Exp. Anim. 60(5), 481–487, 2011

—Original—

Effect of Three Types of Mixed Anesthetic Agents Alternate to Ketamine in Mice

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Abstract: Ketamine is usually used for murine anesthesia in animal experiments with other anesthetics for its sedation and analgesic effects. However, ketamine was categorized as a narcotic drug in Japan on January 1, 2007. After this act came into effect, a narcotic handling license became necessary for using and possessing ketamine. Pentobarbital sodium, which is also used for laboratory animal experiments as Nembutal, is no longer being manufactured. For these reasons, other anesthetic agents that can be used without a license are needed. In this paper, we examined the use of anesthetics other than ketamine and pentobarbital sodium. A combination anesthetic (M/M/B: 0.3/4/5) was prepared with 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol. The anesthetics were administered to male ICR mice by intraperitoneal injection. In order to assess anesthetic depth and duration, we stimulated the mice directly after loss of righting reflexes to recovery of these same reflexes and then recorded four parameters—a tail pinch reflex, a pedal withdrawal reflex in the forelimbs, a pedal withdrawal reflex in the hindlimbs, and corneal reflex. Each parameter was scored, and the anesthetic depth, expressed by the total score, was summed. The surgical anesthesia duration of M/M/B: 0.3/4/5 mg/kg was almost identical to the surgical anesthetic duration with a ketamine and xylazine mixture (80–8 mg/kg). These data suggested that mice can be anesthetized by M/M/B: 0.3/4/5 as an alternate to ketamine. We thus can recommend M/M/B: 0.3/4/5 for murine surgical anesthesia.
Key words: anesthetics, butorphanol, ketamine, medetomidine, midazolam

Introduction

Ketamine has generally been used for murine anesthesia in animal experiments because it has both sedative and analgesic effects [16]. It has been used with a sedatives such as xylazine [1, 4, 5, 7–10, 12, 14, 16, 21, 25]. However, ketamine was categorized as a narcotic drug in Japan on January 1, 2007. After enforcement of the act, a narcotic handling license became necessary for use of ketamine. Additionally, there are strict rules for its purchasing, handling, storage and procedures of record keeping.

In regard to alternatives, pentobarbital sodium cannot be purchased, except for Somnopentyl, because manufacture of Nembutal, which is a type of pentobarbital sodium very commonly used for animal experiments,

(Received 18 February 2011 / Accepted 23 May 2011)
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has been discontinued. Nembutal is no longer available in the market. However, Somnopentyl is an agent authorized as an animal drug, and it has been categorized as a prescription medicine. Therefore, a prescription from a veterinarian is required to use Somnopentyl for animals.

For these reasons, other anesthetic agents that can be used without a license and without involving complicated procedures are needed. In this paper, we examined different anesthetic combinations as substitutes for ketamine and pentobarbital sodium.

Materials and Methods

Animals and housing conditions

We purchased male specific pathogen-free ICR strain mice at 6 weeks of age from Charles River Laboratories Japan Inc. (Yokohama, Japan). Animals were acclimated for at least 7 days before experimental use. Mice 7–9 weeks of age were used for the experiment, and their weights ranged from 32.1–37.9 g (average 33.8 g). All of the animal procedures were conducted in accordance with the guidelines under the Regulations on Animal Experimentation at Osaka University. The animals were housed in stable groups of three mice each in polycarbonate cages with autoclaved bedding (ALPHA-dri, Shepherd Specialty Papers, Watertown, TN, USA). Each cage was provided with reverse-osmosis water delivered by an automatic water supply system and supplied with sterilized food (MF, Oriental Yeast Co., Ltd., Tokyo, Japan). Room temperature was controlled by reheating units inside rooms and was maintained at 23 ± 2°C. The humidity was maintained at 30 to 70%. Animals were maintained on a 12:12-h light:dark cycle (lights on, 8 a.m. to 8 p.m.).

Anesthetic agents

The following anesthetics were evaluated: medetomidine hydrochloride (Domitol, Meiji Seika Pharma Co., Ltd., Tokyo, Japan), which is an alpha 2 adrenoceptor agonist; midazolam (Dormicum, Astellas Pharma Inc., Tokyo, Japan), which is a benzodiazepine derivative; butorphanol (Vetorphale, Meiji Seika Pharma Co., Ltd.); ketamine hydrochloride (Ketalar, Sankyo Lifetech Co., Ltd., Tokyo, Japan); xylazine (Celactar, Bayer, Ltd., Tokyo, Japan) [19]; and two types of pentobarbital sodium (Nembutal, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan; Somnopentyl, Kyoritsu Seiyaku Co., Ltd., Tokyo Japan), both of which are barbiturates. These anesthetics were kept at room temperature.

