species is necessary from the viewpoint of biological diversity protection. For these reasons, we consider that establishment of an appropriate anesthetic is also important for surgical or non-surgical treatment of voles. However, there are only a few reports regarding the effects of anesthesia on voles [6, 20, 32]. In the present study, we assessed the effects of Somnopentyl as well as a combination anesthetic in voles by means of anesthetic scores and also evaluated the anesthetic time and frequency of respiratory in *Microtus montebelli*. The body weight of experimental animals was evaluated at 1, 3, 5, 7 and 14 days after administration of the anesthetic agents. Finally, we examined possibility of influence of different anesthetics on production of offspring.

MATERIALS AND METHODS

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

Ethics statement: The animal experimental protocol was approved by the Animal Experimental Committee of the Nippon Veterinary and Life Science University. All of procedures complied with the Guidelines for Proper Conduct of Animal Experiments published by the Science Council of Japan. The animals were treated humanely throughout the experiments, and maximum care was taken to minimize pain of experimental animals.

Animals: The original Japanese field voles included in this study were derived from wild and maintained for over 30 years by outbreeding. The first experiment of this study included 9 each of female and male voles, and the second experiment included 36 female and 8 male voles. The animals were housed in TPX cages (CLEA Japan, Inc., Tokyo, Japan), with 2–3 voles in each cage, under a light cycle (14 L:10 D). Bedding was provided in each cage and changed once a week. The temperature and humidity of animal room were maintained at 22 ± 2°C and 50 ± 5%, respectively. The voles were fed herbivore pellets (ZC; Oriental East Co., Ltd., Tokyo, Japan) and cubed hay. Food and water were provided *ad libitum*.

Media: The hormones of pregnant mare serum gonadotropin (PMSG; Serotropin® 1000; Aska Pharmaceutical, Tokyo, Japan) and human chorionic gonadotropin (hCG; Gonatropin® 1,000; Aska Pharmaceutical) were diluted in 0.9% saline solution. Gonadotropin-releasing hormone (GnRH; Buserelin acetate; Kyoritsu Seiyaku Corporation, Tokyo, Japan) was dissolved in 20% polyvinylpyrrolidone (PVP K-30; molecular weight 40,000; 20% PVP-GnRH).

The oocytes were collected in 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/m*l* (0.1%) polyvinyl alcohol and 50 μg/m*l* gentamicin, as described by Kimura and Yanagimachi [13].

Cryoprotectant agent (CPA) for cryopreservation of spermatozoa used R18S3 [27]. Briefly, CPA was prepared by dissolving 4.5 g of skimmed milk powder in MilliQ water to attain a final volume of 75 m*l*. This mixture was centrifuged at 15,000 × *g* at 10°C for 90 min until a translucent supernatant was obtained. Then, R18S3 was prepared by dissolving 18 g of raffinose in 50 m*l* of above supernatant (30–35°C) to attain a final volume of 100 m*l*; the solution was sterilized using a 0.22 μm filter and stored at −20°C until use.

In vitro fertilization (IVF) was performed using the Human Tubal Fluid (HTF) medium, composed of 101.6 mM NaCl, 4.69 mM KCl, 0.37 mM KH_2PO_4, 0.20 mM $MgSO_4$-$7H_2O$, 25.0 mM $NaHCO_3$, 2.04 mM $CaCl_2$-$2H_2O$, 21.4 mM sodium lactate, 0.33 mM Na-pyruvate, 2.78 mM glucose, 4 mg/m*l* bovine serum albumins (BSA) and 50 μg/m*l* gentamicin, as described by Quinn *et al.* [23]. Before use, a 100 mM stock solution of hypotaurine (Sigma Aldrich, St. Louis, MO, U.S.A.) was prepared in HTF medium, as described by Wakayama *et al.* [32]; this stock solution was diluted to a final concentration of 1 mM (1 mM hypotaurine-HTF) and equilibrated overnight under 5% CO_2 in air at 37°C.

Anesthetic agents: The following anesthetics were evaluated in the present study—medetomidine hydrochloride (Dorbene® Vet, Kyoritsu Seiyaku Corporation), an alpha 2-adrenoceptor agonist; midazolam (midazolam injection, Sandoz, Yamagata, Japan), a benzodiazepine derivative; butorphanol (Vetorphale, Meiji Seika Pharma Co., Ltd., Tokyo, Japan); and pentobarbital sodium (Somnopentyl, Kyoritsu Seiyaku Corporation), a barbiturate. All anesthetics were stored at room temperature.

Preparation of the anesthetic agents: All of the anesthetic agents were diluted in sterile 0.9% saline solution. The dosage of Somnopentyl was set at 40 mg/kg, and 1 m*l* of Somnopentyl was diluted in 10 m*l* of sterile saline before use.

