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Anesthetic Effects of a Mixture of Medetomidine, Midazolam and Butorphanol in Two Strains of Mice

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Abstract: The combination of ketamine and xylazine is a widely used anesthetic for laboratory animals. However, due to an abuse problem in Japan, ketamine has been specified as a narcotic since 2007. Instead of using ketamine, Kawai *et al.* reported an injectable formula with an equivalent effect to the mixture of ketamine and xylazine [11]. The mixture of 0.3 mg/kg body weight (b.w.) medetomidine (Med.), 4.0 mg/kg b.w. midazolam (Mid.), and 5.0 mg/kg b.w. butorphanol (But.) produced an anesthetic duration of around 40 min in outbred ICR mice. However, the anesthetic effect of the mixture for inbred mice strains remains unknown. Therefore, we examined anesthetic effects of the mixture of Med., Mid., and But. in the BALB/c and C57BL/6J strains. After intraperitoneal injection into mice, right front paw, left hind paw, and tail pinch reflexes as well as corneal and righting reflexes were observed. Every 5 min, we scored each reflex category as 0 for reaction or 1 for no reaction. As long as the total score was at least 4 out of 5, we considered the mixture as putting a mouse in a surgical anesthetic state. The mixture produced an anesthetic duration of more than 45 min in both strains of mice. These results indicate that the mixture of Med., Mid., and But. can be a useful and effective anesthesia for the BALB/c and C57BL/6J strains of inbred mice as well as outbred ICR mice.

Key words: anesthetic, butorphanol, inbred mice, medetomidine, midazolam

Introduction

The mixture of ketamine and xylazine has long been a popular combination as an injectable anesthetic for use in laboratory animals in Japan and around the world [6, 8, 20]. However, in Japan, ketamine has been designated as a narcotic drug due to an abuse problem. Therefore, using ketamine for animal experiments has become burdensome because users have to register to get a license as a researcher of narcotic drugs.

Instead of using ketamine, Kawai *et al.* reported a new injectable anesthetic with an effect in mice equivalent to the combination of ketamine and xylazine [11]. A mixture of two tranquilizers, medetomidine (Med.) and midazolam (Mid.), with butorphanol (But.), a nonnarcotic analgesic, produced a sufficient anesthetic duration about 40 min in ICR mice.

Inbred mice such as the BALB/c and C57BL/6 strains have often been used for animal experiments. The BALB/c strain is used for immunological or oncological

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experiments [1, 7, 16]. The C57BL/6 strain is also used for oncological experiments or as a wild type of genetically engineered mice [13, 18, 21].

Voipio *et al.* reported that there were some strain differences for anesthetic effects in mice [17]. Mulder's experiment indicated strain differences, but no sex differences for anesthetic duration in inbred mice [12]. However, the anesthetic effects of the new mixture in the BALB/c and C57BL/6 strains and any strain-related differences remain unknown.

Therefore, this study examined the anesthetic effects of the mixture of Med., Mid., and But. in BALB/c and C57BL/6J mice at 8, 12, 16, and 20 weeks of age. In addition, we examined the effective period of the mixture after mixing and refrigeration. The results might indicate a new effective anesthesia for inbred laboratory mice.

Materials and Methods

Animals and housing conditions

Twelve male (26.3 ± 1.3 g) (b.w., mean \pm SD) and 12 female (21.9 ± 1.9 g) BALB/c mice and 13 male (23.8 ± 2.6 g) and 10 female (19.8 ± 1.5 g) C57BL/6J mice were used in the first experiment. In the second experiment, we used 6 male BALB/c mice (25.2 ± 0.8 g). Animal care and experimental procedures were approved by the Animal Research Committee of Shimane University and conducted according to the Regulations for Animal Experimentation at Shimane University.