Preparation of the anesthetics

Five combinations of anesthetics were examined, and the combinations and doses are listed in Table 1. All agents were diluted in sterilized water. In addition, they were filtered by 0.2-um filters (S-2502, Kurabo Industries Ltd., Bio-Medical Department, Osaka, Japan) and stored at 4°C in a dark refrigerator. K/X: 80/8 was prepared with ketamine hydrochloride at a dose of 80.0 mg/kg and xylazine at a dose of 8.0 mg/kg [1, 4, 5, 7–10, 12, 14, 16, 21, 25]. Shortly afterwards, 1.60 ml of ketamine hydrochloride was added to 0.40 ml of xylazine resulting in up to a volume 10.00 ml with sterilized water. K/X: 60/6 was prepared with ketamine hydrochloride at a dose of 60.0 mg/kg and xylazine at a dose of 6.0 mg/kg. Briefly, 1.20 ml of ketamine hydrochloride was added to 0.30 ml of xylazine, resulting in up to a volume 10.00 ml with sterilized water. PA: 50 was prepared with pentobarbital sodium A (Nembutal) at a dose of 50.0 mg/kg, and PB: 50 was prepared with pentobarbital sodium B (Somnopentyl) at a dose of 50.0 mg/kg [1, 15, 25]. Practically, 1.00 ml of Nembutal was prepared to a volume 10.00 ml with sterilized water, and 0.77 ml of Somnopentyl was prepared to a volume 10.00 ml with sterilized water. M/M/B: 0.3/4/5 was prepared with medetomidine hydrochloride at a dose of 0.3 mg/kg, midazolam at a dose of 4.0 mg/kg and butorphanol at a dose of 5.0 mg/kg. Concretely, 0.30 ml of medetomidine hydrochloride was mixed with 0.80 ml of midazolam and 1.00 ml of butorphanol and adjusted to a volume of 10.00 ml with sterilized water. The dosage of the combination of M/M/B was constructed as reported in consideration of doses for animal species [2, 6, 8, 13, 17, 18, 20, 24, 26]. The concentration ratio of M/M/B (0.3/4/5 mg/kg) was determined by preliminary experiments. The prepared anesthetic agents were administered to mice at a volume of 0.01 ml/g of body weight.

Table 1. Concentrations and doses of anesthetics that were used in this study

Agent	Product	Product concentration (mg/ml)	Agent dose (mg/kg)	Administered volume (ml/kg)	Volume in 10-ml water solution (ml)
M/M/B: 0.3/4/5	Medetomidine	1.0	0.3	0.30	0.30
	Midazolam	5.0	4.0	0.80	0.80
	Butorphanol	5.0	5.0	1.00	1.00
K/X: 80/8	Ketamine	50.0	80.0	1.60	1.60
	Xylazine	20.0	8.0	0.40	0.40
K/X: 60/6	Ketamine	50.0	60.0	1.20	1.20
	Xylazine	20.0	6.0	0.30	0.30
PA: 50	Pentobarbital A	50.0	50.0	1.00	1.00
PB: 50	Pentobarbital B	64.8	50.0	0.77	0.77

Abbreviations: M/M/B: 0.3/4/5 = medetomidine 0.3 mg/kg : midazolam 4.0 mg/kg : butorphanol 5.0 mg/kg. K/X: 80/8 = ketamine 80 mg/kg : xylazine 8 mg/kg. K/X: 60/6 = ketamine 60 mg/kg : xylazine 6 mg/kg. PA: 50 = pentobarbital sodium A (Nembutal) 50 mg/kg. PB: 50 = pentobarbital sodium B (Somnopentyl) 50 mg/kg.

