Studies on the Anesthetic Effects of a Mixture of Medetomidine, Midazolam and Butorphanol, and Antagonism by Atipamezole in Small Rodents

マウス・ラットにおけるメデトミジン、ミダゾラム、ブトルファノールの

三種混合麻酔薬の麻酔効果とアチパメゾールによる拮抗作用に関する研究

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CONTENTS

GENERAL INTRODUCTION

The mixture of ketamine (KET) and xylazine (XYL) has long been a popular combination as an injectable anesthetic for use in laboratory animals in Japan and around the world [22, 24, 50]. However, in Japan, KET has been designated as a narcotic drug due to an abuse problem in 2007. Therefore, using KET for animal experiments has become burdensome because users have to register to get a license as a researcher of narcotic drugs.

On the other side, pentobarbital (PENT) has been most widely used as an injectable anesthetic for laboratory animals for several decades in Japan. However, PENT causes severe cardiovascular and respiratory depression and has poor analgesic activity [14]. Therefore, PENT should be substituted for another anesthetics.

Instead of using KET or PENT, Kawai *et al.* reported a new injectable anesthetic with an effect in mice equivalent to the combination of KET and XYL [28]. A mixture of two tranquilizers, medetomidine (MED) and midazoram (MID), with butorphanol (BUT), a nonnarcotic analgesic, produced a sufficient anesthetic duration about 40 minutes (min) in ICR mice.

Inbred mice such as the BALB/c and C57BL/6 strains have often been used for animal experiments [1, 23, 35, 47]. However, the anesthetic effects of the new mixture in the BALB/c and C57BL/6 strains and any strain–related differences remain unknown.

Therefore, in the first study, we examined the anesthetic effects of the

mixture of MED, MID, and BUT in both male and female BALB/c and C57BL/6J mice strains at 8, 12, 16, and 20 weeks of age. In addition, we examined the effective period of the mixture after mixing and refrigeration.

Intraperitoneal (IP) injection of drugs has been widely used in laboratory animals including mice [19], because subcutaneous (SC) injection induces drug effects more slowly and weakly than IP injection [18]. Intravenous (IV) injection produces drug effects more quickly and predictably [13]. Another injectable route of the mixture of MED, MID, and BUT may induce different anesthetic effects, because three drugs have different pharmacological mechanism [11, 17] and there may be additive or synergistic effects from each drug.

In the second study, we assessed the anesthetic effects of the anesthetic mixture administered by SC and IV injection compared to IP administration. During the experiment, we measured vital signs using a pulse oximeter just before and after administration of the anesthetic mixture, because the parameters such as Oxygen (O_2) saturation, heart rate, and respiratory rate are related to the anesthetic condition of mice under anesthesia [16].

Atipamezole (ATI) is a synthetic α_2-adrenergic receptor antagonist that can antagonize an α_2-adrenergic receptor agonist, MED [12]. After administration of the anesthetic mixture, injection of ATI causes mice a rapid recovery from anesthesia. However, neither an appropriate dosage nor an optimum injection timing of ATI after administration of the anesthetic mixture are clear. Then, we examined how the timing of injection and a dosage of ATI affected recovery

from anesthesia in mice after administration of the anesthetic mixture.

Several studies have shown that there are different anesthetic effects among different rat strains [2, 10]. However, the differences in the effects of the anesthetic mixture of MED, MID, and BUT in rats remain unclear.

In the third study, we first examined effects of the anesthetic mixture using Wistar (WST), Sprague-Dawley (SD), and Fischer 344 (F344) rat strains. We reported the efficacy of ATI with a suitable dosage and timing in mice in the second study. However, neither the appropriate dosage nor the optimum injection timing of ATI after administration of the anesthetic mixture is clear in rats. Then, we examined how the timing of injection and a dosage of ATI affected recovery from anesthesia in rats after administration of the anesthetic mixture.

During the experiment, we measured vital signs such as O_2 saturation, heart rate, and respiratory rate in the same method as the second study. To investigate differences among the 3 rat strains without administration of an anesthetic, we also measured vital signs just before and after administration of physiological saline (saline) for 90 min.

These results of 3 studies may indicate many important information about a new effective anesthesia for laboratory mice and rats.

CHAPTER 1

Anesthetic effects of a mixture of medetomidine, midazolam, and butorphanol in two strains of mice

1.1 Introduction

The mixture of KET and XYL has long been a popular combination as an injectable anesthetic for use in laboratory animals in Japan and around the world [22, 24, 50]. However, in Japan, KET has been designated as a narcotic drug due to an abuse problem. Therefore, using KET for animal experiments has become burdensome because users have to register to get a license as a researcher of narcotic drugs.

Instead of using KET, Kawai *et al.* reported a new injectable anesthetic with an effect in mice equivalent to the combination of KET and XYL [28]. A mixture of two tranquilizers, MED and MID with 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 experiments [1, 23, 44]. The C57BL/6 strain is also used for oncological experiments or as a wild type of genetically engineered mice [35, 47, 51].

Voipio *et al.* reported that there were some strain differences for anesthetic effects in mice [46]. Mulder's experiment indicated strain differences, but no sex differences for anesthetic duration in inbred mice [32]. 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.

1.2 Materials and Methods

1.2.1 Animals and housing conditions

Twelve male (26.3 ± 1.3 g) (b.w., mean ± 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 x L 320 x 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 ± 2℃) and humidity (55 ± 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.

1.2.2 Experimental procedure

The experiment was conducted during daytime (PM 2:00-5:00). 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 administered by IP injection at 0.1 ml/10 g b.w./mouse. After administration, the mice were kept on a heater pad (Heater Mat KN-475®, Natsume Seisakusho, Co., Ltd., Tokyo, Japan) maintained at approximately 38℃.

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 IV injection of sodium pentobarbital (80 mg/kg b.w.) (Somnopentyl®, Kyoritsu Seiyaku Corporation, Tokyo, Japan).

1.2.3 Measuring anesthetic scores

Each anesthetic score was measured using the modified grading system described by Kawai [28]. 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. The duration for which a mouse showed a score of at least 4 was decided to be the anesthetic duration.

1.2.4 Respiratory rate

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

1.2.5 Drug preparation

The anesthetic was prepared as a mixture of 3 drugs: MED (Domitor® Nippon Zenyaku Kogyo Co., Ltd., Tokyo, Japan), MID (Dormicum®, Astellas Pharma Inc., Tokyo, Japan), and BUT (Vetorphale®, Meiji Seika Kaisha, Ltd., Tokyo, Japan) at a clean bench in a sterile manner. We mixed 0.3 mg of MED, 4 mg of MID, and 5 mg of BUT /kg b.w./mouse 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℃) 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, a 1 tube of the mixture was taken out from the refrigerator and kept at 38℃ until administration.

1.2.6 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 seconds was converted by multiplication to data for a 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.

1.3 Results

In this experiment, all mice recovered from anesthesia, and no death of animals was observed.

1.3.1 Body weight changes

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).

1.3.2 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 2 strains. In addition, there were no significant differences between male and female mice of 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).

1.3.3 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).

1.3.4 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.

1.4 Discussion

The combination of KET and XYL has long been a popular combination as an injectable anesthetic for laboratory animals [22, 24, 50]. KET works through inhibiting N-methyl D-aspartic acid (NMDA) receptors to induce analgesic effects [15]. XYL is an α_2-adrenoceptor agonist [11]. Looking for an injectable mixture as an alternative to using KET, Kawai *et al.* chose a mixture of MED, MID, and BUT [28]. MED is an α_2-adrenergic agonist like XYL [11]. MID is a benzodiazepine receptor agonist that produces sedation [11]. BUT acts at opioid κ-receptors to produce analgesic effects; it is an opioid μ-receptor antagonist, which means it is not a narcotic [17]. This mixture has been used as an anesthesia for dogs [25, 38], monkeys [27], and African lions in a zoo [49].

Kawai *et al.* reported that the mixture produced sufficient anesthetic for a duration of around 40 minutes in outbred male ICR mice [28]. 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/6J 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 administration, the anesthetic score of each mouse varied 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 *et al.* reported that there were some strain differences for anesthetic effects of the combination of KET and MED in mice [46]. 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 KET and promazine [32]. 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. Gonsenhauser *et al.* reported that one reason of the strain difference under anesthesia connected to the difference in respiratory pattern and its variability [20]. They surmised that the neural substrate for differences, at least partly, exists within subcortical structure generating the breathing pattern. In our study, we also found the different respiratory rate between 2 strains. Raekallio *et al.* reported that high levels of diurnal and stress-related corticosterone levels might affect the distribution and metabolism of MED in rabbits [39]. Unfortunately, we didn't measure corticosterone levels of 2 strains. However, the tendency for a longer anesthetic duration and higher anesthetic scores of C57BL/6J compared with BALB/c mice might relate to different stress-related

corticosterone levels in 2 strains. On the other hand, there were no significant differences between the 2 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 [37]. They theorized the reason of sensitivity for diazepam in female mice related to higher hepatic enzymatic activities in male compared to those in female rats quoted from the study by Watanabe *et al.* [48]. 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 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 *et al.* reported a difference in bronchial responsiveness among inbred mouse strains [9]. Bronchial hyper responsiveness 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 3 drugs, when kept in a refrigerator at 4℃, showed the same efficacy for at least 8 weeks after mixing.

In summary, this study showed the anesthetic effects of a mixture of MED, MID, and BUT in BALB/c and C57BL/6J strain 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.

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.

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

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

Data are presented as means ± 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.

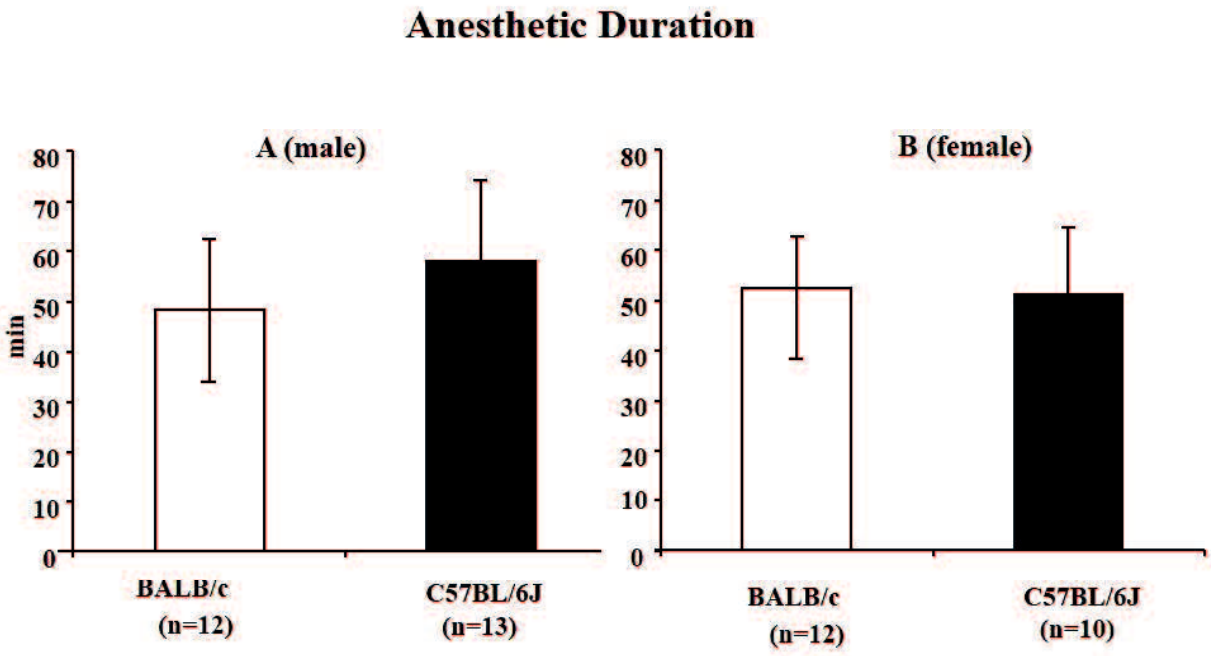

Fig. 1. Anesthetic duration of male BALB/c and C57BL/6J mice (A) and female BALB/c and C57BL/6J mice (B). Data are presented as means ± SD. Differences between strains and sexes were analyzed using the unpaired Student's-t test. A p value less than 0.05 was considered to be statistically significant.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

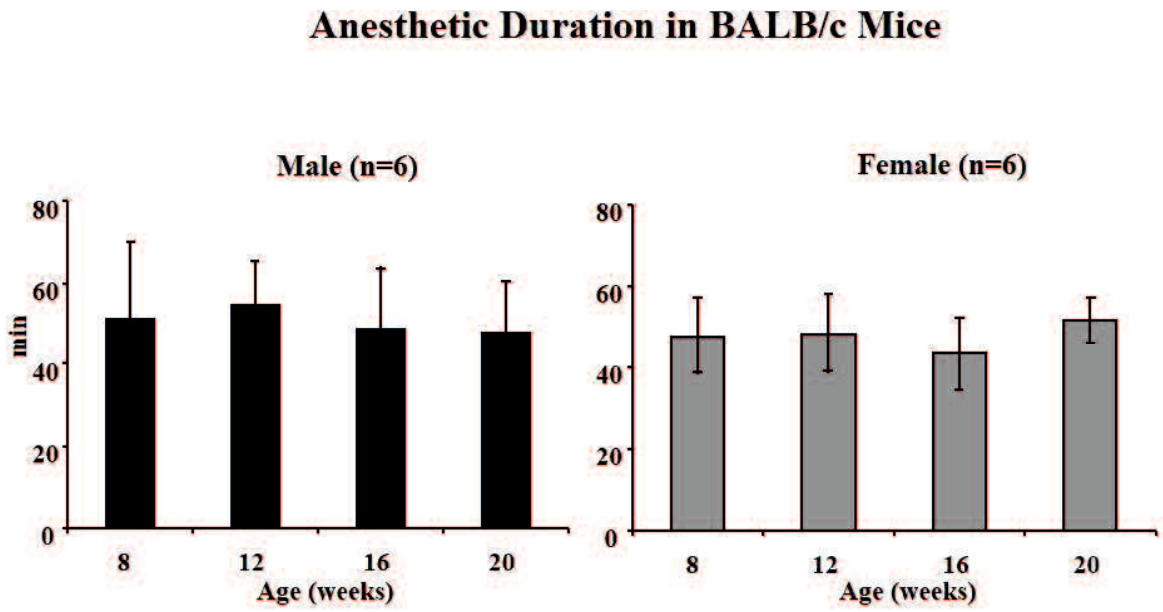

Fig. 2. Anesthetic duration of male (n=6) and female (n=6) BALB/c mice at 8, 12, 16, and 20 weeks of age. Data are presented as means ± SD. Differences in anesthetic duration of each group of mice from 8 to 20 weeks were compared by Dunnett's test. A p value less than 0.05 was considered to be statistically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