The combinations of three anesthetics were prepared using each stock solution; before use, 0.75 m*l* of medetomidine hydrochloride, 2 m*l* of midazolam and 2.5 m*l* of butorphanol were combined, and the volume was adjusted to 25 m*l* with saline solution. M/M/B:0.3/4/5 combination was prepared to achieve a dosage of 0.3 mg/kg medetomidine hydrochloride, 4.0 mg/kg midazolam and 5.0 mg/kg butorphanol. Similarly, the combinations of M/M/B:0.15/2/2.5 and M/M/B:0.23/3/3.75 were also prepared to achieve dosages of 0.15 mg/kg medetomidine hydrochloride, 2 mg/kg midazolam, and 2.5 mg/kg butorphanol and 0.23 mg/kg medetomidine hydrochloride, 3.0 mg/kg midazolam and 3.75 mg/kg butorphanol, respectively. Atipamezole (Atipame®, Kyoritsu Seiyaku Corporation), an antagonist of medetomidine, was diluted in saline solution to achieve dosages of 0.3, 0.15 and 0.23 mg/kg [15]. The anesthetic agents thus prepared were administered to voles at volumes of 0.01 m*l*/g of body weight. Buprenorphine (Lepetan® injection 0.2 mg, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) diluted in saline solution, was administered as an analgesic with Somnopentyl, at a dosage of 0.05 mg/kg.

Administration of anesthetic agents and handling of the animals: A total of 36 voles were randomly separated into 4 groups. All experiments were performed in animal room during light hours (07:00 to 21:00). The weight of each vole

was measured before anesthesia, in order to determine the appropriate volume of anesthetic for intraperitoneal injection. After the induction of anesthesia, the voles were returned to cages until loss of righting reflex. Following this, voles were laid on their backs on heating pads, in a dorsal recumbent position without fixation, in order to record the anesthetic scores as described below. Upon scoring, the voles were returned to their cages after the administration of antagonist or recovery of righting reflex. The recovery of righting reflex was defined by the ability of animal to take a few steps.

Measurement of the anesthetic scores: The anesthetic scores were measured using the modified grading system described by Kawai *et al.* [11] and Kirihara *et al.* [14]. The measurements were based on the assessment of five reflex reactions, the first of which was body righting reflex — when a vole was laid on its back, and it was given a score of 1 if it failed to right itself and a score of 0 if it did; the second was the corneal reflex—when at least one side of one of the eyes of a vole was gently stimulated by air using a Pasteur pipette with a silicone nipple at distance of 1 cm from eye, the animal was given a score of 1 if it did not close its eyelid and a score of 0 if it did; the third was tail reflex—when tail of a vole was gently pinched with forceps, it was given a score of 1 if its tail did not move and a score of 0 if it did; the fourth was front paw reflex—when a front paw of a vole was gently pinched with forceps, it was given a score of 1 if it did not move and a score of 0 if it did; and the fifth was hind paw reflex—when a hind paw of a vole was gently pinched with forceps, it was given a score of 1 if it did not move and a score of 0 if it did. The total anesthetic score was graded from 0 to 5. A total score of 3–5 was considered to indicate level of surgical anesthesia of a vole. The duration for which a vole exhibited a score of at least 3 was defined as duration of anesthesia. The stimulation was repeated every 10 min after administration of anesthetic agent. The duration of anesthesia with combination anesthetics was evaluated until 30 min following administration.

Frequency of respiratory: With the animals laid on their backs, the frequency of respiratory was visually evaluated for each animal for 20 sec every 10 min.

Time-related parameters of anesthesia: Immobilization time (the duration for which animals remained immobile) was defined as the period from loss of righting reflex to its recovery. Induction time was defined as the time from loss of righting reflex to initiation of surgical anesthesia. The duration of anesthesia was defined as duration for which the animals were under surgical anesthesia. Emergence time was defined as the time from the end of surgical anesthesia to recovery of righting reflex.

Vasectomy: Male voles were anesthetized with a combination of M/M/B:0.23/3/3.75. Only those animals that exhibited loss of righting reflex after administration of M/M/B:0.23/3/3.75 were subjected to surgery (8/10; 80.0%). When the paw-withdrawal reflex could no longer be observed, voles were shaved at the midventral incision site. They were placed on heating pads, and rectal body temperature was maintained at 36–38°C. The skin was disinfected with 70% ethanol. The vas deferens was identified, and approximately 0.5 cm of duct was removed by cauterization using an electrosurgical scalpel (South Pointe Surgical Supply, Inc., Coral Springs, FL, U.S.A.). The wound was closed using No. 3 silk thread (Sirakawa Co., Ltd., Tokyo, Japan) at abdominal wall and a clip (Reflex skin closure system; 9 mm; CellPoint Scientific, Inc., Gaithersburg, MD, U.S.A.) at skin. The vasectomized voles were then administered 0.23 mg/kg atipamezole. After 10 days, vasectomized voles were mated with fertile females, in order to confirm their sterility.