Three or 4 mice were housed in a TPX cage (KN-600[®], W 220 \times L 320 \times H 135 mm, Natsume Seisakusho, Co., Ltd., Tokyo, Japan) under a strict light cycle (light on at 7:00 and off at 19:00). Autoclaved bedding (Pure Chip[®], Shimizu Laboratory Supplies, Co., Ltd., Kyoto, Japan) was provided for each cage and changed once a week.

The animal room was maintained at a constant temperature ($23 \pm 2^\circ\text{C}$) and humidity ($55 \pm 10\%$). The mice were given a standard diet (MF[®], Oriental Yeast Co., Ltd., Tokyo, Japan) and filtered tap water by an automatic water supply system *ad libitum*. BALB/c and C57BL/6J mice were purchased at 6 weeks of age from a commercial supplier (CLEA Japan, Inc., Tokyo, Japan) and habituated for 2 weeks in the animal room before starting the experiment.

Experimental procedure

The experiment was conducted during daytime (14:00–17:00). The experimental room was controlled

so that it had the same temperature and humidity as the animal room. The mice were weighed before receiving anesthesia. The anesthetic was injected intraperitoneally at 0.1 ml/10 g b.w./mouse. After the injection, the mice were kept on a hot plate (Heater Mat KN-475[®], Natsume Seisakusho, Co., Ltd., Tokyo, Japan) maintained at approximately 38°C.

In the first experiment, an anesthetic score for each mouse was measured every 5 min until the mouse was completely recovered from anesthesia. Six male and female mice of each strain repeated the experiment every 4 weeks until 20 weeks of age.

In the second experiment, we measured anesthetic duration of 6 male BALB/c mice every 2 weeks until 8 weeks. The anesthetic mixture made on the first day was kept in a refrigerator and used throughout the experiment.

After finishing the experiment, the mice were euthanatized by intravenous injection of sodium pentobarbital (80 mg/kg b.w.) (Somnopentyl[®], Kyoritsu Seiyaku Corporation, Tokyo, Japan).

Measuring anesthetic scores

Each anesthetic score was measured using the modified grading system described by Kawai [11]. Measurement was based on 5 reflexes. The first was a body-righting reflex: when a mouse was put on its back, it was given a score of 1 if it did not get up and a score of 0 if it did. The second was a corneal reflex: when a mouse's eyes were gently stimulated by air using a Pasteur pipette with a silicone nipple 1 cm from its eyes, it was given a score of 1 if it did not move its eyelids and a score of 0 if it did. The third was a tail reflex: when a mouse's tail was gently and suddenly pinched with atraumatic 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 a front paw reflex: when a mouse was gently and suddenly pinched with atraumatic forceps between the second and third fingers of its right front paw, it was given a score of 1 if its paw did not move and a score of 0 if it did. The fifth was a hind paw reflex: when a mouse was gently and suddenly pinched with atraumatic forceps between the second and third finger of the left hind paw, it was given a score of 1 if its paw 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 4 or 5 was considered to indicate a surgical anesthetic level in mice. The duration for which a mouse showed a score of at least 4 was decided to be the anesthetic duration.

Table 1. Changes in body weight (g) of both strains of male and female mice from 8 weeks to 20 weeks of age

Strain	Sex	Age			
		8 weeks	12 weeks	16 weeks	20 weeks
BALB/c	Male	26.3 ± 1.3	28.3 ± 1.2 [§]	30.0 ± 1.3 [§]	30.3 ± 0.6 [§]
BALB/c	Female	21.9 ± 1.9*	23.7 ± 1.0*	25.4 ± 1.7*	25.6 ± 1.5* [§]
C57BL/6J	Male	23.8 ± 2.6 [#]	27.7 ± 2.7 [§]	29.2 ± 2.5 [§]	29.6 ± 3.1 [§]
C57BL/6J	Female	19.8 ± 1.5* [#]	22.1 ± 2.5*	23.5 ± 2.1* [§]	24.2 ± 2.6* [§]

Differences between strains and sexes were analyzed using an unpaired Student's *t*-test. Differences in each group of mice from 8 to 20 weeks were compared by Dunnett's test. Data are presented as means ± SD. **P*<0.05 compared with male mice. [#]*P*<0.05 compared with BALB/c. [§]*P*<0.05 compared with the body weight at 8 weeks of age.