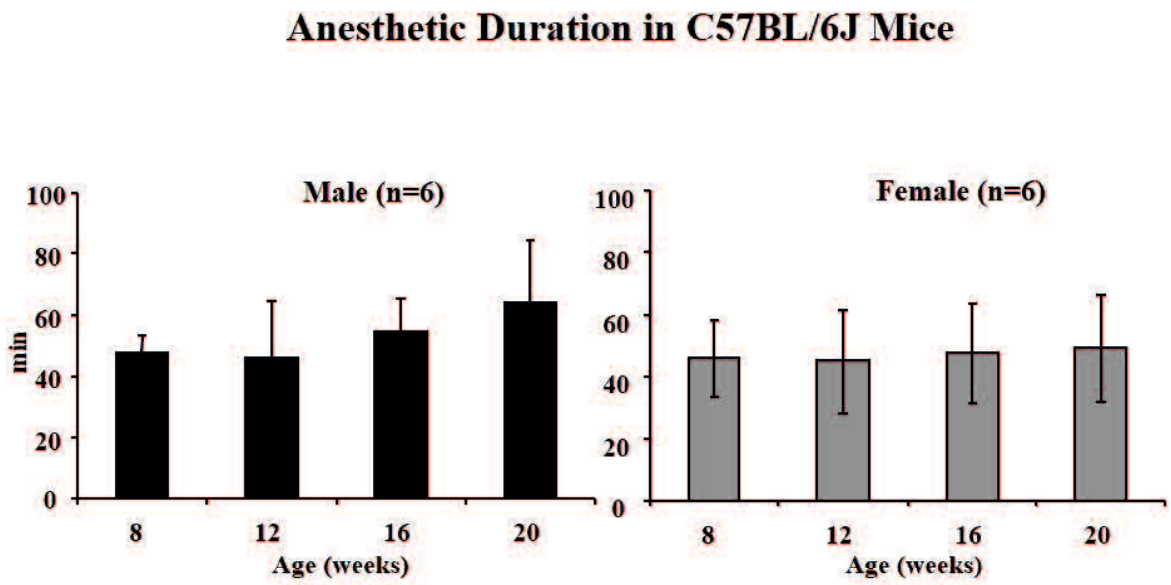

Fig. 3. Anesthetic duration of male (n=6) and female (n=6) C57BL/6J mice at 8, 12, 16, and 20 weeks of age. Data are presented as means ± SD. Differences in anesthetic duration of each group of mice from 8 to 20 weeks were compared by Dunnett's test. A *p* value less than 0.05 was considered to be statistically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

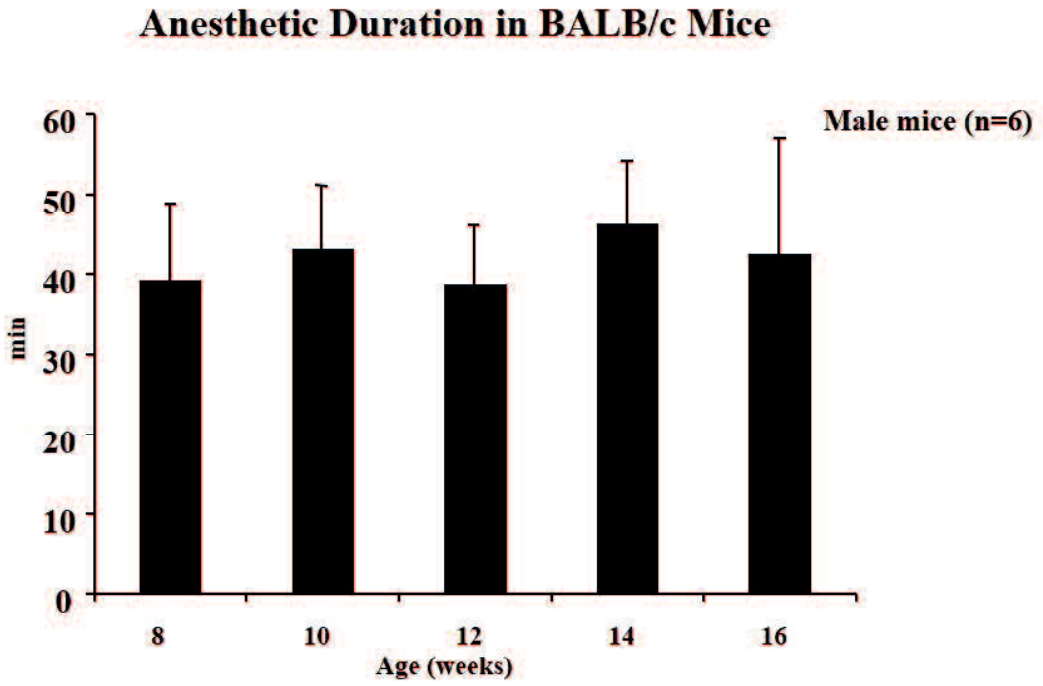

Fig. 4. Anesthetic duration of male (n=6) BALB/c mice at 8, 12, 16, and 20 weeks of age after preparation of a drug. Data are presented as means ± SD. The efficacy of the preserved mixture of anesthetic was compared by Dunnett's test. A p value less than 0.05 was considered to be statistically significant. There were no significant differences from 8 through 20 weeks.

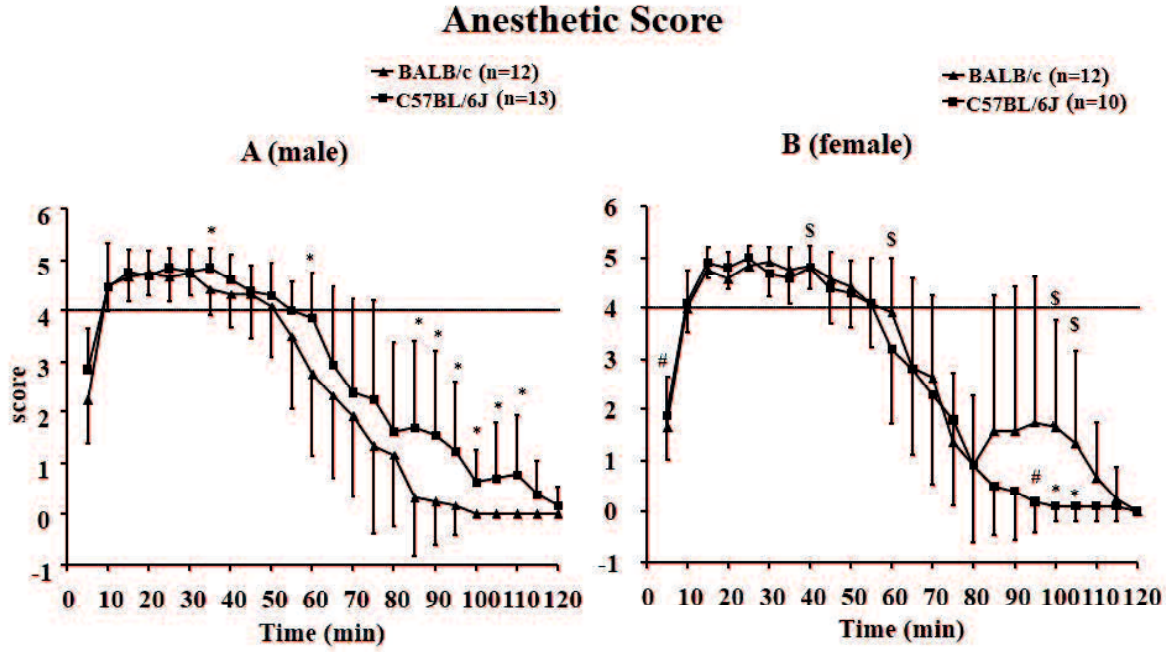

Fig. 5. Time course changes in the anesthetic scores of male BALB/c (n=12) and C57BL/6J (n=13) mice (A) and female BALB/c (n=12) and C57BL/6J (n=10) mice (B). Data are presented as means ± SD. Differences between strains and sexes were analyzed using the unpaired Student's t-test. A p value less than 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.

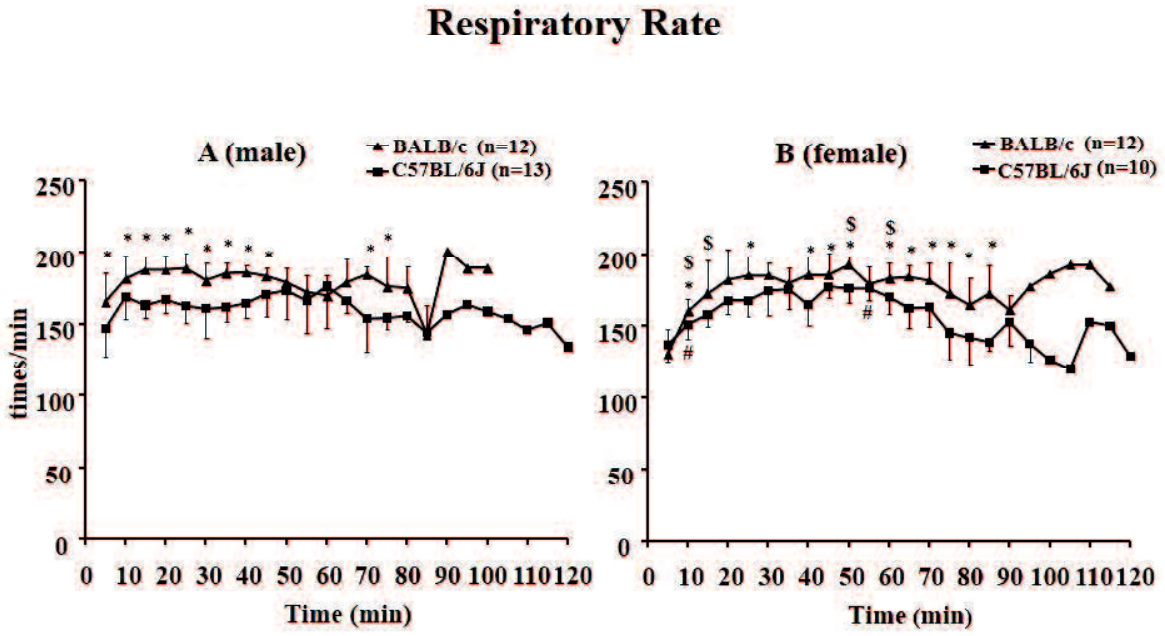

Fig. 6. Time course changes in respiratory rates of male BALB/c (n=12) and C57BL/6J (n=13) mice (A), and female BALB/c (n=12) and C57BL/6J (n=10) mice (B). Data are presented as means ± SD. Differences between strains and sexes were analyzed using the unpaired Student's-t test. A p value less than 0.05 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.

CHAPTER 2

Anesthetic effects of a three-drugs mixture –comparison of administrative routes and antagonistic effects of atipamezole in mice–

2.1 Introduction

An anesthetic mixture of MED, MID, and BUT has been introduced recently [28]. This anesthetic mixture was reported to produce anesthetic duration of around 40 minutes in ICR mice. We reported that this anesthetic mixture produced almost the same anesthetic effects of ICR mice in both male and female BALB/c and C57BL/6J strains [29].

IP injection of drugs has been widely used in laboratory animals including mice [19], because SC injection induces drug effects more slowly and weakly than IP injection [18]. IV injection produces drug effects more quickly and predictably [13]. However, rapid IV injection of anesthetic drugs is sometimes lethal. Another injectable route of the mixture of three drugs may induce different anesthetic effects, because 3 drugs have different pharmacological mechanism [11, 17] and there may be additive or synergistic effects from each drug.

In this study, we used an anesthetic score to assess the effects of the anesthetic mixture administered by SC and IV injection compared to IP administration. During the experiment, we measured vital signs just before and after administration of the anesthetic drugs, because the parameters such as O_2 saturation, heart rate, and respiratory rate are related to the anesthetic condition of mice under anesthesia [16].

ATI is a strong antagonist of MED [12]. After administration of the anesthetic mixture, injection of ATI causes mice a rapid recovery from

anesthesia. However, neither an appropriate dosage nor an optimum injection timing of ATI after administration of the anesthetic mixture are clear. Then, we examined how the timing of injection and a dosage of ATI affected recovery from anesthesia in mice after administration of the anesthetic mixture.

2.2 Materials and Methods

2.2.1 Animals and housing conditions

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.

We used 24 male ICR mice in the experiment. The mice were purchased at 5 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. The mice were 7 to 9 weeks of age during the experiment.

Four mice were housed in a TPX cage (KN-600®, W 220 x L 320 x 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 ± 2℃) and humidity (55 ± 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*. The body weight of the animals used for the

experiment was 34.9 g ± 1.3 g (mean ± SD).

2.2.2 Experimental procedure

The experiment was conducted during daytime (PM 1:00-5:00). The experimental room was controlled as the same temperature and humidity as the animal room. The mice were weighed before receiving anesthesia.

In the first experiment, the anesthetic mixture was administered by IP, IV, and SC injection at 0.1 ml/10 g b.w./mouse. We used 8 mice per each injection group and 9 mice for a group of non-anesthesia. The mice were used repeatedly and allowed at least 2 days of rest after experimental use. After the administration of anesthesia, the mouse was kept on a heater pad (Heater Mat KN-475®, Natsume Seisakusho, Co., Ltd., Tokyo, Japan) maintained at approximately 38℃. After injection of the anesthetic mixture, an anesthetic score for each mouse was measured every 5 min until the mouse was completely recovered from anesthesia. At the same time, we measured O_2 saturation, heart rate and respiratory rate using a pulse oximeter.

In the second experiment, we used 4 groups of mice. Drugs were administered using IP injection. Each group of 6 mice was given ATI after administration of the anesthetic mixture. Group 1: 0.3 mg/kg b.w. ATI at 30 min after administration of the anesthetic mixture. Group 2: 1.5 mg/kg b.w. ATI at 30 min after administration of the anesthetic mixture. Group 3: 0.3 mg/kg b.w. ATI at 10 min after administration of the anesthetic mixture. Group 4: 1.5 mg/kg b.w. ATI at 10 min after administration of the anesthetic

mixture. After administration of the anesthetic mixture, an anesthetic score was measured every 5 min. After injection of ATI, an anesthetic score was measured every 1 min.

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

2.2.3 Measuring anesthetic scores

We briefly explain the method of measuring anesthetic scores that we previously described elsewhere [29]. Measurement was based on 5 reflexes. The first was a front paw reflex. The second was a hind paw reflex. The third was a tail reflex. The fourth was a corneal reflex. The fifth was a body-righting reflex. If a mouse showed no reflex, it was given a score of 1. If a mouse reacted, it was given a score of 0. The total anesthetic score was graded from 0 to 5. The duration for which a mouse showed a score of 4 or 5 was decided to be the anesthetic duration.

2.2.4 Measurement of O_2 saturation, heart rate, and respiratory rate

A pulse oximeter (Mouse Ox plus®, STARR Life Sciences Corp., Oakmont, PA, USA) was used to measure O_2 saturation, heart rate, and respiratory rate of mice during the experiment. The day before the experiment, all hair covering both carotid arteries of the mice was removed using an electric shaver and a depilatory under inhalational isoflurane (Escain®, Mylan Seiyaku, Tokyo,

Japan) anesthesia using an anesthetic instrument (KN-1071-I, Natsume Seisakusho, Co., Ltd., Tokyo, Japan).

A sensor clip of the pulse oximeter was placed at the cervical parts of the mice. Then, we recorded O_2 saturation, heart rate, and respiratory rate until each mouse was recovered from anesthesia. Measurement of mice without anesthesia was carried out in a mouse holder.

2.2.5 Drug preparation

The anesthetic mixture was prepared as a mix of three drugs: MED (Domitor®, Nippon Zenyaku Kogyo Co., Ltd., Tokyo, Japan), MID (Dormicum®, Astellas Pharma Inc., Tokyo, Japan), and BUT (Vetorphale®, Meiji Seika Pharma Co., Ltd., Tokyo, Japan). We mixed MED 0.3 mg, MID 4 mg and BUT 5 mg/kg b.w./ mouse and added distilled sterile water (Otsuka sterile water®, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) to adjust it to an administrative volume of 0.1 ml/10 g b.w./mouse. For example, 0.3 ml of Domitor, 0.8 ml of Dormicum, 1.0 ml of Vetorphale, and 7.9 ml of distilled sterile water were mixed to make 10 ml of an experimental anesthetic mixture. The anesthetic mixture was prepared on the day before the experiment and kept in a refrigerator. The mixed drug was allowed to be used up to 1 week after being mixed.

In the second experiment, we made 1.5 mg/kg b.w./ mouse of ATI (Antisedan®, Nippon Zenyaku Kogyo Co., Ltd., Tokyo, Japan) to adjust it to an administrative volume of 0.1 ml/10 g b.w./mouse. To make 10 ml of 1.5 mg/kg of ATI, 0.3 ml of

Antisedan, and 9.7 ml of distilled sterile water were mixed. To make 0.3 mg/kg of ATI, 1 ml of a solution of 1.5 mg/kg of ATI and 4.0 ml of distilled sterile water were mixed. The solution of ATI was also allowed to be used up to 1 week after being made.