Oocyte collection: Female Japanese field voles received 30 IU PMSG followed by either 30 IU hCG or 30 μg/kg of PVP-GnRH after 48 hr. At 10 hr after administration of hCG or PVP-GnRH, the animals were sacrificed by cervical dislocation. The ampullae of oviducts were collected and placed on a watch glass. Under a stereoscope, the cumulus-oocyte complexes (COCs) were collected either by flushing oviduct with 200 μl of H-CZB medium or by releasing them from ampullae using a pair of needles. The COCs were then transferred into 380 μl of 1 mM hypotaurine-HTF insemination medium.

Sperm cryopreservation: Cauda epididymal spermatozoa were collected from male voles. The sample collected from each male was suspended in 1.1 m*l* of CPA and incubated for 15 min under 5% CO_2 in air at 37°C. The suspension was loaded into a 0.25 m*l* straw with 100 μl HTF, followed by the addition of 50 μl of air and 25 μl × 4 columns of the suspension (a total of 100 μl sperm suspension in each straw). Both ends of straw were heat-sealed, and the straw was placed on a polystyrene raft floating at a height of 4 cm from surface of liquid nitrogen (LN_2) for 10 min and then stored in LN_2 for at least 1 week.

In vitro fertilization: In order to thaw frozen spermatozoa, straws were warmed for 1 min in a water bath (37°C) after air exposure for 5 sec. The sperm count of the thawed sperm suspension was adjusted to 2.0×10^7 cells/m*l* with 1 mM hypotaurine-HTF, and the suspension was preincubated for 15 min under 5% CO_2 in air at 37°C. Following this, 20 μl of sperm suspension was added to an insemination drop of IVF medium containing the COCs. Insemination was performed for 3 hr. The final sperm concentration for insemination was 1.0×10^6 cells/m*l*. After insemination, oocytes were separated from spermatozoa and cumulus cells by gentle pipetting.

Embryo transfer: For the induction of pseudopregnancy, female voles were mated with vasectomized male voles, and copulation was confirmed; this was considered as Day 0 (0 day post-coitum, 0 dpc). Only those female voles that had confirmed copulation and exhibited copulatory plugs were used for embryo transfer. Immediately after insemination, the presumable zygotes were transferred into the oviducts of recipient voles (0.5 dpc). The recipients were anesthetized either with a combination of M/M/B:0.23/3/3.75 or Somnopentyl (40 mg/kg). Only those animals that exhibited loss of righting reflex after administration of either of anesthetics were subjected to surgery (Somnopentyl, 18/19 voles, 94.7%; and M/M/B:0.23/3/3.75, 18/20 voles, 90.0%). Once the paw-withdrawal reflex was no longer observed, the voles were shaved at midventral incision site. The animals were placed on heating pads, and rectal body temperature was

Table 1. Number of voles administered anesthesia and rate of survival/death following administration of different anesthetics

Sex	Agent	Week-old (wks)	Total no. of voles examined	Weight (g)[a)]	Administrated volume (m*l*)[a)]	No. of effective voles for anesthesia[b)]	Rate (%)[c)]	
							Viability	Mortality
Female	Somnopentyl	9 to 50	9	30.19 ± 1.28	0.18 ± 0.01	9/9 (100)	5/9 (55.6)[A)]	4/9 (44.4)[C)]
	M/M/B: 0.3/4/5	6 to 51	9	29.19 ± 1.24	0.29 ± 0.01	9/9 (100)	9/9 (100)[B)]	0/9 (0.0)[D)]
	M/M/B: 0.23/3/3.75	9 to 69	9	28.86 ± 1.40	0.28 ± 0.01	7/9 (77.8)	7/7 (100)[B)]	0/7 (0.0)[D)]
	M/M/B: 0.15/2/2.5	7 to 39	9	27.90 ± 1.90	0.27 ± 0.02	7/9 (77.8)	7/7 (100)[B)]	0/7 (0.0)[D)]
Male	Somnopentyl	9 to 28	9	37.67 ± 0.92	0.23 ± 0.01	9/9 (100)	6/9 (66.7)	3/9 (33.3)
	M/M/B: 0.3/4/5	8 to 37	9	40.77 ± 1.80	0.40 ± 0.02	7/9 (77.8)	7/7 (100)	0/7 (0.0)
	M/M/B: 0.23/3/3.75	8 to 30	9	42.18 ± 2.22	0.42 ± 0.02	7/9 (77.8)	7/7 (100)	0/7 (0.0)
	M/M/B: 0.15/2/2.5	11 to 28	9	41.07 ± 2.85	0.41 ± 0.03	6/9 (66.7)	6/6 (100)	0/6 (0.0)

a) Data are presented as the mean ± SEM. b) Effect voles for anesthesia were defined as lost righting reflex. c) Viability was defined as recovery of righting reflex, and mortality was defined as incidence of cardiac arrest. A–D) Different superscripts represent significant differences along the same line ($P<0.05$).