Respiratory rate

When a mouse was put on its back, we visually counted the respiratory rate for 20 s every 5 min until the end of the experiment.

Drug preparation

The anesthetic was prepared as a mixture of three drugs: medetomidine (Domitor[®] Nippon Zenyaku Kogyo Co., Ltd., Tokyo, Japan), midazolam (Dormicum[®], Astellas Pharma Inc., Tokyo, Japan), and butorphanol (Vetorphale[®], Meiji Seika Kaisha, Ltd., Tokyo, Japan) at a clean bench in a sterile manner. We mixed 0.3 mg Med., 4.0 mg Mid., and 5.0 mg/kg b.w./mouse But. and added sterilized distilled water to adjust it to an administrative volume of 0.1 ml/10 g b.w./mouse. Usually, 45 µl of Domitor, 120 µl of Dormicum, 150 µl of Vetorphale, and 1185 µl of sterilized distilled water were mixed to make 1500 µl for an experimental drug. The drug was prepared just before the experiment and kept at the same temperature as the mouse body temperature (38°C) until administration.

In the second experiment, we made 15 ml of the mixture and preserved 1.5 ml of solution in 10 sterilized microtubes, which were kept in a refrigerator for 8 weeks. Two hours before the experiment, one tube of the mixture was taken out from the refrigerator and kept at 38°C until administration.

Statistical analysis

Statistical analysis was conducted using StatView software (Hulinks Inc., Tokyo, Japan). Data are presented as means ± SD. Data for respiratory rate during 20 s was converted by multiplication to data for one minute.

Differences between strains and sexes were analyzed

using the unpaired Student's *t*-test. Differences in anesthetic duration of each group of mice from 8 to 20 weeks and the efficacy of the preserved mixture of anesthetic were compared by Dunnett's test. A *P* value less than 0.05 was considered to be statistically significant.

Results

Body weight changes

In this experiment, all mice recovered from anesthesia, and no death of animals was observed. The body weights of the C57BL/6J strain were smaller than those of BALB/c strain in both male and female mice at 8 weeks of age. In the same strain, there were significant differences in body weights between male and female mice. As they grew from 8 weeks to 20 weeks of age, the body weights of the mice became heavier in the strains (Table 1).

Anesthetic duration

At 8 weeks, the anesthetic durations of the male BALB/c and C57BL/6J mice were 48.3 ± 14.1 and 58.1 ± 16.1 min, respectively. The shortest anesthetic duration of the male BALB/c and C57BL/6J mice was 35 min for both strains. The longest anesthetic durations of male BALB/c and C57BL/6J were 75 and 85 min, respectively. Also, the anesthetic duration of female BALB/c and C57BL/6J mice were 52.5 ± 10.3 and 51.0 ± 13.7 min, respectively. The shortest anesthetic duration of the female BALB/c and C57BL/6J mice were 35 min and 30 min respectively. The longest anesthetic durations of the female BALB/c and C57BL/6J mice were 70 min for both strains. There were no significant differences between the two strains. In addition, there were no significant differences between male and female mice of