Drug preparation was conducted at a clean bench in a sterile manner. Before administration, the drug was kept in the incubator of which temperature is around 37℃.

2.2.6 Statistical analysis

Statistical analysis was conducted using the StatView software (Hulinks Inc., Tokyo, Japan). Graph data are presented as means ± SD until 90 min after drug administration. Body weight differences, anesthetic duration, and recovery time for each experimental group were analyzed using one-way analysis of variance (ANOVA). Time course differences in anesthetic score, O_2 saturation, heart rate, and respiratory rate were analyzed by one-way repeated measures ANOVA. Scheffe's test was used for post hoc analysis. A p value less than 0.05 was considered to be statistically significant.

2.3 Results

All mice used in this experiment were recovered from anesthesia.

2.3.1 First experiment

2.3.1.1 Body weight

There were no significant differences of body weights of the 4 groups in the

male ICR mice (Table 1).

2.3.1.2 Anesthetic duration

The anesthetic durations of IP, SC, and IV injection groups were 45.6 ± 7.8, 56.9 ± 14.1, and 44.4 ± 12.7 min, respectively (Fig. 1). There were no significant differences among the three groups. The shortest anesthetic durations of IP, SC, and IV injection groups were 35, 35, and 25 min, respectively. The longest anesthetic durations of IP, SC, and IV injection were 55, 80, and 70 min, respectively (Table 1). The recovery times (at time when mice return from body-righting reflex) of IP, SC, and IV injection groups were 68.8 ± 7.9, 76.3 ± 16.2 and 62.5 ± 16.3 min, respectively (Fig. 2). The shortest recovery times of IP, SC, and IV injection were 55, 55, and 50 min, respectively. The longest recovery times of IP, SC, and IV injection groups were 80, 105, and 100 min, respectively. (Table 1).

2.3.1.3 Anesthetic score

The anesthetic scores of IP, SC, and IV injection groups at 5 min after the administration of the anesthetic mixture were 2.8 ± 0.7, 3.4 ± 0.7, and 3.8 ± 0.5, respectively. The anesthetic score of IV injection group at 5 min was significantly higher than IP injection group. There were no significant differences of the anesthetic scores between SC and IV injection groups at 5 min. There are no significant differences of the scores among the 3 groups at other time points (Fig. 3).

2.3.1.4 Measurement of O_2 saturation, heart rate, and respiratory rate

1) O_2 saturation

There were no significant differences of O_2 saturation between the 3 groups before drug administration as well as the non-anesthesia group. The non-anesthesia group showed normal O_2 saturation of 95.4 ± 2.1% during 90 min. After the drug administration, O_2 saturations of IP, SC, and IV injection groups showed lower than that of the non-anesthesia group throughout the experiment (excluding at 70, 85, and 90 min). O_2 saturation levels of IP, SC, and IV injection at 5 min after the administration were 83.2 ± 2.7, 78.2 ± 7.6, and 69.7 ± 6.2%, respectively. O_2 saturation of SC and IV injection at 5 min was significantly lower than that of IP injection. O_2 saturation levels of IV injection at 10, 25, 35, 40, 45, 70, and 75 min and SC injection from at 40 to 80 min were significantly lower than that of the IP injection (Fig. 4).

2) Heart rate

There were no significant differences of heart rate between the 3 groups before drug administration as well as the non-anesthesia group. Heart rates of the non-anesthesia, IP, SC, and IV injection groups at 5 min after the administration were 723 ± 93, 574 ± 175, 421 ± 123, and 449 ± 148 beats /min, respectively. Heart rates of 3 injection groups at 5 and 15 min were significantly lower than the non-anesthesia group. Heart rates of IV injection groups at 5 min was significantly lower than the IP injection group (Fig. 5).

3) Respiratory rate

There were no significant differences of respiratory rates between the 3 groups before and after the drug administration as well as the non-anesthesia group (Fig. 6).

2.3.2 Second experiment

2.3.2.1 Body weight

There were no significant differences of body weights of the 4 groups in male ICR mice (Table 2).

2.3.2.2 Recovery time

At 30 min after administration of the anesthetic mixture, the recovery times of mice injected 0.3 mg /kg and 1.5mg /kg of ATI were 3.0 ± 1.1 and 2.5 ± 0.6 min, respectively. There were no significant differences between the 2 dosages of ATI. At 10 min after administration of the anesthetic mixture, the recovery times of mice injected 0.3 mg /kg and 1.5mg /kg of ATI were 6.2 ± 2.0 and 2.5 ± 0.6 min, respectively. The recovery time of 1.5 mg /kg of ATI was significantly shorter than 0.3 mg /kg of ATI at 10 min after administration of the anesthetic mixture (Fig. 7).

2.3.2.3 Anesthetic score

There were no significant differences of anesthetic scores between 2 dosages of

ATI at 30 min after the administration of the anesthetic mixture. At 10 min after administration of the anesthetic mixture, the anesthetic scores of ATI (0.3 mg/kg) were significantly higher than those of ATI (1.5mg /kg) from 2 to 6 min after injection (Fig. 8).

2.3.2.4 O_2 saturation

There were no significant differences of O_2 saturation levels between 2 dosages of ATI when injected at 30 min after administration of the anesthetic mixture. At 10 min after administration of the anesthetic mixture, the O_2 saturation level of ATI (1.5 mg/kg) was significantly higher than ATI (0.3 mg/kg) at 2 and 3 min after injection (Fig. 9).

2.4 Discussion

The anesthetic mixture of MED, MID, and BUT for mice has been introduced recently [28]. Originally this mixture has been used as anesthesia for dogs [25, 38, 45], monkeys [27, 33], and African lions in a zoo [49]. This anesthetic mixture was reported to produce anesthetic duration of around 40 minutes in ICR mice [28]. We reported that this anesthetic mixture produced almost the same anesthetic effects in both male and female BALB/c and C57BL/6J strains [29]. Both mice studies described above used IP injection to administer the anesthetic mixture. IP injection of drugs has been widely used in mice [19], because SC injection induces drug effects more slowly and weakly than IP injection [18]. IV injection produces drug effects more quickly and predictably

[13]. However, rapid IV injection of anesthetic drugs is sometimes lethal.

In this study, the first experiment indicated that there were no significant differences of anesthetic duration among the 3 different injection routes (Fig. 1), although the IV injection group showed a quick increase and decrease of anesthetic scores after administration of the anesthetic mixture (Fig. 3). The anesthetic score of the IV injection group was significantly higher than the IP injection group at 5 min. From 10 to 90 min, the 3 injection groups did not show statistically different anesthetic scores. However, our scoring method to estimate anesthetic depth could not measure an anesthetic score of over 5. As the result of O_2 saturation levels showed, IV injection may have worked more strongly than IP injection during the earlier period after injection. At 5 min after administration of the anesthetic mixture, the IV injection showed significantly lower O_2 saturation level (69.7 ± 6.2 %) compared with IP (83.2 ± 2.7 %) (Fig. 4). We slowly and carefully administered the anesthetic mixture by IV route. Then, when careless and rapid IV injection is carried, it may cause death.

Unexpectedly, SC injection showed a tendency to produce longer anesthetic duration compared to IP injection (Fig. 1). Claassen reported that IP injection technique has a failure rate of 10-20% [8]. An IP injection is made through the abdominal wall into the peritoneal cavity and there is no visual confirmation that the injection has been correctly administered [19]. Compared to IP injection, inspectional failures are easily detected with SC and IV injection routes. In our study, all SC and IV injections were conducted successfully with

visual confirmation. Burnside *et al.* reported that there were no significant differences of anesthetic durations between IP and SC injection for a mixed drug of KET and MED in mice [6]. Their study said that SC injection may be considered preferable to prevent additional stress to animals, as well as potential damage to internal organs that may occur by IP injection. On the other side, an improper IV injection is lethal. Therefore, we recommend SC injection of the anesthetic mixture compared to IP and IV injection, although there are no significant differences of anesthetic duration among the 3 injection routes.

It is very difficult to explain the precise mechanism as to why there were no significant differences of anesthetic duration among the 3 different injection routes. The 3 drugs each have a different pharmacological mechanism. MED is an α_2-adrenargic agonist to produce sedative and analgesic effects [11]. MID is a benzodiazepine receptor agonist to produce sedation [11]. BUT acts at opioid κ-receptors to produce analgesic effects, but it is an opioid μ-receptor antagonist [17]. Salonen *et al.* reported that there was synergistic interaction between dexmedetomidin (α_2-adrenargic agonist) and MID in rats [40]. In our study, MED may work synergistically to improve effects when mixed with MID and BUT. The result of the second experiment showed that the injection of ATI (1.5 mg/kg) caused mice to have perfect recovery from anesthesia within 10 min after administration of the anesthetic mixture. At this time point, usually pharmacological effects of MID and BUT still remain after a single administration.

Heart rates of anesthesia injection groups were significantly decreased at 5 or 15 min after administration compared to the non-anesthesia group. However, during the anesthesia after 20 min of administration of the anesthetic mixture, heart rates of each anesthetic injection group showed a stable condition (Fig. 5). Respiratory rate did not affect the difference of any injection route or the non-anesthesia condition. Therefore, O_2 saturation is a suitable parameter to estimate anesthetic depth and condition under anesthesia for laboratory animals.

ATI is an α_2-adrenargic antagonist, then it antagonizes the effect of MED [12]. At 30 min after the anesthetic mixture, injection of ATI at 0.3 mg/kg and 1.5 mg/kg had almost the same rapid recovery time from anesthesia (Fig. 7). However, at 10 min after administration of the anesthetic mixture, injection of ATI at 0.3 mg/kg needed more time to recover from anesthesia compared to ATI at 1.5 mg/kg (Fig. 7). Baker *et al.* reported that there were no significant differences of recovery times after receiving 5 mg/kg of ATI at 10 min and 40 min after administration of a combination of KET (75 mg/kg) and MED (1 mg/kg) [3]. Our data also showed no significant differences of recovery times at 10 min and 30 min after administration of the anesthetic mixture when administered ATI at 1.5 mg/kg (Fig. 7). In the study of Baker *et al.*, a 5-times higher dosage of ATI (5 mg/kg) than MED (1 mg/kg) was used. We also used a 5-times higher dosage of ATI (1.5 mg/kg) than MED (0.3 mg/kg). When mice are administered an anesthetic mixture to have surgery for around 30 min, ATI at 0.3 mg/kg is a large enough dosage to recover from anesthesia. ATI at 1.5

mg/kg is suitable to allow mice to recover from anesthesia quickly. O_2 saturation levels after administration of ATI 1.5 mg/kg also indicated a quick recovery from anesthesia (Fig. 9).

In summary, our study indicated that an anesthetic mixture of MED, MID, and BUT produced almost same anesthetic duration by IP, SC, and IV injection in ICR mice. SC injection of the anesthetic mixture is a recommended route compared to IP or IV injection, because there are some technical risks for IP injection, and IV method is sometimes lethal and not easy for injection. This anesthetic mixture is a useful drug to have a MED antagonist; ATI which helps mice quickly recover from anesthesia. These results may contribute to the welfare of laboratory animals.

Table 1. Body weight (g), anesthetic duration (min), and recovery time of intraperitoneal (IP), subcutaneous (SC), intravenous (IV), and non-anesthesia groups (non) of male ICR mice

		Body Weight (g)	Anesthetic duration (min)			Recovery time (min)		
Route	n.	Mean ± SD	Mean ± SD	Shortest	Longest	Mean ± SD	Shortest	Longest
IP	8	34.7 ± 0.9	45.6 ± 7.8	35	55	68.8 ± 7.9	55	80
SC	8	34.9 ± 0.5	56.9 ± 14.1	35	80	76.3 ± 16.2	55	105
IV	8	35.4 ± 0.9	44.4 ± 12.7	25	70	62.5 ± 16.3	50	100
non	9	34.8 ± 1.4	—	—	—	—	—	—

Data are presented as means ± SD, as well as the shortest and longest time of injection groups. Differences between each experimental groups were analyzed by one-way ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. There were no significant differences of body weights among the 4 groups, as well as no significant differences of anesthetic durations and recovery time of 3 injection groups.

Table 2. Second experiment procedure and body weight of mice

		Body weight (g)	Mixed drug	Atipamezole		Injection timing after anesthetic mixture (min)
Group	n.	Mean ± SD	Route	Route	Concentration	
1	6	34.3 ± 2.2	IP	IP	0.3 mg/kg	30
2	6	34.4 ± 1.3	IP	IP	1.5 mg/kg	30
3	6	34.6 ± 1.4	IP	IP	0.3 mg/kg	10
4	6	34.3 ± 0.6	IP	IP	1.5 mg/kg	10

Data of body weight are presented as means ± SD. Differences between each experimental group were analyzed by one-way ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. There were no significant differences of body weights among the 4 groups.

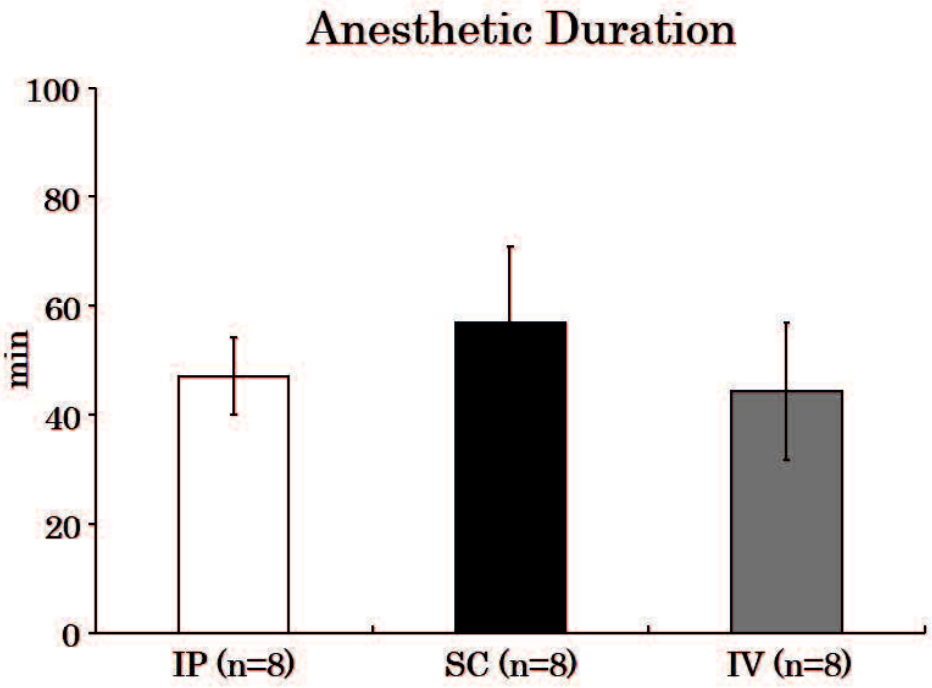

Fig. 1. Anesthetic duration of IP, SC, and IV groups injected the anesthetic mixture in male ICR mice. Data are presented as means ± SD. Differences between each injection group were analyzed by one-way ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. There were no significant differences among the 3 groups.