maintained at 36–38°C. The skin was disinfected with 70% ethanol. The exposed fat pad was clipped using a clamp, and oviducts and ovaries were retained outside the body cavity. Under a stereomicroscope, pronuclear stage-embryos with air were transferred into the ampulla of oviduct. The wound was closed using No. 3 silk thread at the abdominal wall and a clip at skin. Voles anesthetized with the M/M/B combination were then administered 0.23 mg/kg atipamezole, which acts as an antagonist of medetomidine, while those anesthetized with Somnopentyl were administered 0.05 mg/kg buprenorphine. At the end of surgery, voles were returned to their cages and placed on heating pads. At 21 dpc, the survival rate of offspring was recorded, and at 23 dpc, weight and sex of offspring were recorded.

Statistical analysis: Statistical analysis was performed using the Statcel 3 software (OMS Ltd., Saitama, Japan). The data obtained by measurements after the administration of anesthetic agents were presented as the mean value ± standard error of the mean (SEM). All of the data expressed as rates were analyzed using χ^2-test. The duration, anesthetic scores and frequency of respiratory were analyzed by one-way analysis of variance (ANOVA). Multiple comparisons was performed using the Tukey-Kramer procedure. The number of offspring as well as the weight of offspring and placenta was analyzed using Student's *t*-test. The differences in body weight among the voles included in this study were analyzed by one-way repeated measures ANOVA. A *P* value less than 0.05 was considered to be statistically significant.

RESULTS

Duration of anesthesia: The results of evaluation of the duration of anesthesia are shown in Table 1. In female voles, the mean rates of induction of anesthesia were 100% with Somnopentyl and M/M/B:0.3/4/5 and 77.8% with M/M/B:0.23/3/3.75 and M/M/B:0.15/2/2.5. The mean rate of mortality was significantly higher with Somnopentyl than with the other anesthetics ($P<0.05$), and the all the animals that were administered one of three combinations of M/M/B survived.

In male voles, the mean rates of induction of anesthesia with different anesthetics were as follows, in a decreasing order: Somnopentyl, 100%; M/M/B:0.3/4/5, 77.8%; M/M/B:0.23/3/3.75, 77.8%; and M/M/B:0.15/2/2.5, 66.7%. The mean rate of mortality was significantly higher with Somnopentyl than with the other three anesthetics, and all male voles that were administrated one of three combinations of M/M/B survived.

Time-related parameters of anesthesia: The results of evaluation of time-related parameters of anesthesia are shown in Table 2. In female voles, the mean time for onset of loss of righting reflex in all groups was less than 5 min. Therefore, observation of the parameters was possible after 5 min of administration of the anesthetics. The mean induction time of M/M/B:0.15/2/2.5 was longer compared to those of the rest of the anesthetics. While Somnopentyl exhibited the shortest mean duration of anesthesia (24.2 ± 3.0 min), M/M/B:0.3/4/5 exhibited the longest (29.6 ± 0.3 min). The mean emergence time with M/M/B:0.3/4/5 was similar to that with M/M/B:0.23/3/3.75 (5.7 ± 2.4 and 5.8 ± 3.4 min, respectively); however, M/M/B:0.15/2/2.5 exhibited the shortest mean emergence time (1.8 ± 0.9 min). Furthermore, while the mean immobilization time with M/M/B:0.3/4/5 was the longest, that with Somnopentyl was the shortest. However, there were no significant differences in the mean immobilization times among four groups of animals.

In male voles, the mean time for onset of loss of righting reflex in all groups was less than 5 min. The mean duration of anesthesia with Somnopentyl was the longest (28.8 ± 3.5 min), while that with M/M/B:0.15/2/2.5 was the shortest (24.2 ± 2.5 min). The mean emergence time with M/M/B:0.15/2/2.5 was the shortest (0.5 ± 0.5 min) of four groups. The mean immobilization time with M/M/B:0.3/4/5 was the longest (34.7 ± 4.6 min), while that with M/M/B:0.15/2/2.5 was shortest (25.2 ± 2.4 min). However, there were no significant differences in the mean immobilization times among four groups of animals.