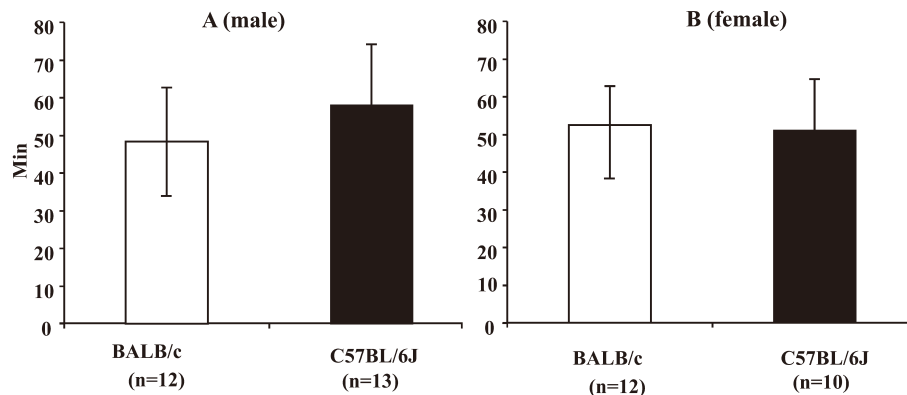


Fig. 1. Anesthetic duration of male BALB/c and C57BL/6J mice (A) and female BALB/c and C57BL/6J mice (B). Data are presented as means \pm SD. Differences between strains and sexes were analyzed using the unpaired Student's *t*-test. A *P* value less than 0.05 was considered to be statistically significant. There were no significant differences between the groups.

the same strain (Fig. 1, Table 2).

Anesthetic duration in the two strains showed no significant differences from 8 weeks to 20 weeks of age in both male and female mice (Fig. 2 and Fig. 3).

The anesthetic duration of the preserved drug did not change for 8 weeks after making the drug and storing it in a refrigerator (Fig. 4).

Anesthetic score

In male mice, C57BL/6J mice showed a significantly higher anesthetic score at 35, 60, 85, 90, 95, 100, 105, and 110 min compared with BALB/c mice. On the other hand, in female mice, C57BL/6J mice showed a significantly lower anesthetic score at only 100 and 105 min compared with BALB/c mice.

In C57BL/6J mice, male mice had significantly higher scores at 5 and 95 min compared with female mice. In BALB/c mice, female mice received a significantly higher anesthetic score at 40, 60, 100, and 105 min compared with male mice (Fig. 5).

Respiratory rate

In both male and female mice, the respiratory rate of BALB/c mice significantly differed from that of C57BL/6J mice (Fig. 6).

In both strains, male mice showed a significantly higher respiratory rate compared with female mice at 10, 15, 50, and 60 min in BALB/c mice, and at 10 and 55 min in C57BL/6J mice.

Table 2. Anesthetic duration of male BALB/c (n=12) and C57BL/6J (n=13) mice and female BALB/c (n=12) and C57BL/6J (n=10) mice

Strain	Sex	Anesthetic duration (min)		
		Mean \pm SD	Shortest	Longest
BALB/c	Male	48.3 \pm 14.1	35	75
BALB/c	Female	52.5 \pm 10.9	35	70
C57BL/6J	Male	58.1 \pm 16.1	35	85
C57BL/6J	Female	51.0 \pm 13.7	30	70

Data are presented as means \pm SD, as well as the shortest and longest time of each group. Differences between strains and sexes were analyzed using the unpaired Student's *t*-test. A *P* value less than 0.05 was considered to be statistically significant. There were no significant differences between the groups.

Discussion

The combination of ketamine and xylazine has long been a popular combination as an injectable anesthetic for laboratory animals [6, 8, 20]. Ketamine works through inhibiting N-methyl D-aspartic acid (NMDA) receptors to induce analgesic effects [5]. Xylazine is an α_2 -adrenoceptor agonist [4]. Looking for an injectable mixture as an alternative to using ketamine, Kawai *et al.* chose a mixture of Med., Mid., and But. [11]. Med. is an α_2 -adrenergic agonist like xylazine [4]. Mid. is a benzodiazepine receptor agonist that produces sedation [4]. But. acts at opioid κ -receptors to produce analgesic effects; it is an opioid μ -receptor antagonist, which means it is not a narcotic [3]. This mixture has been used as an anesthesia for dogs [9, 15], monkeys [10], and African lions in a zoo [19].