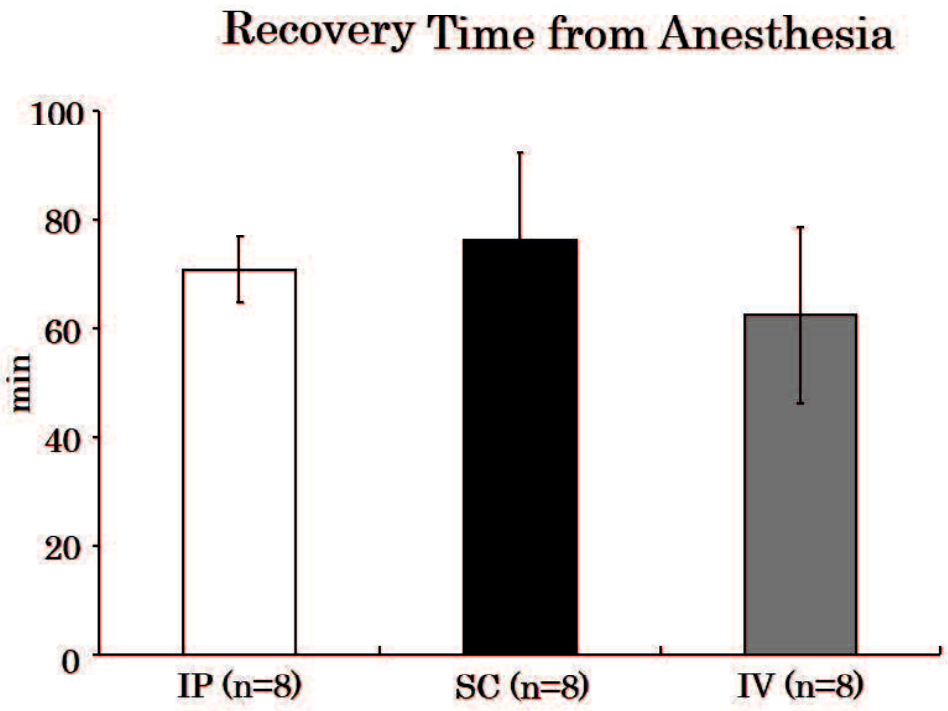

Fig. 2. Recovery time from anesthesia of IP, SC, and IV groups injected the anesthetic mixture in male ICR mice. Data are presented as means ± SD. Differences between each injection group were analyzed by one-way ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. There were no significant differences among the 3 groups.

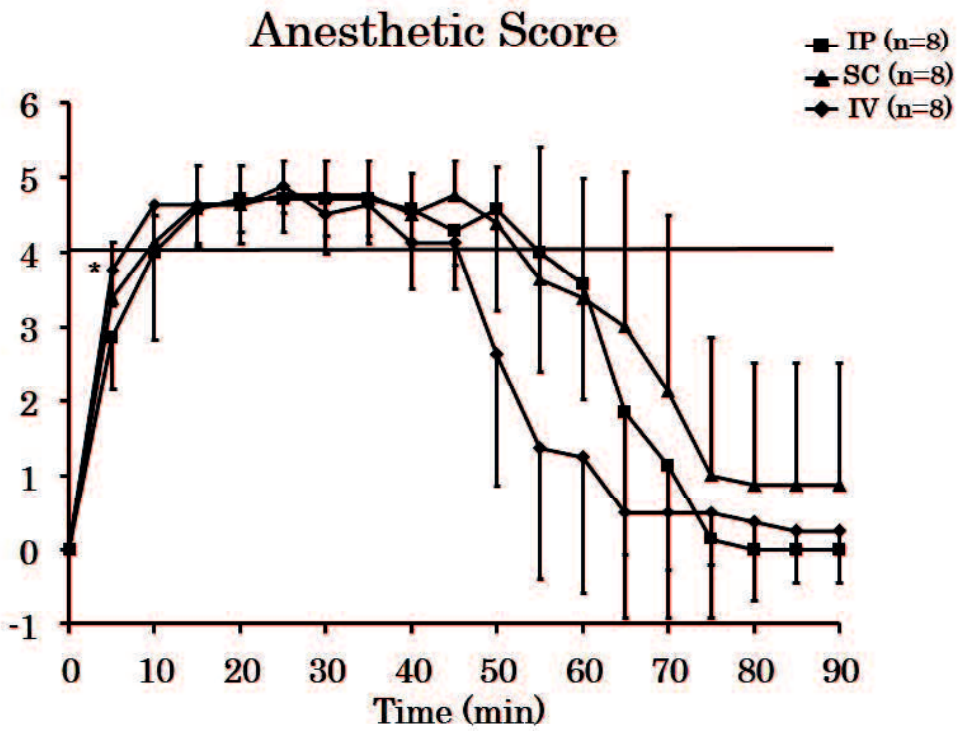

Fig. 3. Anesthetic score of IP, SC, and IV groups injected the anesthetic mixture in male ICR mice. Data are presented as means ± SD. Differences between each injection group were analyzed by one-way repeated measures ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. *p<0.05 compared with the IP injection group.

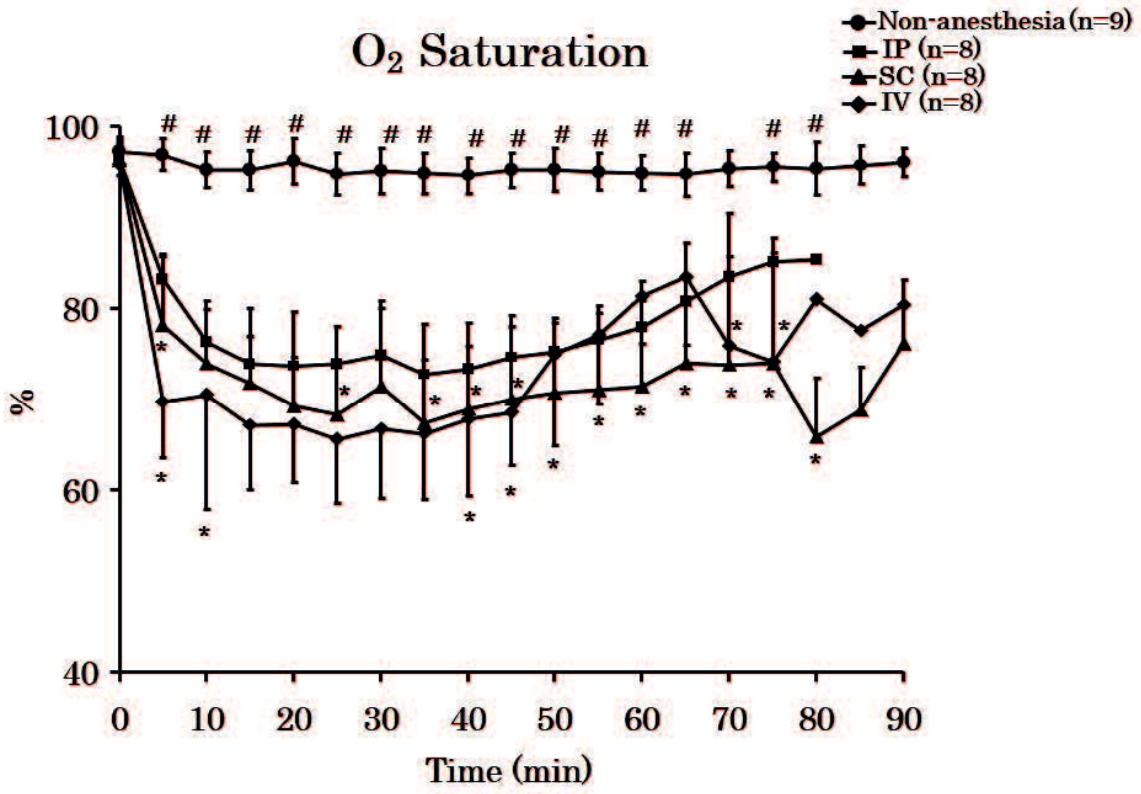

Fig. 4. O_2 Saturation of IP, SC, and IV groups injected the anesthetic mixture, and the non–nesthesia group in male ICR mice. Data are presented as means ± SD. Differences between each group were analyzed by one-way repeated measures ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. *p<0.05 compared with the IP injection group. #p<0.05 compared with the IP, SC, and IV injection groups.

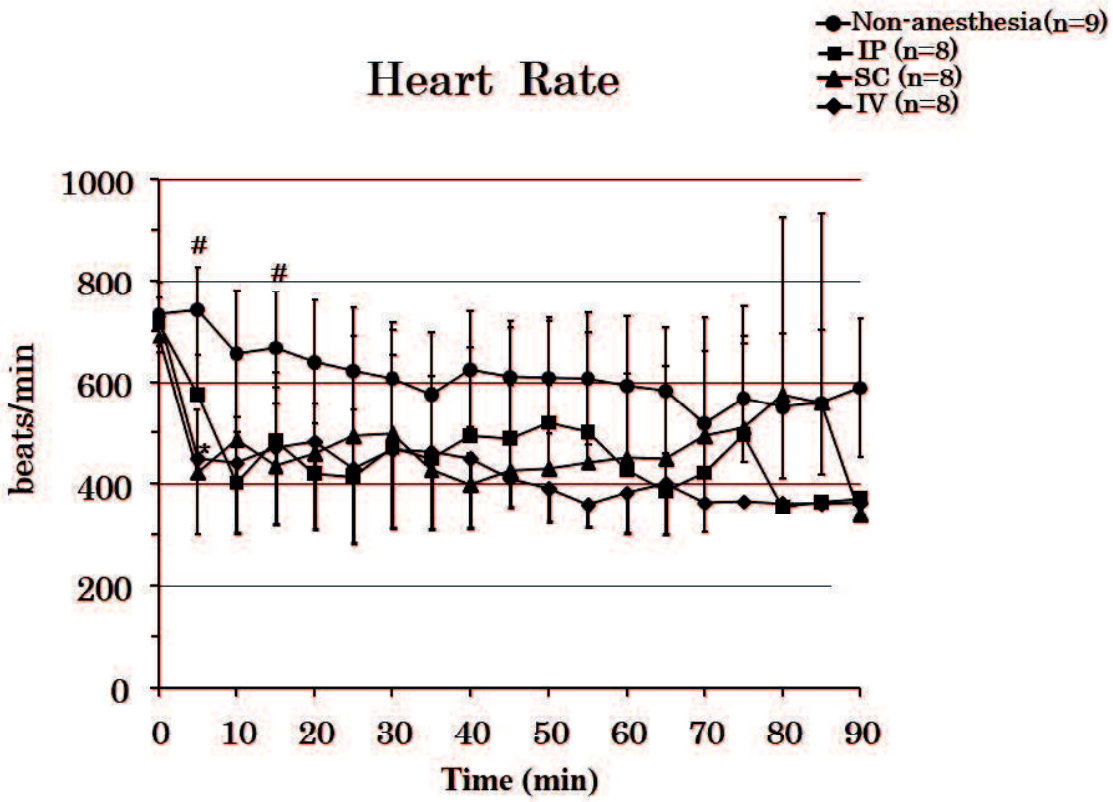

Fig. 5. Heart Rate of IP, SC, and IV groups injected the anesthetic mixture, and the non–anesthesia group in male ICR mice. Data are presented as means ± SD. Differences between each group were analyzed by one-way repeated measures ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. *p<0.05 compared with the IP injection group. #p<0.05 compared with the IP, SC, and IV injection groups.

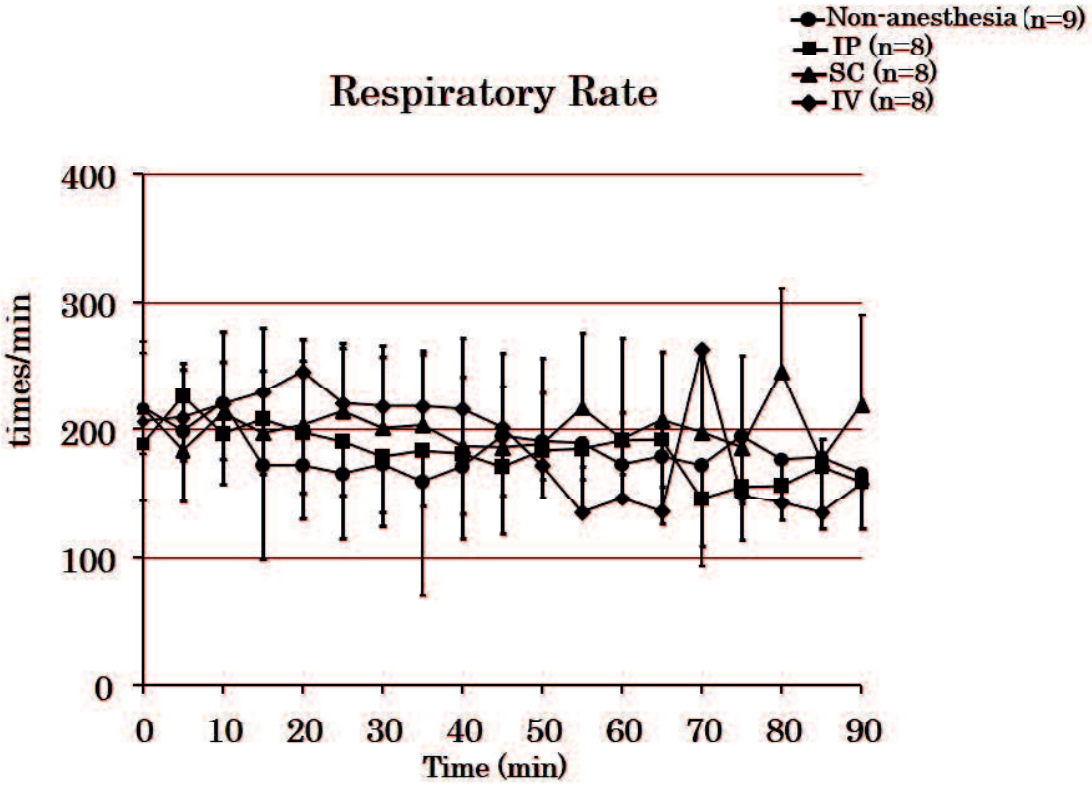

Fig. 6. Respiratory Rate of IP, SC, and IV groups injected the anesthetic mixture, and the non–anesthesia group in male ICR mice. Data are presented as means ± SD. Differences between each group were analyzed by one-way repeated measures ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. There were no significant differences among the 4 groups.

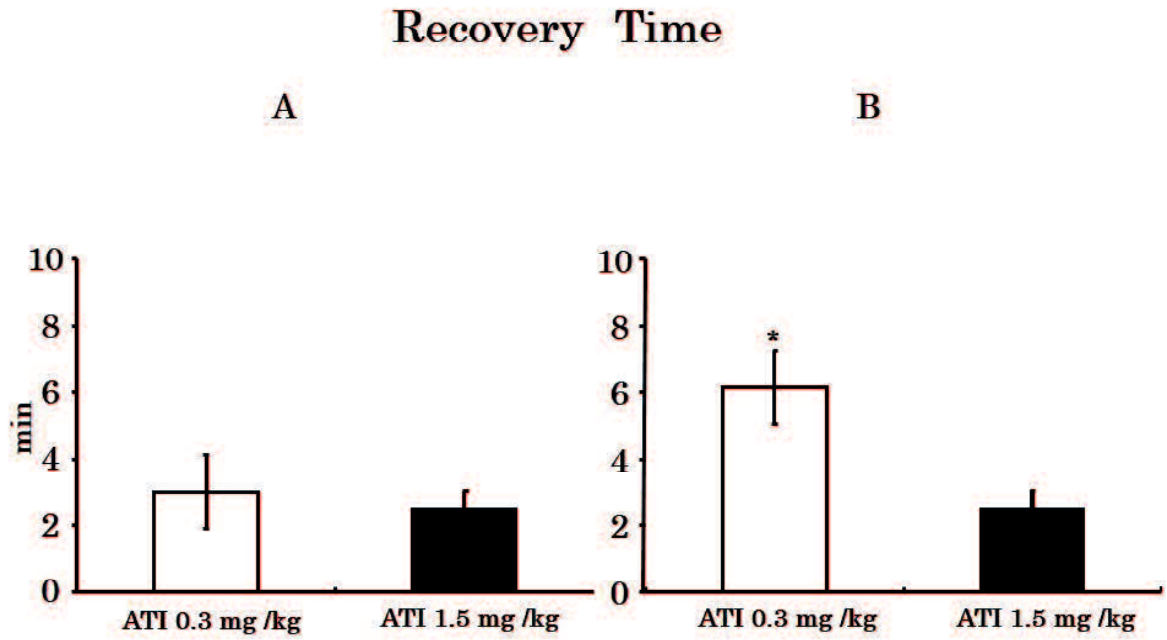

Fig. 7. Recovery time from anesthesia by IP injection of ATI (0.3 mg/kg or 1.5 mg/kg) at 30 min (A) and 10 min (B) after the administration of the anesthetic mixture in male ICR mice. Data are presented as means ± SD. Differences between each group were analyzed one-way ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. *p<0.05 compared with the other groups.