Body weight: The results of evaluation of body weight are shown in Table 3. None of the voles included in this experiment died. Among the female voles, body weight of animals anesthetized with Somnopentyl tended to decrease on Day 1, although it recovered subsequently after several days. No

Table 2. Comparison of the induction emergence and immobilization times and duration of anesthesia with different anesthetic agents

Sex	Agent	Total no. of voles examined	Loss of righting reflex (min)[a]	Induction time (min)[a,b)]	Duration of anesthesia (min)[a,c)]	Emergence time (min)[a,d)]	Immobilization time (min)[a,e)]
Female	Somnopentyl	5	2.8 ± 0.4	0.8 ± 0.4	24.2 ± 3.0	-	25.0 ± 2.7
	M/M/B: 0.3/4/5	9	2.7 ± 0.5	0.7 ± 0.2	29.6 ± 0.3	5.7 ± 2.4	35.3 ± 2.2
	M/M/B: 0.23/3/3.75	7	4.1 ± 0.8	0.9 ± 0.3	26.9 ± 2.8	5.8 ± 3.4	32.7 ± 4.4
	M/M/B: 0.15/2/2.5	7	4.6 ± 0.8	2.0 ± 0.7	26.7 ± 2.0	1.8 ± 0.9	30.1 ± 2.2
Male	Somnopentyl	9	5.0 ± 0.7	1.2 ± 0.3	28.8 ± 3.5	-	30.0 ± 3.3
	M/M/B: 0.3/4/5	7	3.0 ± 1.0	1.0 ± 0.0	27.9 ± 2.1	6.0 ± 3.6	34.7 ± 4.6
	M/M/B: 0.23/3/3.75	7	3.0 ± 0.4	0.7 ± 0.2	27.3 ± 2.7	1.5 ± 0.4	29.9 ± 3.2
	M/M/B: 0.15/2/2.5	6	4.2 ± 0.3	0.8 ± 0.3	24.2 ± 2.5	0.5 ± 0.5	25.2 ± 2.4

a) Time is expressed in min and presented as the mean ± SEM. b) Induction time was defined as duration between loss of righting reflex and duration of anesthesia. c) The duration of anesthesia was defined as duration between induction and emergence times. d) Emergence time was defined as duration between the end of surgical anesthesia and recovery of righting reflex. e) Immobilization time was defined as duration between loss of righting reflex and recovery of righting reflex.

Table 3. Changes in the mean body weight (g) of female and male voles treated with different anesthetics

Sex	Agent	Total no. of voles examined	Weight (g)[a)]					
			Day 0	Day 1	Day 3	Day 5	Day 7	Day 14
Female	Somnopentyl	5	28.88 ± 2.08	27.23 ± 2.76	27.59 ± 2.46	26.83 ± 1.90	26.63 ± 1.85	27.39 ± 2.24
	M/M/B: 0.3/4/5	9	29.19 ± 1.24	29.54 ± 1.12	29.13 ± 1.12	29.47 ± 1.16	28.52 ± 1.01	28.89 ± 1.00
	M/M/B: 0.23/3/3.75	9	28.86 ± 1.40	28.42 ± 1.40	28.38 ± 1.46	28.63 ± 1.41	28.72 ± 1.30	29.30 ± 1.37
	M/M/B: 0.15/2/2.5	9	27.90 ± 1.90	27.84 ± 2.22	27.18 ± 1.67	28.81 ± 2.10	28.84 ± 2.11	28.93 ± 2.11
Male	Somnopentyl	6	38.39 ± 1.44	38.03 ± 1.92	39.23 ± 1.40	39.56 ± 1.35	39.70 ± 1.77	40.77 ± 1.72
	M/M/B: 0.3/4/5	9	40.77 ± 1.89	39.14 ± 1.59	40.43 ± 1.79	41.07 ± 1.79	41.43 ± 1.99	42.31 ± 1.73
	M/M/B: 0.23/3/3.75	9	41.15 ± 2.45	39.70 ± 2.14	40.61 ± 2.22	40.94 ± 2.11	41.74 ± 2.11	43.49 ± 2.16
	M/M/B: 0.15/2/2.5	9	41.07 ± 2.79	40.49 ± 2.77	40.82 ± 2.81	41.69 ± 2.90	41.98 ± 2.89	41.50 ± 2.91

a) Data are presented as the mean ± SEM.

changes in body weight were observed in the other groups. There were no significant differences in body weight among four groups.

In male voles, body weight of animals anesthetized with M/M/B:0.3/4/5 tended to decrease on Day 1; however, there were no significant differences in body weight among four groups.

Anesthetic scores: In female voles (Fig. 1A), M/M/B: 0.3/4/5 and M/M/B:0.23/3/3.75 exhibited significantly higher anesthetic scores at 0 to 20 min after administration than Somnopentyl (P<0.05); M/M/B:0.15/2/2.5 exhibited a significantly lower anesthetic score at 0 to 20 min after administration than M/M/B:0.3/4/5 (P<0.05). At 0 min after administration, M/M/B:0.3/4/5 exhibited a significantly higher anesthetic score than M/M/B:0.23/3/3.75 (P<0.05), and at 10 min after administration, M/M/B:0.15/2/2.5 exhibited a significantly higher anesthetic score than Somnopentyl (P<0.05). There were no significant differences in the anesthetic scores among four groups at rest of time points.