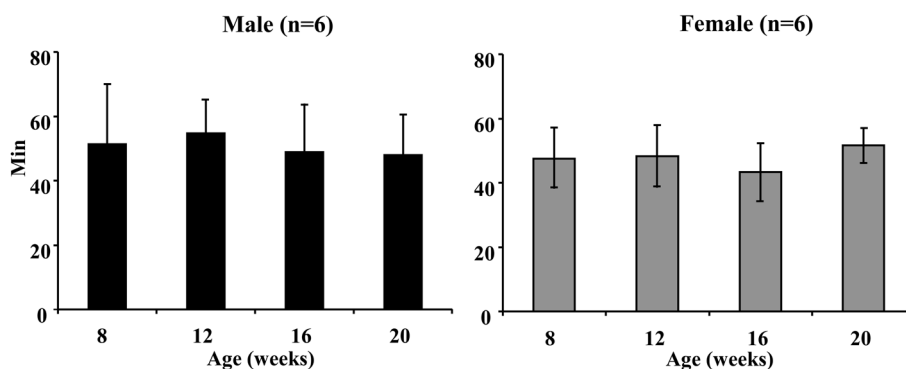


Fig. 2. Anesthetic duration of male (n=6) and female (n=6) BALB/c mice at 8, 12, 16, and 20 weeks of age. Data are presented as means \pm SD. Differences in anesthetic duration of each group of mice from 8 to 20 weeks were compared by Dunnett's test. A *P* value less than 0.05 was considered to be statistically significant. There were no significant differences at any week in either the male or female mice.

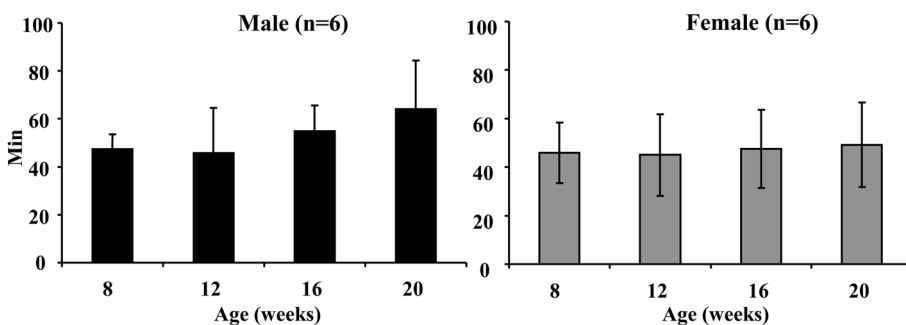


Fig. 3. Anesthetic duration of male (n=6) and female (n=6) C57BL/6J mice at 8, 12, 16, and 20 weeks of age. Data are presented as means \pm SD. Differences in anesthetic duration of each group of mice from 8 to 20 weeks were compared by Dunnett's test. A *P* value less than 0.05 was considered to be statistically significant. There were no significant differences at any week in either the male or female mice.

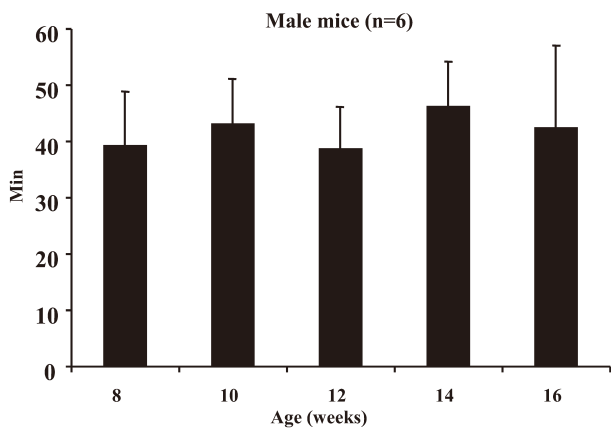


Fig. 4. Anesthetic duration of male (n=6) BALB/c mice at 8, 10, 12, 14, and 16 weeks of age after preparation of a drug. Data are presented as means \pm SD. The efficacy of the preserved mixture of anesthetic was compared by Dunnett's test. A *P* value less than 0.05 was considered to be statistically significant. There were no significant differences from 8 through 16 weeks.