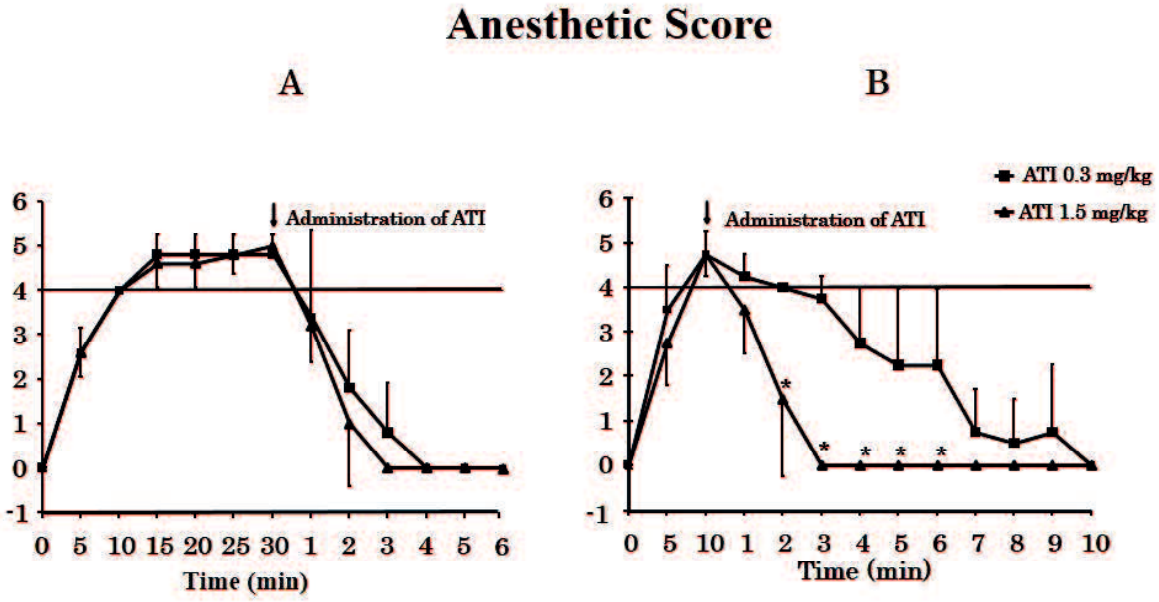

Fig. 8. Anesthetic score of male ICR mice injected ATI (0.3 mg / kg or 1.5 mg/kg) 30 min (A) or 10 min (B) after the administration of the anesthetic mixture. Data are presented as means ± SD. Differences between each injection group were analyzed by ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. *p<0.05 compared with the ATI (0.3 mg/kg) injection group 10 min after the administration of the anesthetic mixture.

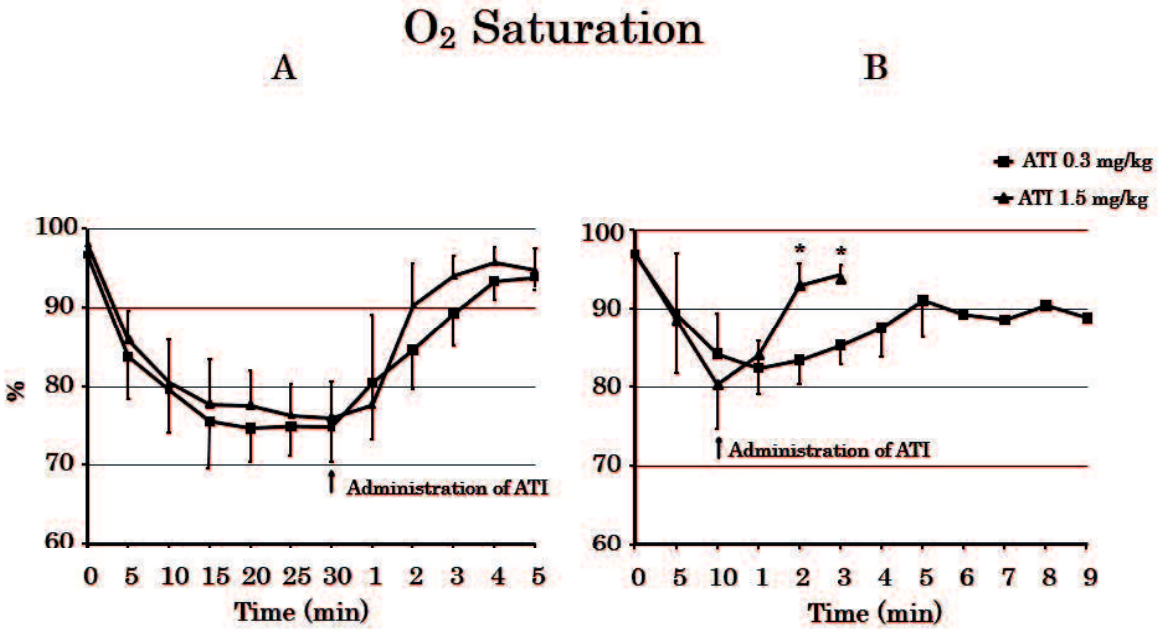

Fig. 9. O_2 Saturation of male ICR mice injected ATI (0.3 mg / kg or 1.5 mg/kg) at 30 min (A) or 10 min (B) after the administration of the anesthetic mixture. Data are presented as means ± SD. Differences between each injection group were analyzed by ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. *p<0.05 compared with the ATI (0.3 mg/kg) injection group 10 min after the administration of the anesthetic mixture.

CHAPTER 3

Effects of an anesthetic mixture of medetomidine, midazolam, and butorphanol in rats–strain difference and antagonism by atipamezole

3.1 Introduction

The anesthetic mixture of MED, MID, and BUT has been recently introduced [28]. This anesthetic mixture was reported to produce an anesthetic duration of around 40 minutes in ICR mice. We reported that it produced closely similar anesthetic effects in both male and female BALB/c and C57BL/6J strains [29]. We also demonstrated that there were no significant differences in anesthetic duration among IP, SC, and IV injection route groups in mice [30]. Several studies have shown that there are different anesthetic effects among different rat strains [2, 10]. However, the differences in the effects of the anesthetic mixture of MED, MID, and BUT in rats remain unclear. In the present study, we first examined effects of the anesthetic mixture using the WST, SD, and F344 rat strains. At the 57th Annual Meeting of the Japanese Association for Laboratory Animal Science (2010, Kyoto, Japan), Pang *et al.* showed an anesthetic duration of about 60 min after administration of an anesthetic mixture of MED 0.15 mg, MID 2 mg, and BUT 2.5 mg/kg b.w./rat [36]. Therefore, we used exactly the same dosage of anesthetic mixture as Pang *et al.* used.

ATI is a synthetic α_2-adrenergic receptor antagonist that can antagonize an α_2-adrenergic receptor agonist, MED [12]. After administration of the anesthetic mixture, injection of ATI caused rapid recovery from anesthesia. We reported the efficacy of ATI with a suitable dosage and timing in mice [30]. However, neither the appropriate dosage nor the optimum injection timing of

ATI after administration of the anesthetic mixture is clear in rats.

In this study, we used the anesthetic score to assess the effects of the anesthetic mixture administered to the 3 different rat strains. During the experiment, we measured vital signs just before and after administration of the anesthetic mixture because parameters such as O_2 saturation, heart rate, and respiratory rate are related to the anesthetic condition of rats under anesthesia [16]. To investigate differences among the 3 rat strains without administration of an anesthetic, we also measured vital signs just before and after administration of saline for 90 min.

3.2 Materials and Methods

3.2.1 Animals and housing conditions

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.

We used 8, 8, and 6 male rats of the WST, SD, and F344 strains, respectively, in the first experiment. In the second experiment, we used the same 8 male rats of the WST strain repeatedly after allowing them to rest for at least 2 days after drug administration. The rats were purchased at 5 weeks of age from a commercial supplier (Japan SLC, Inc., Shizuoka, Japan) and habituated for 2 weeks in the animal room before starting the experiment. The rats were 7 to 9 weeks of age during the experiment.

Two or 3 rats were housed per TPX cage (KN-601-T®, W 270×L 440×H 187

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 twice a week.

The animal room was maintained at a constant temperature (23 ± 2℃) and humidity (55 ± 10%). The rats were given a standard diet (MF®, Oriental Yeast Co., Ltd., Tokyo, Japan) and filtered tap water by an automatic water supply system *ad libitum*.

3.2.2 Experimental procedure

The experiment was conducted during daytime (13:00-17:00). The experimental room was controlled at the same temperature and humidity as the animal room. The rats were weighed before receiving saline or the anesthetic mixture.

In the first experiment, saline or the anesthetic mixture was administered by IP injection at 0.5 ml/100 g b.w./rat. We used 8 (WST and SD) or 6 (F344) rats per each strain group. After administration of the anesthetic mixture, the rats were kept on a heater pad (Heater Mat KN-475®, Natsume Seisakusho, Co., Ltd., Tokyo, Japan) maintained at approximately 38 ℃. When saline was administered, rats were kept in a rat holder. After administration of the anesthetic mixture, the anesthetic score for each rat was measured every 5 min until the rat was completely recovered from anesthesia. At the same time, we measured O_2 saturation, heart rat, and respiratory rate using a pulse oximeter.

In the second experiment, we used 4 groups of rats. We used the same 8 male rats of the WST strain repeatedly after allowing them to rest for at least 2 days after drug administration. Drugs were administered by IP injection. Each group of 8 rats was given ATI after administration of the anesthetic mixture. The rats in group 1 were administered 0.15 mg/kg b.w. ATI at 30 min after administration of the anesthetic mixture. The rats in group 2 were administered 0.75 mg/kg b.w. ATI at 30 min after administration of the anesthetic mixture. The rats in group 3 were administered 0.15 mg/kg b.w. ATI at 10 min after administration of the anesthetic mixture. The rats in group 4 were administered 0.75 mg/kg b.w. ATI at 10 min after administration of the anesthetic mixture. After administration of the anesthetic mixture, the anesthetic score was measured every 5 min. After injection of ATI, the anesthetic score was measured every 1 min.

The rats were euthanatized by IV injection of sodium pentobarbital (80 mg/kg b.w.) (Somnopentyl®, Kyoritsu Seiyaku Corporation, Tokyo, Japan) after completion of the experiment.

3.2.3 Measurement of anesthetic scores

The method of measuring the anesthetic scores was previously described elsewhere [29]. The score was based on 5 reflexes. The first was a front paw reflex, the second was a hind paw reflex, the third was a tail reflex, the fourth was a corneal reflex, and the fifth was a body righting reflex. If a rat showed no reflex, it was given a score of 1. If it reacted, it was given a score of 0. The

total anesthetic score was graded from 0 to 5. A rat that showed a score of 0 to 3 was not considered to be anesthetized. A rat that showed a score of 4 or 5 was considered to be anesthetized, and the duration for which at a rat showed a score of 4 or 5 was considered the anesthetic duration. The time required for the anesthetic score to return to 0 was considered the recovery time.

3.2.4 Measurement of O_2 saturation, heart rate, and respiratory rate

A pulse oximeter (Mouse Ox Plus®, STARR Life Sciences Corp., Oakmont, PA, USA) was used to measure O_2 saturation, heart rate, and respiratory rate of rats during the experiment. The day before the experiment, all hair covering both carotid arteries of each rat was removed using an electric shaver and a depilatory under inhalational isoflurane (Escain®, Mylan Seiyaku, Tokyo, Japan) anesthesia using an anesthetic instrument (KN-1071-I, Natsume Seisakusho Co., Ltd., Tokyo, Japan).

A sensor clip of the pulse oximeter was placed at the cervical parts of the rat. Then, we recorded O_2 saturation, heart rate, and respiratory rate until 90 min after administration of saline. After administration of the anesthetic mixture, we recorded vital signs until the time when each rat was completely recovered from anesthesia.

3.2.5 Drug preparation

Saline (Otsuka Normal Saline®, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) and drugs were purchased. The anesthetic mixture was

prepared as a mix of three drugs: MED (Domitor®, Nippon Zenyaku Kogyo Co., Ltd., Tokyo, Japan), MID (Dormicum®, Astellas Pharma Inc., Tokyo, Japan), and BUT (Vetorphale®, Meiji Seika Pharma Co., Ltd., Tokyo, Japan). We mixed MED of 0.15 mg, MID of 2 mg, and BUT of 2.5 mg/kg b.w./rat and added distilled sterile water (Otsuka Distilled Water®, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) to adjust the mixture to an administrative volume of 0.5 ml/100 g b.w./rat. For example, 0.3 ml of Domitor, 0.8 ml of Dormicum, 1.0 ml of Vetorphale, and 7.9 ml of distilled sterile water were mixed to make 10 ml of an experimental anesthetic mixture. The anesthetic mixture was prepared on the day before the experiment and kept in a refrigerator. The mixed drug was allowed to be used up to 1 week after being mixed.

In the second experiment, we made 0.75 mg/kg b.w./rat of ATI (Antisedan®, Nippon Zenyaku Kogyo Co., Ltd., Tokyo, Japan) and adjusted it to an administrative volume of 0.5 ml/100 g b.w./rat. To make 10 ml of 0.75 mg/kg of ATI, 0.3 ml of Antisedan and 9.7 ml of distilled sterile water were mixed. To make 0.15 mg/kg of ATI, 2 ml of a solution of 0.75 mg/kg of ATI and 8 ml of distilled sterile water were mixed. The solution of ATI was also allowed to be used up to 1 week after being made.

Drug preparation was conducted at a clean bench in a sterile manner. Just before administration, the drug was warmed in an incubator set at a temperature of around 37℃.

3.2.6 Statistical analysis

Statistical analysis was conducted using the StatView software (Hulinks Inc., Tokyo, Japan). Graph data are presented as means ± SD until 90 min after drug administration except for a graph of the anesthetic scores. Body weight differences, anesthetic duration, and recovery time for each experimental group were analyzed using one-way analysis of variance (ANOVA). Time course differences in anesthetic score, O_2 saturation, heart rate, and respiratory rate were analyzed by one-way repeated measures ANOVA. Scheffe's test was used for post hoc analysis. A p value less than 0.05 was considered to be statistically significant.

3.3 Results

All rats used in this experiment recovered from anesthesia.

3.3.1 First experiment

3.3.1.1 Body weight

The body weights of the SD (n=6), WST (n=6), and F344 (n=6) strains before saline was administered were 296.6 ± 8.0, 264.4 ± 6.6, and 187.8 ± 3.7 g, respectively. Significant differences in body weights were recorded among the 3 rat strains administered saline (Table 1). The body weights of the SD (n=8), WST (n=8), and F344 (n=6) strains before the anesthetic mixture was administered were 279.4 ± 17.7, 261.2 ± 19.6, and 168.9 ± 18.6 g, respectively. The body weight of the F344 strain was significantly lighter than those of the other 2 rat strains administered the anesthetic mixture (Table 1).

3.3.1.2 Anesthetic duration

The anesthetic durations of the SD, WST, and F344 strains were 44.4 ± 14.0, 56.9 ± 6.5, and 45.0 ± 13.8 min, respectively (Fig. 1). There were no significant differences among the 3 strains. The shortest anesthetic durations of the SD, WST, and F344 strains were 25, 45, and 30 min, respectively. The longest anesthetic duration of each strain was 65 min (Table 1).