In male voles (Fig. 1B), M/M/B:0.3/4/5 exhibited a significantly higher anesthetic score at 0 to 30 min after administration than Somnopentyl (P<0.05), while M/M/B:0.23/3/3.75 exhibited a significantly higher anesthetic score at 0 to 20 min after administration than Somnopentyl (P<0.05). At 10 min after administration, M/M/B:0.15/2/2.5 exhibited a significantly higher anesthetic score than Somnopentyl (P<0.05). At 30 min after administration, M/M/B:0.15/2/2.5 exhibited a significantly lower anesthetic score than M/M/B:0.3/4/5 (P<0.05). There were no significant differences in anesthetic scores among four groups at rest of the time points.

Frequency of respiratory: In the female voles, frequency of respiratory in Somnopentyl-treated group was remarkably decreased between 0 and 10 min after administration. However, there were no significant differences in the respiratory rates among four groups (Fig. 2A).

In the male voles, Somnopentyl exhibited a significantly higher frequency of respiratory compared with M/M/B:0.3/4/5 and M/M/B:0.15/2/2.5 at 0 min after administration; however, there were no significant differences in frequency of respiratory among four groups at the rest of the time points (Fig. 2B).

Influence of different anesthetics on recipients of embryo transfer: Similar to results presented in Table 1, the rate of mortality with Somnopentyl was significantly higher compared to those with the M/M/B combinations (P<0.05; Table 4). The rates of pregnancy in voles administered M/M/B combinations tended to be higher compared to that with Somnopentyl; the birth rate with Somnopentyl was higher compared to those with M/M/B combinations, although the differences were insignificant. The rate of abor-

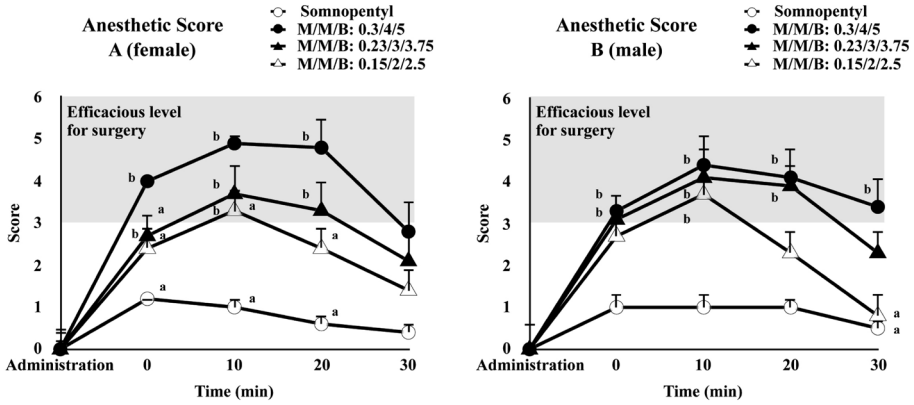

Fig. 1. Time course changes in the anesthetic scores of the female (A) and male (B) voles. Open circle (○), Somnopentyl; solid circle (●), M/M/B:0.3/4/5; solid triangle (▲), M/M/B:0.23/3/3.75; open triangle (△), M/M/B:0.15/2/2.5. The gray background indicates the efficacious level for surgery. The data are presented as the mean ± SEM. The differences between anesthetics were analyzed by one-way ANOVA. A P value less than 0.05 was considered to be statistically significant. a, $P<0.05$ compared with M/M/B:0.3/4/5 at each time point. b, $P<0.05$ compared with Somnopentyl at each time point. A score of more than 3 was defined as indicating significant anesthesia.

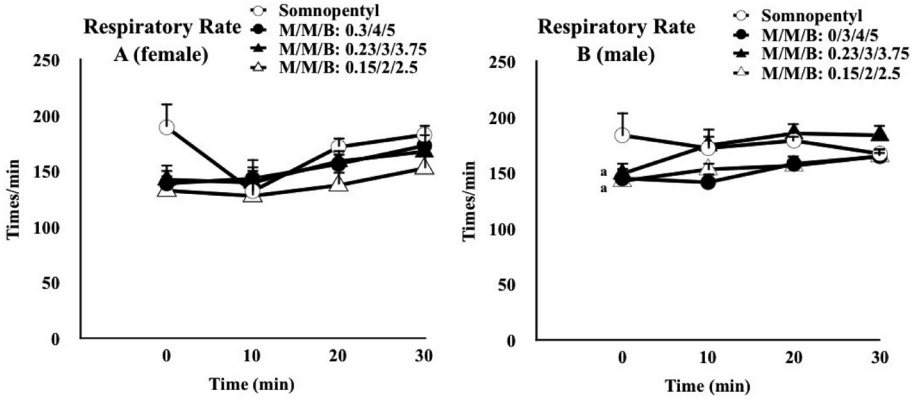

Fig. 2. Time course changes in the frequency of respiration in female (A) and male (B) voles. Open circle (○), Somnopentyl; solid circle (●), M/M/B:0.3/4/5; solid triangle (▲), M/M/B:0.23/3/3.75; open triangle (△), M/M/B:0.15/2/2.5. The data are presented as the mean ± SEM. The differences between anesthetics were analyzed by one-way ANOVA. A P value less than 0.05 was considered to be statistically significant. a, $P<0.05$ compared with Somnopentyl at each time point.