Kawai *et al.* reported that the mixture produced sufficient anesthetic for a duration of around 40 min in outbred male ICR mice [11]. However, the anesthetic effects of the mixture for other strains of mice are not clear. The present paper is the first study to indicate the anesthetic effects of the mixture for inbred laboratory mice such as the BALB/c and C57BL/6 strains. When we compared the anesthetic duration of ICR mice described by Kawai *et al.* to our results, the data of the BALB/c and C57BL/6 strains were very similar; however, the anesthetic duration was about 10 min longer than ICR mice. Inbred mice might be more sensitive to the mixture of Med., Mid., and But. Also, the anesthetic scores after administration of drugs showed similar changes, even though the scoring method in our study and Kawai's study were slightly different. Forty minutes after injection, the anesthetic score of each mouse varied

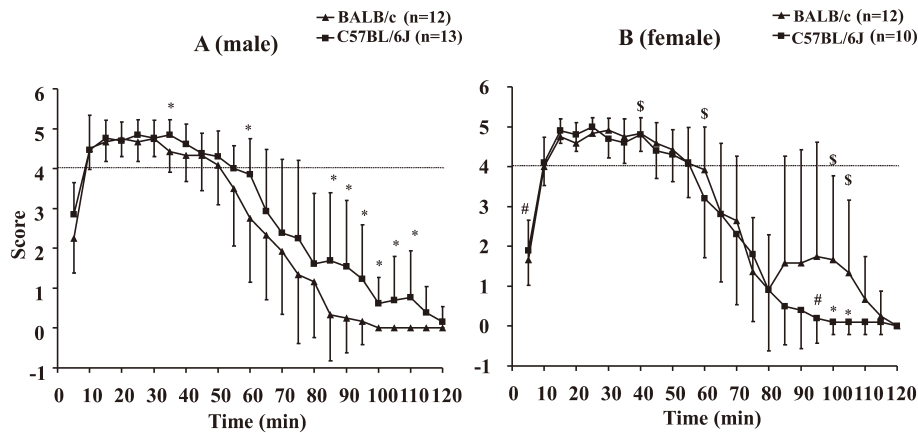


Fig. 5. Time course changes in the anesthetic scores of male BALB/c (n=12) and C57BL/6J (n=13) mice (A) and female BALB/c (n=12) and C57BL/6J (n=10) mice (B). Data are presented as means \pm SD. Differences between strains and sexes were analyzed using the unpaired Student's *t*-test. A *P* value less than 0.05 was considered to be statistically significant. **P*<0.05 compared with BALB/c mice. #*P*<0.05 compared with male C57BL/6J mice. \$*P*<0.05 compared with male BALB/c mice.

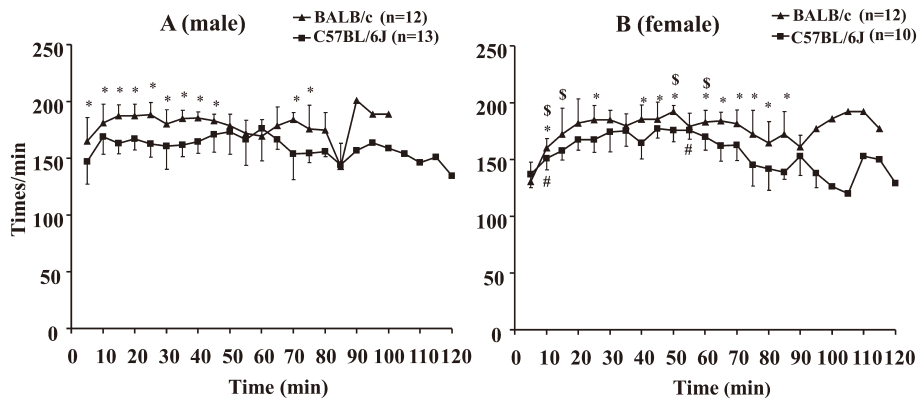


Fig. 6. Time course changes in respiratory rates of male BALB/c (n=12) and C57BL/6J (n=13) mice (A) and female BALB/c (n=12) and C57BL/6J (n=10) mice (B). Data are presented as means \pm SD. Differences between strains and sexes were analyzed using the unpaired Student's *t*-test. A *P* value less than 0.05 was considered to be statistically significant. **P*<0.05 compared with C57BL/6J mice. #*P*<0.05 compared with male C57BL/6J mice. \$*P*<0.05 compared with male BALB/c mice.