3.3.1.3 Recovery time

The recovery times of the SD, WST, and F344 strains were 101.9. ± 20.0, 86.3 ± 16.9, and 72.5 ± 6.9 min, respectively (Fig. 2). The recovery time of the SD strain was significantly longer than those of the other 2 strains. The shortest recovery times of the SD, WST, and F344 strains were 75, 70, and 60 min, respectively. The longest recovery times of SD, WST, and F344 strains were 125, 115, and 80 min, respectively (Table 1).

3.3.1.4 Anesthetic score

There were no significant differences in anesthetic scores among the 3 rat strains during anesthesia (anesthetic scores of 4 or 5), but 80 min after drug administration, the anesthetic score of the F344 strain was significantly lower than that of the SD strain (0.0 ± 0.0 vs. 2.0 ± 1.3) (Fig. 3).

3.3.1.5 Measurement of O_2 saturation, heart rate, and respiratory rate

1) O_2 saturation

There were no significant differences in O_2 saturation among the 3 rat strains before administration of saline or the anesthetic mixture (Fig. 4). After administration of saline, the 3 strains showed no significant differences in O_2 saturations over the course of 90 min (Fig. 4A).

From 25 to 80 min after administration of the anesthetic mixture, the O_2 saturation of the WST strain was significantly lower than that of the F344 strain (excluding that at 30 min). It was also lower than that of the SD strain from 60 min to 80 min after administration (excluding that at 70 min) (Fig. 4B).

2) Heart rate

There were no significant differences in heart rate among the 3 rat strains before administration of saline or the anesthetic mixture (Fig. 5). From 15 to 90 min after administration of saline, the heart rate of the SD strain was significantly lower than that of the F344 strain, and it was lower that of the WST strain from 40 to 90 min after administration. At 35, 45, and 65 min and 80 to 90 min after administration of saline, the heart rate of the WST strain was significantly lower than that of the F344 strain (Fig. 5A).

From 15 to 65 min after administration of the anesthetic mixture, the heart rate of the SD strain was significantly lower than that of the F344 strain, and it was also lower than that of the WST strain from 35 to 55 min after administration (excluding that at 45 min), respectively.

3) Respiratory rate

There were no significant differences in respiratory rate among the 3 rat strains before administration of saline or the anesthetic mixture (Fig. 6). From 35 min to 75 min after administration of saline, the respiratory rate of the SD strain was significantly higher than that of the F344 strain (excluding that at 50 and 65 min), and it was also higher than that of the WST strain from 35 to 75 min after administration (excluding those at 50, 60, and 65 min) (Fig. 6A).

At 55 and 60 min after administration of the anesthetic mixture, the respiratory rate of the SD strain was significantly lower than that of the WST strain (Fig. 5B).

3.3.2 Second experiment

3.3.2.1 Body weight

There were no significant differences in the body weights of the 4 groups in male WST rats (Table 2).

3.3.2.2 Recovery time

At 30 min after administration of the anesthetic mixture, the recovery times of the rats injected 0.15 mg/kg and 0.75 mg/kg of ATI were 5.9 ± 2.2 and 4.0 ± 1.1 min, respectively. There were no significant differences between the 2 dosages of ATI (Fig. 7A).

At 10 min after administration of the anesthetic mixture, the recovery times of the rats injected 0.15 mg/kg and 0.75 mg/kg of ATI were 10.3 ± 3.3 and 3.6 ± 0.7 min, respectively. The recovery time for 0.15 mg/kg of ATI was

significantly longer than that for 0.75 mg/kg of ATI at 10 min after administration of the anesthetic mixture (Fig. 7B).

3.4 Discussion

An anesthetic mixture of MED, MID, and BUT for laboratory mice has been recently introduced [28-30]. Originally, this mixture was used as anesthesia for dogs [25, 38, 45], monkeys [27, 33] and African lions in a zoo [49]. Kawai *et al.* reported that it produced an anesthetic duration of around 40 min in ICR mice [28]. We demonstrated that it produced closely similar anesthetic effects in both male and female BALB/c and C57BL/6J strains [29]. We also demonstrated that there were no significant differences in anesthetic duration among the 3 different (IP, IV, and SC) injection routes in mice [30].

There are only a few studies for the anesthetic mixture of MED, MID, and BUT in rats. Konno *et al.* reported the efficacy of this anesthetic mixture for endotracheal intubation in rats [31]. Bellini *et al.* evaluated the efficacy of an anesthetic mixture of MED, MID, and BUT compared with MED only or a combination of MED and BUT using rats [4]. Several studies have shown that there are different anesthetic effects among different rat strains. Siller-Matula *et al.* reported that WST strain rats were more sensitive to long-lasting anesthesia with isoflurane as compared with SD rats [41]. Avsaroglu *et al.* used 8 inbred strains to compare anesthetic responses for several anesthetics and found some strain differences [2]. However, the differences in the effects of the anesthetic mixture of MED, MID, and BUT in rats remain unclear.

In the present study, we first examined effects of the anesthetic mixture using 2 outbred strains (WST and SD) and 1 inbred F344 strain. These strains are very common and often used for rat experiments. We found that there were no significant differences in anesthetic duration after administration of the anesthetic mixture among the 3 strains, although the WST strain had a tendency for a longer anesthetic duration compared with the other 2 strains (Fig. 1). Otherwise, the recovery time for the SD strain was significantly longer than those for the other 2 strains (Fig. 2). After an anesthetic duration of around 45 min, the anesthetic score of the F344 strain quickly decreased, and the score reached 0 at 80 min. Compared with the F344 strain, the SD and WST strains needed more time (125 and 115 min, respectively) to reach an anesthetic score of 0 (Fig. 3). In this experiment, the mean body weight of the F344 strain (168.9 g) was lighter than those of the SD (279.4 g) and WST (261.2 g) strains (Table 1). The anesthetic mixture was administered according to body weight (0.5 ml/100 g b.w./rat). However, one reason for the rapid recovery from anesthesia for the F344 strain may be related to its lighter body weight as compared with the heavier SD and WST strains. Also, Avsaroglu *et al.* reported that the F344 strain slept a significantly shorter amount of time after administration of MED than other inbred strains [2]. Therefore, we guessed that the F344 strain would recover quickly from anesthesia as compared with the other 2 strains.

In our previous study using 2 mouse strains, we recorded no significant differences in anesthetic effects for the anesthetic mixture between male and

female mice [29]. Several studies have reported sex differences in anesthesia for rats [7, 52]. However, further exploration of the sex differences for rats using the anesthetic mixture will require more experiments and research.

We measured the vital signs of the 3 strains without anesthesia because there were some differences in physiological parameters among various strains [5, 21, 43]. After administration of saline, there were no significant differences in O_2 saturation among the 3 strains (Fig. 4A). After administration of saline, the heart rate of the SD strain was significantly lower than that of the other 2 strains (Fig. 5A). Furthermore, after administration of saline, the respiratory rate of the SD strain was significantly higher than that of the other 2 strains (Fig. 6A). However, the normal ranges for the heart rate and respiratory rate in several rat strains were 260-450 beats/min and 66-115 times/min, respectively [34, 42]. Therefore, in spite of statistical differences in heart rate and respiratory rate among the 3 different strains after administration of saline, it is not an important issue compared with the heart rate and respiratory rate after administration of the anesthetic mixture.

All 3 rat strains in this experiment showed lower O_2 saturation after administration of the anesthetic mixture (Fig. 4B). In particular, the WST strain showed significantly lower O_2 saturation than the F344 strain. O_2 saturation is one of the parameters used to estimate anesthetic depth and condition under anesthesia in laboratory animals [16]. According to the changes in O_2 saturation after administration of the anesthetic mixture, the WST strain seemed to be in a deeper anesthetic condition from 40 to 80 min

after administration compared with the 2 other strains. However, our results showed that there were no significant differences in anesthetic duration among the 3 different strains (Fig. 1). One reason for this discrepancy may be related to our scoring method for estimation of anesthetic depth because it cannot measure an anesthetic score over 5.

After administration of the anesthetic mixture, all rat strains showed lower heart rates compared with those after administration of saline (Fig. 5B). However, we found that heart rate differences among the 3 strains were reduced and stable under anesthesia (Fig. 5).

After administration of the anesthetic mixture, the respiratory rate of the SD strain was lower than that after administration of saline (Fig. 6). However, the respiratory rates of the WST and F344 strains after administration of the anesthetic mixture were not different from those after administration of saline (Fig. 6). After administration of the anesthetic mixture, the respiratory rates of 3 different strains were almost same and were stable during the rest of the experiment (Fig. 6B).

In our previous mice study, we showed an anesthetic duration of about 46 min after IP administration of the anesthetic mixture [30]. In the present rat experiment, we demonstrated almost the same anesthetic duration, about 49 min, after IP administration in 3 different rat strains. When we compared the cardiorespiratory influences of the anesthetic mixture between mice and rats, we found similar tendencies. Both rats and mice showed reduced O_2 saturation and a reduced heart rate after administration of the anesthetic mixture. We

did not find any respiratory rate changes after administration of the anesthetic mixture in either mice or rats.

ATI is a synthetic α_2-adrenergic receptor antagonist that can antagonize an α_2-adrenergic receptor agonist, MED [12]. After administration of the anesthetic mixture, injection of ATI caused rapid recovery from anesthesia. However, neither the appropriate dosage nor optimum injection timing of ATI after administration of the anesthetic mixture is clear in rats.

We previously reported the efficacy of ATI with a suitable dosage and timing in mice [30]. In this previous mouse experiment, we used the same dosage of ATI as MED (0.3 mg/kg) and a 5-times higher dosage of ATI (1.5 mg/kg) than MED and chose injection timings of 10 and 30 min after administration of the anesthetic mixture. Therefore, we used the same dose as MED (0.15 mg/kg) and a 5-times higher dose (0.75 mg/kg) than MED and injection timings of 10 and 30 min after administration of the anesthetic mixture in our rat experiment.

At 30 min after administration of the anesthetic mixture, injection of ATI at 0.15 mg/kg and 0.75 mg/kg resulted in almost the same rapid recovery time (Fig. 7A). On the other hand, at 10 min after administration of the anesthetic mixture, injection of ATI 0.15 mg/kg resulted in the need for more time to recover from anesthesia compared with ATI 0.75 mg/kg (Fig. 7B). Baker *et al.* reported that there were no significant differences in recovery times after receiving 5 mg/kg of ATI at 10 min and 40 min after administration of a combination of KET (75 mg /kg) and MED (1 mg/kg) in mice [3]. Our previous

study using mice also showed no significant differences in recovery times at 10 min and 30 min after administration of the anesthetic mixture when ATI was administered at 1.5 mg/kg, a 5-times higher dosage than that of MED (0.3 mg/kg) [30]. In a study by Jang *et al.* using SD rats, a 5-times higher dosage of ATI than MED was injected at 70 min after administration of a combination of MED (0.2, 0.4 or 0.8 mg/kg) and KET (60 mg/kg) [26]. They reported that a 5-times higher dosage of ATI caused reversal of anesthesia within 10 min after administration of MED and KET. However, Jang *et al.* theorized that ATI at a dosage 5-times higher than the MED might not be adequate for satisfactory reversal of anesthesia induced by MED and KET in rats [26]. In our rat study, we also used a 5-times higher dosage of ATI (0.75 mg/kg) than MED (0.15 mg/kg) and observed quick recovery, in 5 min, from anesthesia.

When ATI at a dosage 5-times higher than the dosage of MED is used, a combination of MED and KET may result in the need for more time to recover from anesthesia compared with in the case of administration of the anesthetic mixture. At 10 min after administration of the anesthetic mixture, rats injected with ATI (0.15 mg/kg) at the same dosage as MED (0.15 mg/kg) needed more time (10.3 ± 3.3 min) to recover from anesthesia as compared with the time needed by mice (6.2 ±3.3 min) [30].

Regarding the safety of ATI, all rats injected with ATI (0.75 mg/kg) perfectly recovered from anesthesia and showed normal values for O_2 saturation, heart rate, and respiratory rate. In this experiment, we used rats repeatedly and confirmed that the parameters described above were normal before proceeding

to the next injection experiment. In our previous study using mice, we injected a 5-times higher dosage of ATI (1.5 mg/kg) than MED (0.3 mg/kg) and found no significant changes in health condition [30]. Baker *et al.* reported that there were no mortalities and no weight loss or reduced water intake at 24 and 48 hours after administration of ATI (5 mg/kg) in mice [3]. Also, Jang *et al.* injected ATI (4 mg/kg) in rats and reported rapid increases in respiratory rate, but there was no mention of health problems after recovering from anesthesia [26]. Therefore, we suggest that 0.75 mg/kg is a safe and proper dosage of ATI for helping rats quickly recover from anesthesia at both 10 and 30 min after administration of the anesthetic mixture.

In summary, our study indicated that an anesthetic mixture of MED, MID, and BUT produced a closely similar anesthetic duration in 3 different rat strains: SD, WST, and F344. The anesthetic mixture of MED, MID, and BUT is a useful anesthetic that can be antagonized with an antagonist such as ATI to help rats quickly recover from anesthesia. These results may contribute to the welfare of laboratory animals.

Table 1. Body weight (g), anesthetic duration (min), and recovery time (min) of the SD, WST, and F344 rat strains.

		Body weight (g) (mean ± SD)			Anesthetic duration (min)			Recovery time (min)		
Strain	n.	Saline	n.	Anesthetic mixture	Mean ± SD	Shortest	Longest	Mean ± SD	Shortest	Longest
SD	6	296.6 ± 8.0$^{\#}$	8	279.4 ± 17.7$^{\$}$	44.4 ± 14.0	25	65	101.9 ± 20.0	75	125
WST	6	264.4 ± 6.6$^{\#}$	8	261.2 ± 19.6$^{\$}$	56.9 ± 6.5	45	65	86.3 ± 16.9*	70	115
F344	6	187.8 ± 3.7$^{\#}$	6	168.9 ± 18.6	45.0 ± 13.8	30	65	72.5 ± 6.9*	60	80

Differences between each strain were analyzed by one-way ANOVA followed by Scheffe's test. A p value less than 0.05 was considered statistically significant. *p<0.05 compared with the SD strain. \$$p$<0.05 compared with the F344 strain. #p<0.05 compared with the other strains.

Table 2. Body weights (g) of the 4 groups of male WST rats. Injection route, concentration of atipamezole, and injection timing after administration of the anesthetic mixture are shown in addition to body weights in this table

		Body weight (g)	Anesthetic mixture	Atipamezole		Injection timing after anesthetic
Group	n.	Mean ± SD	Route	Route	Concentration	mixture (min)
1	8	270.9 ± 21.4	IP	IP	0.15 mg/kg	30
2	8	257.0 ± 27.9	IP	IP	0.75 mg/kg	30
3	8	267.6 ± 22.7	IP	IP	0.15 mg/kg	10
4	8	264.0 ± 22.5	IP	IP	0.75 mg/kg	10

Data for body weight are presented as means ± SD. Differences between experimental groups were analyzed by one-way ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant.