tion with Somnopentyl was significantly higher compared to those with M/M/B combinations ($P<0.05$). Consequently, the rate of implantation with Somnopentyl was significantly higher compared to that with M/M/B combinations ($P<0.05$), since the values of rate of implantation included the values of both rate of abortion and birth. Finally, voles produced offspring irrespective of the type of anesthetic administered, and there were no significant differences in the data of offspring among four groups (Table 5).

DISCUSSION

Somnopentyl had been used as an anesthetic for surgeries in voles in our previous experiments. Although the concentration of Somnopentyl used in these experiments was lower than that used for other rodents, the death of voles was observed during surgical procedures, such as embryo transfer or vasectomy (data not shown), indicating that voles might have a higher sensitivity to anesthesia than the other rodents. Therefore, we believed that it was necessary to identify a more appropriate anesthetic for experiments involving voles.

In recent studies, a combination anesthetic composed of medetomidine, midazolam and butorphanol (M/M/B) has been used in animal experiments involving mice and rats [11, 14–16]; this anesthetic has also been used in dogs, monkeys, sea lions, red foxes, ibexes and African lions [8–10, 18, 22, 25, 33]. Many reports indicate that M/M/B is relatively safe and reliable. For these reasons, M/M/B was evaluated in the present study as an alternative anesthetic agent to Somnopentyl; additionally, the appropriate concentration of

Table 4. Results of embryo transfer experiments in voles with different anesthetics

Agent[a)]	Age	No. of recipient females	Viability (%)[b)]	Mortality (%)[b)]	Pregnancy rate (%)[c)]	No. of transferred embryos	Birth rate (%)[d)]	Abortion rate (%)[d)]	Implantation rate (%)[e)]
Somnopentyl	13 to 47	18	12 (66.7)[A)]	6 (33.3)[C)]	7 (58.3)	124	27 (21.8)	18 (14.5)[E)]	45 (36.3)[G)]
M/M/B	14 to 34	18	18 (100)[B)]	0 (0.0)[D)]	12 (66.7)	202	34 (16.8)	14 (6.9)[F)]	48 (23.8)[H)]

a) The anesthetics administered were 40 mg/kg Somnopentyl and M/M/B:0.23/3/3.75. b) Viability and mortality rates were calculated according to the number of recipient females. c) The rates of pregnancy were calculated according to the number of surviving individuals. d) The number of implantation was defined as total of number of birth and abortion. e) The birth, abortion and implantation rates were calculated according to the number of transferred embryos. A–H) Different superscripts represent significant differences along the same line ($P<0.05$).

Table 5. Data of the offspring after embryo transfer with different anesthetics

Agent[a)]	No. of offspring		Of offspring (%)[c)]		Weight (g) of[b)]		Sex (%)[d)]	
	Total	Average[b)]	Viability	Mortality	Offspring	Placenta	Female	Male
Somnopentyl	27	5.4 ± 1.3	23 (85.2)	4 (14.8)	3.09 ± 0.13	0.2 ± 0.03	13 (56.5)	10 (43.5)
M/M/B	34	3.8 ± 0.8	29 (85.3)	5 (14.7)	3.19 ± 0.09	0.24 ± 0.02	14 (48.3)	15 (51.7)

a) The anesthetics administered were 40 mg/kg Somnopentyl and M/M/B:0.23/3/3.75. b) Data of the average number of offspring and weight are presented as the mean values ± SEM. c) Viability and mortality rates were calculated according to the total number of offspring. d) The distribution of sex was calculated according to the number of surviving offspring.

M/M/B for induction of anesthesia in voles was also evaluated. Since it has been reported that pentobarbital (Somnopentyl) causes severe adverse cardiorespiratory reactions and has poor analgesic action [29], we adjusted its dosage at 40 mg/kg, which is a relatively low dosage in mice. Although a previous study had adjusted the dosage of Somnopentyl in voles to 35 mg/kg [32], in our preliminary experiments, the administration of Somnopentyl at a dosage of 35 mg/kg did not induce sufficient loss of righting reflex in Japanese field voles, and high rates of mortality were observed at a dosage of 45 mg/kg (data not shown). Therefore, dosage of Somnopentyl was set at 40 mg/kg in the present study.