Administration of anesthetics and animal handling

Each experimental group of 15 mice were separated into 5 sets with each set consisting of 3 randomly chosen mice. All manipulations were carried out in the experimental room after transfer from the animal holding room. After weighing the animals, the appropriate volumes of anesthetic agents were administered by intraperitoneal injection in the lower left quadrant of each mouse under manual restraint [3]. Each animal was placed back in its home cage until it lost its righting reflex. After loss of righting reflex, the animals were lied on their back on a Micro-Temp II 747 heated platform (40–42°C, Cincinnati Sub-Zero Products, Inc., Cincinati, OH, USA) and laid in dorsal recumbency without fixation. Each animal breathed room air for the duration of the experiment and was returned to its home cage at recovery of righting reflex. After that, all animals were observed for at least one additional hour to record any abnormal behavior. Recovery of righting reflex was defined as the time when the animals first righted themselves and began to walk two or three steps.

Parameters of anesthesia

To assess the depth and duration of anesthesia, four parameters were used, i.e., the tail pinch reflex, pedal withdrawal reflex in forelimbs, pedal withdrawal reflex in hindlimbs and corneal reflex. For the tail pinch reflex, excluding the vein, 6 locations of the proximal tail were slightly pinched using atraumatic forceps. For the pedal withdrawal reflex, the interdigital webbing of both side limbs were slightly pinched using atraumatic forceps and pulled. The pedal withdrawal reflex was assessed in both forelimbs and hindlimbs. For the corneal reflex, air was blown at the animal's cornea using a Pasteur pipette (IK-PAS-9P, Iwaki Glass, Funabashi, Japan; AGC TECHNO GLASS Co., Ltd., Funabashi, Japan) with a 2-ml silicone nipple (6-356-02, AS ONE Corporation, Osaka, Japan).

Tests and observations

A series of observations, simple tests, and reflex measurements were carried out on each individual mouse to evaluate the depth and duration of anesthesia. To reduce sources of variation in response to the stimuli, all reflex tests were carried out and assessed by the same operator. The stimulation was repeated every 5 min after administration of an anesthetic agent. The stimulation was repeated from loss of righting reflex to recovery of righting reflex. When a reflex reaction to stimulation was observed, the score was 0. When a reflex reaction to stimulation was not observed, the score was 1. Each parameter was scored, and the anesthetic depth was expressed by total score calculated for each mouse. A score of 3 or more was defined as surgical anesthesia. Furthermore, the time of loss of righting reflex and the time of recovery of righting reflex were recorded.

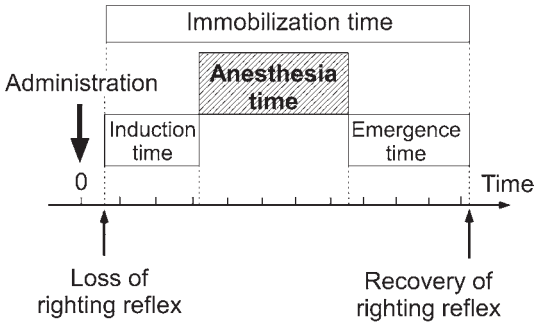

Fig. 1. Time-related parameters for the anesthesia recorded in this study.

Table 3. Duration of immobilization and anesthesia

Agent	Duration of immobilization (min)	Duration of anesthesia (min)
M/M/B:0.3/4/5	52.00 ± 14.73	40.00 ± 13.23
K/X: 80/8	40.83 ± 3.00	28.33 ± 7.64
K/X: 60/6	26.75 ± 6.88	15.00 ± 5.00
PA: 50	43.42 ± 11.14	–
PB: 50	23.00 ± 3.38	–

The immobilization time was defined as the duration between the loss of righting reflex and recovery of righting reflex. The anesthesia time was defined as the duration between the commencement of surgical anesthesia and the end of surgical anesthesia. Time courses are expressed in minutes and presented as means ± SD (n=3). See Table 1 for abbreviations.