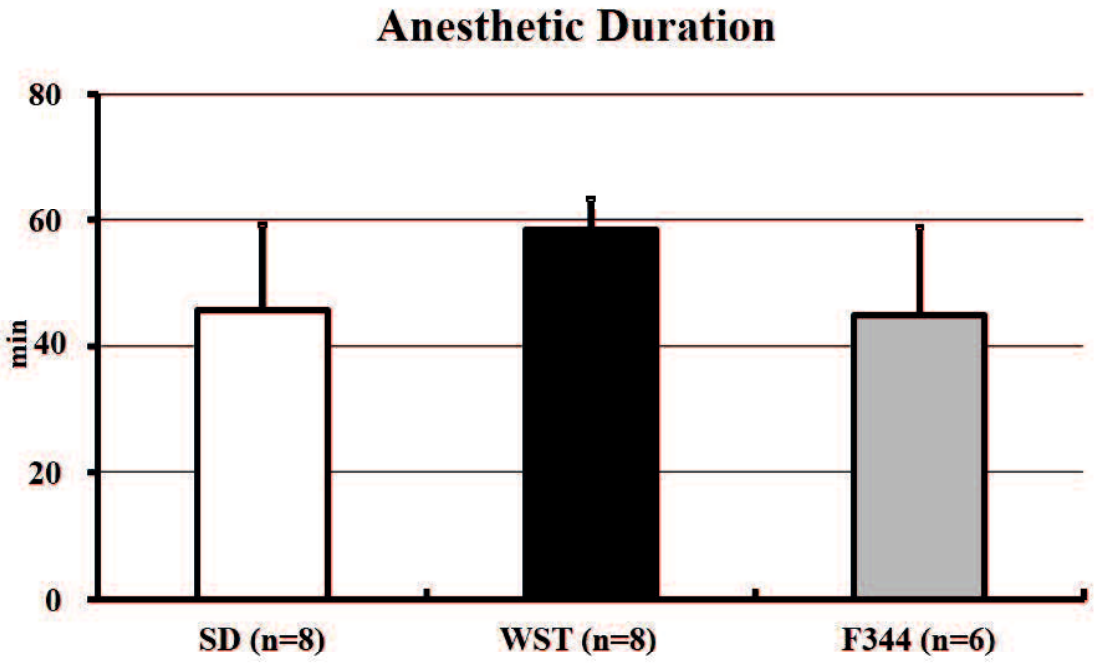

Fig. 1. Anesthetic duration of the SD, WST, and F344 rat strains administered the anesthetic mixture. Data are presented as means ± SD. Differences between strains were analyzed by one-way ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant.

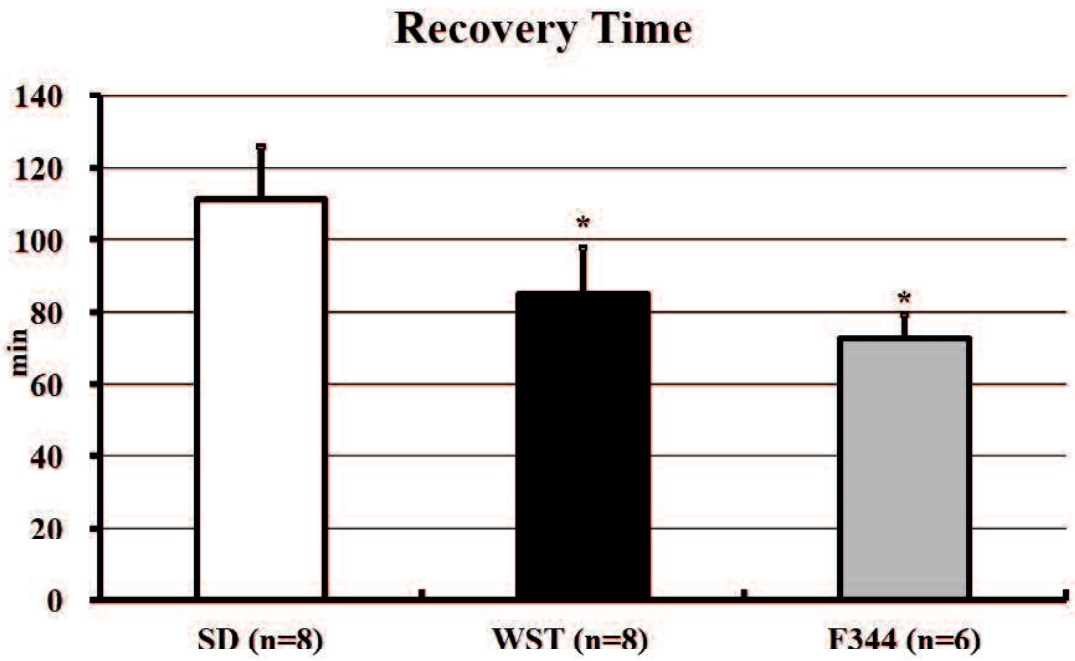

Fig. 2. Recovery times of the SD, WST, and F344 rat strains administered the anesthetic mixture. Data are presented as means ± SD. Differences between strains were analyzed by one-way ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. *p<0.05 compared with the SD strain.

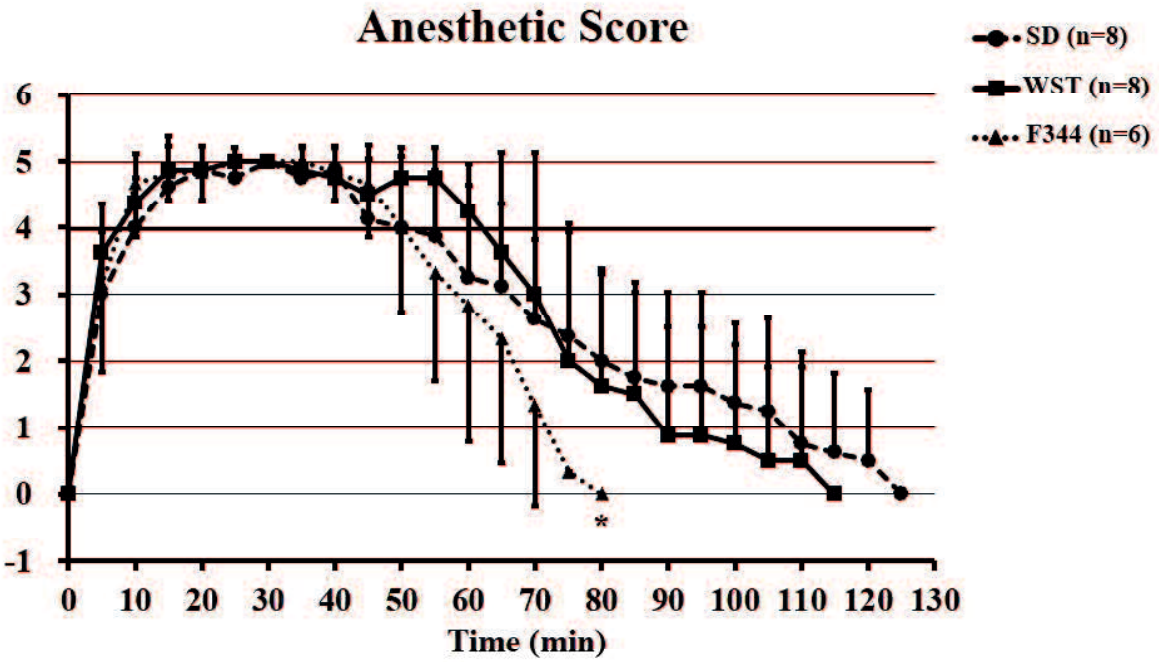

Fig. 3. Time courses of the anesthetic score of the SD, WST, and F344 rat strains administered the anesthetic mixture. Data are presented as means ± SD. Differences between strains were analyzed by one-way repeated measures ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. *p<0.05 compared with the SD strain.

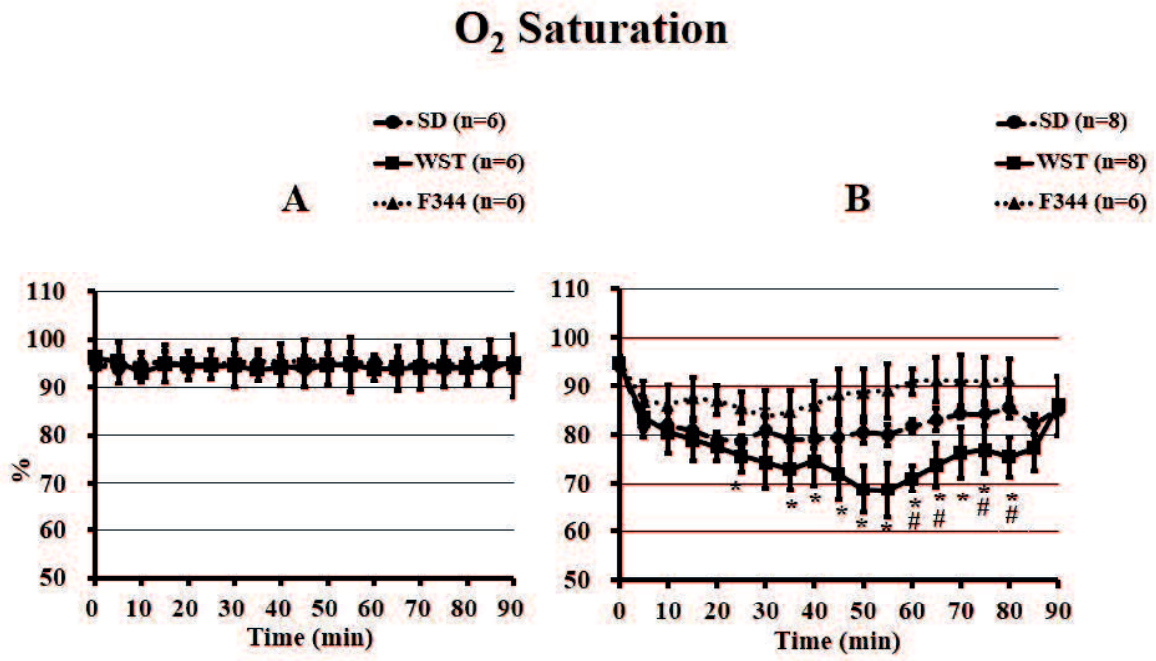

Fig. 4. Time courses of O_2 saturation of the SD, WST, and F344 rat strains administered saline (A) or the anesthetic mixture (B). Data are presented as means ± SD. Differences between strains were analyzed by one-way repeated measures ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. *p<0.05 compared with the F344 strain. #p<0.05 compared with the SD strain.

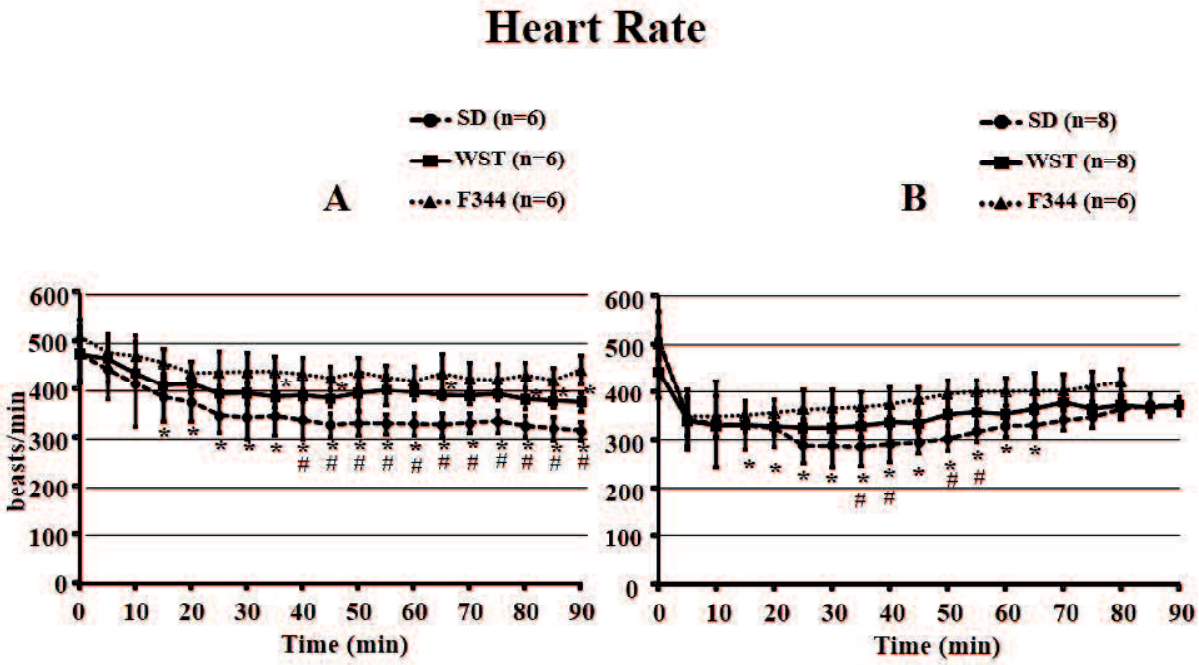

Fig. 5. Time courses of heart rate of the SD, WST, and F344 rat strains administered saline (A) or the anesthetic mixture (B). Data are presented as means ± SD. Differences between strains were analyzed by one-way repeated measures ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. *p<0.05 compared with the F344 strain. #p<0.05 compared with the WST strain.

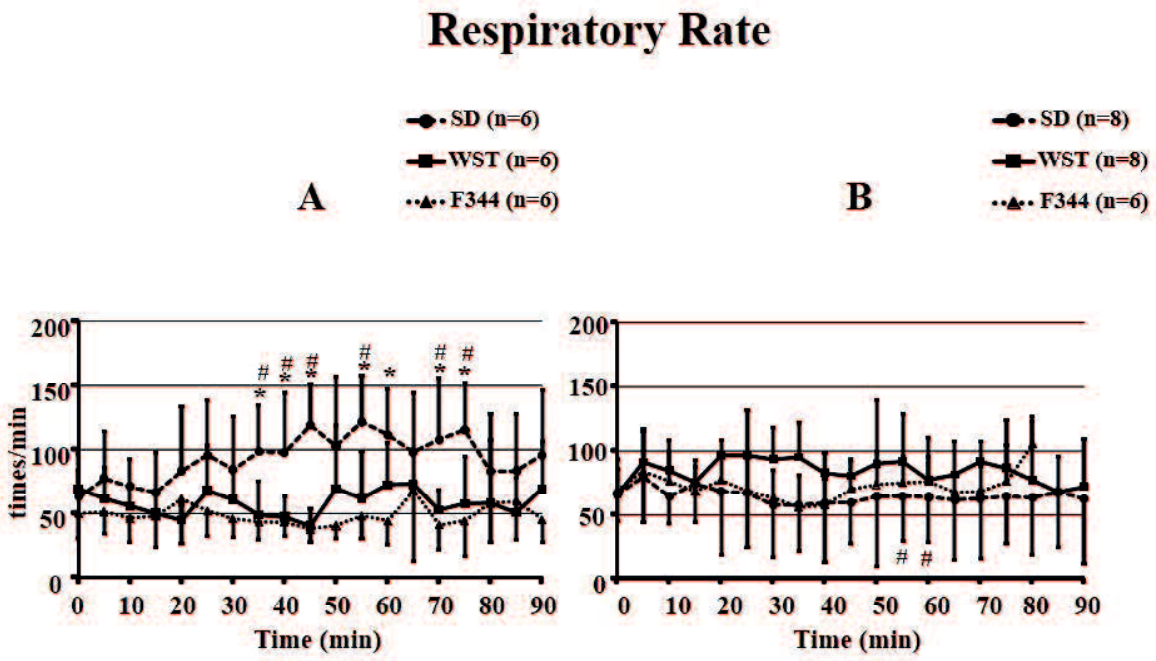

Fig. 6. Time courses of respiratory rate of the SD, WST, and F344 rat strains administered saline (A) or the anesthetic mixture (B). Data are presented as means ± SD. Differences between strains were analyzed by one-way repeated measures ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. *p<0.05 compared with the F344 strain. #p<0.05 compared with the WST strain.