Upon the administration of Somnopentyl, reflex parameters of voles were continuously recorded after loss of righting reflex; the average scores were less than 3, suggesting that Somnopentyl did not induce anesthesia to an adequate depth. This result also indicates that intraperitoneal injection of 40 mg/kg Somnopentyl is inadequate for performing surgical procedures in voles, which is in accordance with findings of previous reports indicating that 50 mg/kg Somnopentyl failed to induce an adequate depth of anesthesia in rodents [3, 24, 26].

Each of drugs included in the combination anesthetic M/M/B exhibits different pharmacological mechanisms and effects. Medetomidine is an alpha$_2$-adrenergic agonist that induces a sedative analgesic effect [30, 31]. Midazolam is a benzodiazepine receptor agonist that causes sedation [12]. Butorphanol is an opioid μ-receptor antagonist and consequently, a non-narcotic drug, that acts on opioid κ-receptors to induce an analgesic effect [7]. Atipamezole is an alpha$_2$-adrenergic antagonist that reverses the effects of medetomidine [4]. In the groups anesthetized with M/M/B combinations in the present study, the average scores of each of reflex parameters reached 3 in both female and male voles. Additionally, M/M/B combinations induced adequate depths of anesthesia and exhibited survival rates of 100%. These results indicate that intra-peritoneal injection of M/M/B might be adequate for induction of anesthesia during surgical procedures in voles. However, disrupted respiratory rhythm and apneic states were observed in 77.8% of voles anesthetized with M/M/B:0.3/4/5 (data not shown). These symptoms of respiratory distress were observed among voles of all ages anesthetized at this concentration and were similar to those exhibited by voles anesthetized with Somnopentyl, which also exhibited a high rate of mortality. These symptoms were observed even after the administration of atipamezole, although they seemed to resolve in a time-dependent manner. Furthermore, most of the voles anesthetized with M/M/B:0.3/4/5 did not show active movements after recovery of righting reflex and crouched in their cages. However, these symptoms were not observed after administration of atipamezole in voles anesthetized with M/M/B:0.23/3/3.75. Although there was no significant difference in the mean emergence time between M/M/B:0.3/4/5 and M/M/B:0.23/3/3.75 in female voles (Table 2), that in the male voles was extended from 1.5 ± 0.4 min with M/M/B:0.23/3/3.75 to 6.0 ± 3.6 min with M/M/B:0.3/4/5. In both female and male voles, the mean anesthetic score of M/M/B:0.15/2/2.5 was lower compared to that of the other M/M/B combinations. Regarding duration of deep anesthesia, although M/M/B:0.23/3/3.75 exhibited a shorter anesthetic duration than M/M/B:0.3/4/5, we believe that this duration is adequate for completion of surgical procedures, such as embryo transfer and vasoligation, which are generally completed within approximately 25 min after induction of anesthesia in both female and male voles. Therefore, we concluded that the concentration of M/M/B:0.23/3/3.75 is adequate for induction of anesthesia during surgery in voles.

Although a previous study had reported no differences in the duration of anesthesia between female and male mice [14], differences in sensitivity to anesthesia were observed between female and male voles in the present study. The immobilization times with M/M/B combinations in male voles tended to be shorter compared to those in the female voles;

however, this trend was reversed in the case of Somnopentyl. This result may be attributed to the differences in tolerance to anesthesia with M/M/B between female and male voles. Then, the result of Somnopentyl might account for the differences in body weight between female and male voles; however, the M/M/B combinations also induced different effects in the sexes at each of the dosages evaluated in the present study. Moreover, in female voles, a rapid decrease in respiratory rate was observed with Somnopentyl from 0 to 10 min after administration. Differences in bronchial responsiveness have been reported among inbred mouse strains [2]. Similarly, bronchial hyper-response induced by Somnopentyl might induce a higher respiratory rate in female voles compared to that induced by M/M/B.

Finally, we examined the effects of different anesthetics on production of vole offspring derived from *in vitro* fertilized oocytes. The results indicated that animal death due to Somnopentyl occurred during surgical embryo transfer, and the corresponding rate of mortality was similar to result of Table 1, which indicates that the problem was not caused by maneuvering of the researcher during embryo transfer. On the other hand, all of the voles anesthetized with M/M/B:0.23/3/3.75 survived; however, there were no significant differences in rates of pregnancy between M/M/B:0.23/3/3.75 and Somnopentyl. Moreover, the rate of abortion in Somnopentyl-treated group was significantly higher compared to those of the M/M/B combination-treated groups. These results strongly indicate that M/M/B could be an alternative to Somnopentyl, which causes adverse effects in patients, including pain and stress during surgery.

In conclusion, we demonstrated the advantageous effects of administration of a combination anesthetic comprising medetomidine, midazolam and butorphanol in female and male Japanese field voles. The findings of the present study provide basic information for achieving appropriate anesthetic concentrations, thus contributing to the development of experiments and improvement of the laboratory animal welfare with regard to the genus *Microtus*.

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