Table 2. The commencement and end of surgical anesthesia and the induction and emergence of anesthesia

Agent	Loss of righting reflex (min)	Commencement of surgical anesthesia (min)	End of surgical anesthesia (min)	Recovery of righting reflex (min)	Induction time (min)	Emergence time (min)
M/M/B:0.3/4/5	2.67 ± 0.58	8.33 ± 2.89	35.00 ± 13.23	54.67 ± 15.01	5.67 ± 2.31	11.33 ± 0.58
K/X: 80/8	2.50 ± 1.10	6.67 ± 2.89	25.00 ± 10.00	43.33 ± 2.52	4.17 ± 2.95	11.67 ± 10.07
K/X: 60/6	2.58 ± 1.21	8.33 ± 2.89	10.00 ± 5.00	29.33 ± 5.77	5.75 ± 1.88	11.00 ± 5.00
PA: 50	3.25 ± 0.43	–	–	46.67 ± 11.50	–	–
PB: 50	2.67 ± 2.67	–	–	25.67 ± 2.08	–	–

The commencement of surgical anesthesia was the end of the induction time. The end of surgical anesthesia was defined as the end of the anesthesia time. The induction time was defined as the duration between the loss of righting reflex and the commencement of surgical anesthesia. The emergence time was defined as the duration between the end of surgical anesthesia and the recovery of righting reflex. Time courses are expressed in minutes and presented as means ± SD (n=3). See Table 1 for abbreviations.

Time-related parameters of anesthesia (Fig. 1)

Immobilization time (time during which the animals made no movements) was defined as the time span between loss of righting reflex and recovery of righting reflex. Induction time was considered to be the time from loss of righting reflex to start of surgical anesthesia. Duration of anesthesia was the time span of surgical anesthesia. Emergence time was considered to be the time from the end of surgical anesthesia to recovery of righting reflex.

Data analysis

All experimental data were analyzed using Excel (Microsoft, Tokyo, Japan).

Results

K/X (ketamine/xylazine) mixed agents

The loss of righting reflex times of K/X: 80/8 and K/X: 60/6 were both less than 5 min (Table 2). Therefore, observation of the four parameters was possible after 5 min of administration. The induction times of K/X: 80/8 and K/X: 60/6 were the same (Table 2). Furthermore, the emergence time of K/X: 80/8 was similar to that of K/X: 60/6 (Table 2). The anesthesia time of K/X: 60/6 was less than 10 min, whereas the anesthesia time of K/X: 80/8 was more than 20 min (Table 3).

PA and PB (pentobarbital sodium)

The loss of righting reflex times of PA: 50 and PB: 50 were both less than 5 min (Table 2). Therefore, observation of the four parameters was possible from 5 min of

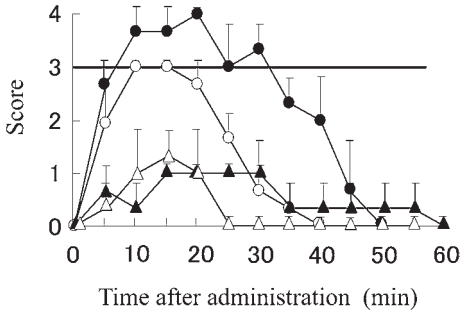

Fig. 2. The anesthetic scores for the four kinds of agents. Solid circle (●): K/X: 80/8. Open circle (○): K/X: 60/6. Solid triangle (▲): PA: 50. Open triangle (△): PB: 50. Data represent means ± SD of 3 mice. A score of more than 3 was defined as surgical anesthesia. See Table 1 for abbreviations.

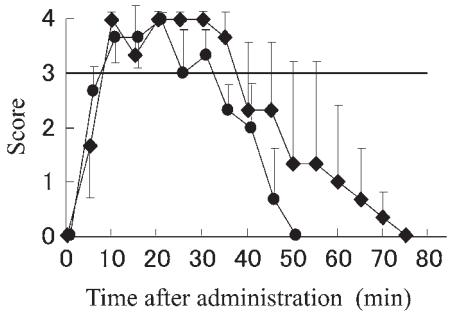

Fig. 3. The anesthetic scores for M/M/B: 0.3/4/5 and K/X: 80/8. Solid circle (●): K/X: 80/8. Solid diamond (◆): M/M/B: 0.3/4/5. All values represent the means ± SD of 3 mice. A score of more than 3 was defined as surgical anesthesia. See Table 1 for abbreviations.

administration. However, surgical anesthesia was not achieved because the score did not reach 3 (Fig. 2).