significantly. For example, some mice were perfectly awake, while other mice still had not regained their righting reflex and/or tail reflex.

The data of this study showed that there were no significant differences in anesthetic duration between strains and sexes. However, the differences between strains and sexes when using mixed drugs remain controversial. Voipio reported that there were some strain differences for anesthetic effects of the combination of ketamine and medetomidine in mice [17]. Mulder's experiment using C57BL/6 and DBA/2 mice showed strain differences,

but no sex differences for the anesthetic duration of a combination of ketamine and promazine [12]. We found that in male mice, in spite of no significant differences in anesthetic duration between strains, C57BL/6J mice showed a tendency for a longer anesthetic duration and higher anesthetic scores compared with BALB/c mice. On the other hand, there were no significant differences between the two female strains. When we compared male and female mice, the anesthetic score at 5 min indicated that male C57BL/6J had a significantly higher score (2.8 ± 0.6) compared with female C57BL/6J mice (1.9 ± 0.9).

The male BALB/c mice (2.3 ± 0.9) also showed a higher score than the female BALB/c mice (1.7 ± 1.0), but the difference was not statistically significant (Fig. 5). Then we found that male mice of both strains have a tendency to go into an anesthetic condition faster than female mice. On the other hand, Podhorna *et al.* reported that diazepam was more effective in female C57BL/6 mice than male mice [14]. The difference between our results and those of Podhorna may be due to our use of a mixture of several drugs, as each mechanism of a drug works differently. Thus, effectiveness may also vary for each mixture of some drugs.

Most mice reached a sufficient surgical anesthetic state at 10 min after injection of the mixture. However, some mice still showed a hind paw reflex or a corneal reflex at 10 min. Fifteen minutes after injection, all mice reached a state of surgical anesthesia. After reaching the surgical anesthetic state, some mice occasionally still had a corneal reflex. Usually, the corneal reflex disappeared when the mice entered a deeper anesthetic state. Then, we decided that an anesthetic score of at least 4 or 5 was indicative of the surgical anesthetic state. Forty-five minutes after injection, the rank order of recovery from anesthesia was hind paw > front paw > tail > body-righting reflex. It seems that legs are more sensitive to stimulus than the tail when mice are anesthetized.

We also found that there were no significant differences for anesthetic duration from 8 weeks to 20 weeks of age in males and females of both strains. This could be a beneficial result for researchers because the period from 8 weeks to 20 weeks is the most useful period for mouse experiments.

The respiratory rate of BALB/c mice was higher than that of C57BL/6J mice in both male and female mice during the experiment. Duguet reported a difference in bronchial responsiveness among inbred mouse strains [2]. Bronchial hyperresponsiveness in BALB/c mice may cause a higher respiratory rate when compared with C57BL/6J mice during anesthesia.

One problem is that the mixed anesthetic used in this study is not on the market. The stability of the anesthetic effects of the mixture is also important. Our data indicated that the mixture of three drugs, when kept in a refrigerator at 4°C, showed the same efficacy for at least 8 weeks after mixing.

In summary, this study showed the anesthetic effects of a mixture of medetomidine, midazolam, and butor-

phanol in BALB/c and C57BL/6J mice from 8 to 20 weeks of age. The results may offer a new, safe, and effective surgical anesthesia for inbred laboratory mice. In addition, it might also contribute to the welfare of laboratory animals.

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