Alternative anesthetic agent

The data for K/X: 80/8 were used as evaluation criteria of an alternative anesthetic agent because K/X: 80/8 has an anesthetic time of more than 20 min and has both a short induction time and short emergence time. M/M/B: 0.3/4/5 was comparable with K/X: 80/8 (Fig. 3). The induction time of M/M/B: 0.3/4/5 was identical to the induction time of K/X: 80/8 (5.67 ± 2.31, 4.17 ± 2.95; Table 2). The emergence time of M/M/B: 0.3/4/5 was the similar to the emergence time of K/X: 80/8 (11.33 ± 0.58, 11.67 ± 10.07; Table 2). The anesthesia time of M/M/B: 0.3/4/5, however, was longer than the anesthetic time of K/X: 80/8 (40.00 ± 13.23, 28.33 ± 7.64; Table 2, Fig. 3).

Discussion

Midazolam is a water-soluble benzodiazepine [16]. Benzodiazepines can produce marked sedation in rodents, pigs, and primates; however, they are not analgesic and do not produce a true general anesthetic state [16]. In veterinary medicine, midazolam is used in a combination with other agents for anesthesia or anesthetic induction [16]. Medetomidine is an imidazole derivative more potent than xylazine, with higher alpha2-adrenoceptor selectivity [16]. It has been used with ketamine for laboratory rodents [6, 10, 16]. This combination produces moderate surgical anesthesia in mice [6, 10, 16]. Butorphanol, a synthetic opioid agonist-antagonist, is used as an analgesic drug in veterinary medicine [11]. The combination of medetomidine, midazolam, and butorphanol has been reported as a safe and reliable anesthetic drug in the dog [13, 26], sea lion [24] and red fox [2]. However, there are no reports in rodents. In this study, combinations of injectable anesthetic agents (M/M/B) were examined as alternative agents for ketamine in mice.

The combination of ketamine and xylazine was used as a comparison standard for comparison with an injectable agent. This combination was decided based on studies in mice [1, 4, 5, 7–10, 12, 14, 16, 21, 25]. The anesthesia time of K/X: 80/8 was more than 20 min, but the anesthesia time of K/X: 60/6 was less than 20 min. It has been reported that the effect of combinations of ketamine and xylazine depends on the quantities of ketamine and xylazine [1, 4]. On the other hand, the combination injectable agent (M/M/B) was determined by reports that were examined concerning other animal species [2, 13, 24, 26], and the concentration of M/M/B (0.3/4/5 mg/kg) was determined by a preliminary experiment. The induction time of K/X: 80/8 was less than 5 min, and that of M/M/B: 0.3/4/5 was also less than 5 min. Furthermore, the emergence time of K/X: 80/8 was less than 15 min, and that of M/M/B: 0.3/4/5 was also less than 15 min. Consequently, the induction and emer-

gence times of M/M/B: 0.3/4/5 and K/X: 80/8 were both equivalent. Furthermore, postsurgical management seemed to be easier to be controlled for M/M/B: 0.3/4/5 than for K/X: 80/8 because the SD of K/X: 80/8 was larger than that of M/M/B: 0.3/4/5. However, the anesthetic time of M/M/B: 0.3/4/5 was 40 min, while the anesthetic time of K/X: 80/8 was 28 min. M/M/B: 0.3/4/5 had a longer anesthetic time than K/X: 80/8. Therefore, it is preferable to use M/M/B: 0.3/4/5 as an anesthetic instead of ketamine when mice are anesthetized for a short time.

Furthermore, we examined two kinds of pentobarbital sodium (PA: 50 and PB: 50). The reflex parameters were continuously observed after the righting reflex disappeared. The average score did not reach 2. It became clear that anesthesia with PA: 50 and PB: 50 did not reach the depth of surgical anesthesia. Since this was the case, intraperitoneal injection of 50 mg/kg pentobarbital sodium was inadequate for surgical operation. In previous reports, it was also reported that 50 mg/kg pentobarbital sodium did not induce adequate anesthetic depth in rodents [7, 21, 25]. Therefore, we recommend M/M/B: 0.3/4/5 for murine surgical anesthesia.

In previous reports [7, 21, 25], changes in the time course of anesthetic depth were unclear. In our method, in contrast, the change of anesthetic depth was expressed definitely. This was the case because, for determination of anesthetic effect, the reflex reaction was scored every 5 min, and then the anesthetic depth was expressed as the total score. We recommend usage of the total score of four different reflexes as a marker of anesthetic depth.

In addition, the effects of anesthetic agents vary by murine strain [22, 23]. Therefore, further study is necessary to determine the anesthetic effect of M/M/B: 0.3/4/5 in other strains of mice, transgenic mice and knockout mice.

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