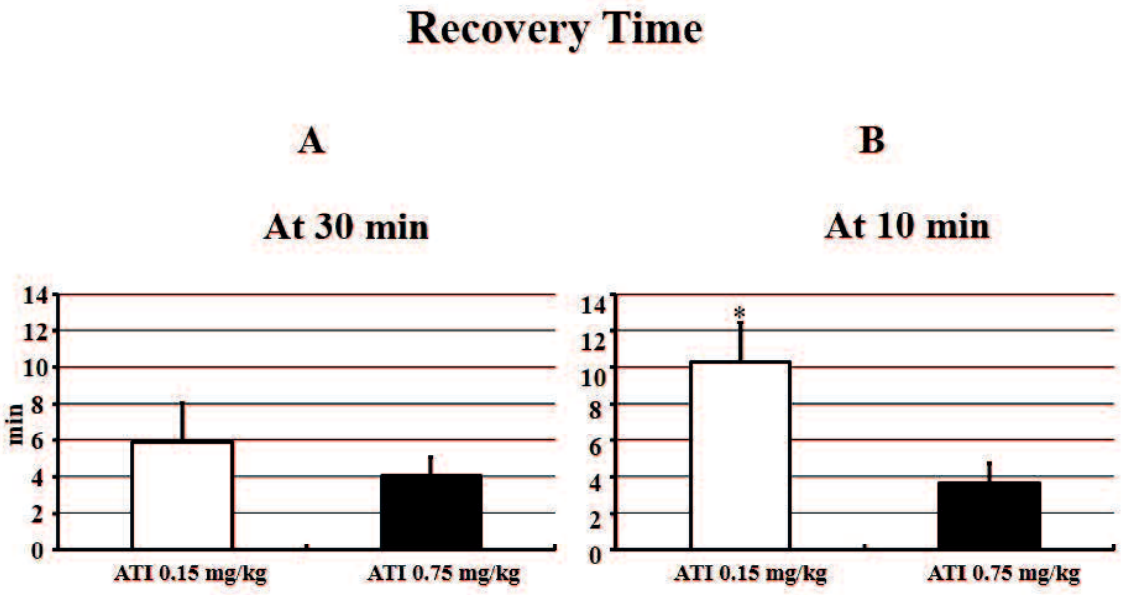

Fig. 7. Recovery time after IP injection of ATI (0.15 mg/kg or 0.75 mg/kg) at 30 min (A) and 10 min (B) after administration of the anesthetic mixture in male WST rats. Data are presented as means ± SD. Differences between groups were analyzed by one-way ANOVA followed by Scheffe's test. A p value less than 0.05 was considered to be statistically significant. *p<0.05 compared with ATI 0.75 mg/kg at 10 min.

GENERAL CONCLUTION

The first study indicated 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/6J 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. The data of this study showed that there were no significant differences in anesthetic duration between strains and sexes. However, 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. We also found that there were no significant differences for anesthetic duration from 8 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 to 20 weeks is the most useful period for mouse experiments. 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℃, showed the same efficacy for at least 8 weeks after mixing.

In the second study, the first experiment indicated that there were no significant differences of anesthetic duration among the three different injection

routes, although the IV injection group showed a quick increase and decrease of anesthetic scores after administration. The anesthetic score of the IV injection group was significantly higher than the IP injection group at 5 min. From 10 to 90 min, the three injection groups did not show statistically different anesthetic scores. However, our scoring method to estimate anesthetic depth could not measure an anesthetic score of over 5. As the result of O_2 saturation levels showed, IV injection may have worked more strongly than IP injection during the earlier period after injection. We slowly and carefully administered the anesthetic mixture by IV route. Then, when careless and rapid IV injection is carried, it may cause death. Unexpectedly, SC injection showed a tendency to produce longer anesthetic duration compared to IP injection. An IP injection is made through the abdominal wall into the peritoneal cavity and there is no visual confirmation that the injection has been correctly administered. Compared to IP injection, inspectional failures are easily detected with SC and IV injection routes. SC injection of the anesthetic mixture is a suitable route compared to IP or IV injection, because there are some technical risks for IP injection, and IV method is sometimes lethal and not easy for injection. Then, we recommend SC injection of the anesthetic mixture compared to IP and IV injection, although there are no significant differences of anesthetic duration among the 3 injection routes.

ATI is an α_2-adrenargic antagonist, then it antagonizes the effect of MED. At 30 min after the anesthetic mixture, administration of ATI at 0.3 mg/kg and 1.5 mg/kg had almost the same rapid recovery time from anesthesia in mice.

However, at 10 min after injection of the anesthetic mixture, administration of ATI (0.3 mg/kg) needed more time to recover from anesthesia compared with ATI (1.5 mg/kg). Our data also showed no significant differences of recovery times at 10 min and 30 min after administration of the anesthetic mixture when administered ATI (1.5 mg/kg).

In the third study, we examined effects of the anesthetic mixture using 2 outbred strains (WST and SD) and 1 inbred F344 strain. We found that there were no significant differences in anesthetic duration after administration of the anesthetic mixture among the 3 strains, although the WST strain had a tendency for a longer anesthetic duration compared with the other 2 strains. The WST strain showed significantly lower O_2 saturation than the F344 strain. O_2 saturation is one of the parameters used to estimate anesthetic depth and condition under anesthesia in laboratory animals. According to the changes in O_2 saturation after administration of the anesthetic mixture, the WST strain seemed to be in a deeper anesthetic condition compared with the 2 other strains. Otherwise, the recovery time for the SD strain was significantly longer than those for the other 2 strains, especially compared to the F344 strain. One reason for the rapid recovery from anesthesia for the F344 strain may be related to its lighter body weight as compared with the heavier SD and WST strains.

In our rat study, we also used a 5-times higher dosage of ATI (0.75 mg/kg) than MED (0.15 mg/kg) and observed quick recovery, in 5 min, from anesthesia. Therefore, we suggest that 0.75 mg/kg is a safe and proper dosage of ATI for helping rats quickly recover from anesthesia at both 10 and 30 min after

administration of the anesthetic mixture.

In summary, our study indicated that the anesthetic effects of the mixture for inbred laboratory mice such as the BALB/c and C57BL/6J strains were similar to those of ICR mice. The anesthetic mixture of MED, MID, and BUT produced almost same anesthetic duration by IP, SC, and IV injection in ICR mice. SC injection of the anesthetic mixture is a recommended route compared to IP or IV injection. There were no significant differences in anesthetic duration after administration of the anesthetic mixture among the 3 rat strains, although the WST strain had a tendency for a longer anesthetic duration compared with the other 2 strains. The anesthetic mixture is a useful drug to have a MED antagonist; ATI which helps mice and rats quickly recover from anesthesia. These results may contribute to the welfare of laboratory animals.

SUMMARY

The purpose of these studies is to clarify the usefulness of the anesthetic mixture of MED, MID, and BUT in laboratory mice and rats.

The results obtains are as follows.

1. The anesthetic mixture of MED, MID, and BUT was useful and effective anesthesia for both of male and female BALB/c and C57BL/6J strains of inbred mice as well as out-bred ICR mice.
2. There were no significant differences for anesthetic effects of the anesthetic mixture from 8 to 20 weeks of age in males and females of both BALB/c and C57BL/6J strains.
3. The anesthetic mixture, when kept in a refrigerator at 4℃, showed the same efficacy for at least 8 weeks after mixing.
4. The anesthetic mixture produced almost same anesthetic duration by IP, SC, and IV injection in ICR mice. However, SC injection of the anesthetic mixture was a recommended route compared to IP or IV injection.
5. The anesthetic mixture produced a closely similar anesthetic duration in SD, WST, and F344 strain rats.
6. ATI which helped mice and rats quickly recover from anesthesia by the anesthetic mixture.

The anesthetic mixture of MED, MID, and BUT is a useful anesthetic that can be antagonized with an antagonist; ATI to help mice and rats quickly

recover from anesthesia. These results may contribute to the welfare of laboratory animals.

REFERENCES

1. Araki, Y., Mukaisyo, K., Sugihara, H., Fujiyama, Y., Hattori, T. 2010. Increased apoptosis and decreased proliferation of colonic epithelium in dextran sulfate sodium-induced colitis in mice. *Oncol. Rep.* 24: 869-884.
2. Avsaroglu, H., van der Sar, A. S., van Lith, H. A., van Zutphen, L. F., Hellebrekers, L. J. 2007. Differences in response to anaesthetics and analgesics between inbred rat strains. *Lab. Anim.* 41: 337-344.
3. Baker, N. J., Schofield, J. C., Caswell, M. D., McLellan, A. D. 2011. Effects of early atipamezole reversal of medetomidine-ketamine anesthesia in mice. *J. Am. Assoc. Lab. Anim. Sci.* 50: 916-920.
4. Bellini, L., Banzato, T., Contier, B., Zotti, A. 2014. Evaluation of three medetomidine-based protocols for chemical restraint and sedation for non-painful procedures in companion rats (*Rattus norvegicus*). *Vet. J.* 200: 456-458.
5. Boyd, R. L., Halderman, L. W., Harris, J. O., Mangos, J. A. 1982. Strain differences in pulmonary function of laboratory rats. *Lab. Anim. Sci.* 32: 42-43.
6. Burnside, W. M., Flecknell, P. A., Cameron, A. I., Thomas, A. A. 2013. A comparison of medetomidine and its active enantiomer dexmedetomidine when administered with ketamine in mice. *BMC. Vet. Res.* 9: 48. doi: 10. 1186/1746-6148-9-78.

7. Ciccone, G. K., Holdcroft, A. 1999. Drugs and sex differences: a review of drugs relating to anaesthesia. *Br. J. Anaesth.* 82: 255-265.

8. Claassen, V. 1994. Neglected factors in pharmacology and neuroscience reserch. p. 46-58. In: Tecniques in the Behavioral and Neural Sciences. vol. 12. ELSEVIER, Amsterdam.

9. Duguet, A., Biya, K., Minshall, E., Gomes, R., Wang, C. G., Taoudi-Benchekroun, M., Bates, J. H., Eidelman, D. H. 2000. Bronchial responsiveness among inbred mouse strains. Role of airway smooth-muscle shortening velocity. *Am. J. Respir. Crit. Care Med.* 161: 839-848.

10. Fender, C., Fujinaga, M., Maze, M. 2000. Strain differences in the antinociceptive effect of nitrous oxide on the tail flick test in rats. *Anesth. Analg.* 90: 195-199.

11. Flecknell, P. A. 2009. Chapter 2. Anaesthesia. p. 23. In: Laboratory Animal Anaesthesia. 3rd ed. ELSEVIER, London.

12. Flecknell, P. A. 2009. Chapter 2. Anaesthesia. p. 24. In: Laboratory Animal Anaesthesia. 3rd ed. ELSEVIER, London.

13. Flecknell, P. A. 2009. Chapter 2. Anaesthesia. p. 63. In: Laboratory Animal Anaesthesia. 3rd ed. ELSEVIER, London.

14. Flechnell, P. A. 2009. Chapter 2. Anaesthesia. p. 65. In: Laboratory Animal Anaesthesia. 3rd ed. ELSEVIER, London.

15. Flecknell, P. A. 2009. Chapter 2. Anaesthesia. p. 68. In: Laboratory Animal Anaesthesia. 3rd ed. ELSEVIER, London.

16. Flecknell, P. A. 2009. Capter 3. Anaesthetic Management. p. 83-87. In: Laboratory Animal Anaesthesia. 3rd ed. ELSEVIER, London.

17. Flecknell, P. A. 2009. Chapter 5. Analgesia and Post-poerative Care. p. 170. In: Laboratory Animal Anaesthesia. 3rd ed. ELSEVIER, London.

18. Fujiwara, M., Kuriyama, K., Hattori, K. 1986. p. 29-30. In: The Textbook of Medical Pharmacology. NANZANDO Co., Ltd., Tokyo.

19. Gaines, D. R., North, D. 2007. Implications of experimental technique for analysis and interpretation of data from animal experiments: outliers and increased variability resulting from failure of intraperitoneal injection procedures. *Lab. Anim.* 41: 312-320.

20. Gonsenhauser, I., Wilson, C. G., Han, F., Strohl, K. P., Dick, T. E. 2004. Strain differences in murine ventilatory behavior persist after urethane anesthesia. *J. Appl. Physiol.* 97: 888-897.

21. Gordon, C. J., Watkinson, W. P. 1995. Strain differences in the laboratory rat: impact on the autonomic, behavioral, and biochemical response to cholinesterase inhibition. *J. Toxicol. Environ. Health.* 45: 59-73.

22. Green, C. J., Knight, J., Precious, S., Simpkin, S. 1981. Ketamine alone and combined with diazepam or xylazine in laboratory animals: a 10 year experience. *Lab. Anim.* 15: 163-170.

23. Hirose, A., Sato, E., Fujii, H., Sun, B., Nishioka, H., Aruoma, O. I. 2007. The influence of active hexose correlated compound (AHCC) on cisplatin-evoked chemotherapeutic and side effects in tumor-bearing mice. *Toxicol. Appl. Pharmacol.* 222: 152-158.

24. Hsu, W. H., Bellin, S. I., Dellmann, H. D., Hanson, C. E. 1986. Xylazine-ketamine-induced anesthesia in rats and its antagonism by yohimbine. *J. Am. Vet. Med. Assoc.* 189: 1040-1043.

25. Itamoto, K., Hikasa, Y., Sakonjyu. I., Itoh, H., Kakuta, T., Takase, K. 2000. Anaesthetic and cardiopulmonary effects of balanced anaesthesia with medetomidine-midazolam and butorphanol in dogs. *J. Vet. Med. A Physiol. Pathol. Clin. Med.* 47: 411-420.

26. Jang, H. S., Choi, H. S., Lee, S. H., Jang, K. H., Lee, M. G. 2009. Evaluation of the anaesthetic effects of medetomidine and ketamine in rats and their reversal with atipamezole. *Vet. Anaesth. Analg.* 36: 319-327.

27. Kalema-Zikusoka, G., Horne, W. A., Levine, J., Loomis, M. R. 2003. Comparison of the cardiorespiratory effects of medetomidine-butorphanol-ketamine and medetomidine-butorphanol-midazolam in patas monkeys (*Erythrocebus patas*). *J. Zoo Wildl. Med.* 34: 47-52.

28. Kawai, S., Takagi, Y., Kaneko, S., Kurosawa, T. 2011. Effect of three types of mixed anesthetic agents alternate to ketamine in mice. *Exp. Anim.* 60: 481-487.

29. Kirihara, Y., Takechi, M., Kurosaki, K., Kobayashi, Y., Kurosawa, T. 2013. Anesthetic effects of a mixture of medetomidine, midazolam and butorphanol in two strains of mice. *Exp. Anim.* 62: 173-180.

30. Kirihara, Y., Takechi, M., Kurosaki, K., Kobayashi, Y., Saito, Y.,

Takeuchi, T. 2015. Anesthetic effects of a three-drugs mixture -comparison of administrative routes and antagonistic effects of atipamezole in mice-. *Exp. Anim.* 64: 39-47.

31. Konno, K., Shiotani. Y., Itano, N., Ogawa, T., Hatakeyama, M., Shioya, K., Kasai, N. 2014. Visible, safe and certain endotracheal intubation using endoscope system and inhalation anesthesia for rats. *J. Vet. Med. Sci.* 76: 1375-1381.

32. Mulder, J. B. 1978. Anesthesia in the mouse using a combination of ketamine and promazine. *Lab. Anim. Sci.* 28: 70-71.

33. Ochi, T., Nishiura, I., Tatsumi, M., Hirano, Y., Yahagi, K., Sakurai, Y., Matsuyama-Fujiwara, K., Sudo, Y., Nishina, N., Koyama, H. 2014. Anesthetic effects of combination of medetomidine-midazoram-butorphanol in cynomolgus monkeys (*Macaca fascicularis*). *J. Vet. Med. Sci.* 76: 917-921.

34. Ohno, M., Hirose, H., Sugano, S. 1987. Alteration in heart rate and conduction time during the growing process of rats, mice and guinea pigs. *Adv. Anim. Cardiol.* 20: 24-32.

35. Okabe, T., Toda, T., Nukitrangsan, N., Inafuku, M., Iwasaki, H., Yanagita, T. 2010. Comparative study of the effect of basal diet formulation, dietary fat and cholesterol levels on the development of aortic atherosclerotic lesions in B6. KOR-Apoeshl mice. *J. Oleo. Sci.* 59: 161-167.

36. Pang, H., Kawai, S., Shioya, K., Katahira, K., Yabuuci, K., Tajima, M., Kurosawa, T. 2010. Short anesthesia with a combination of three

injectable anesthetic in rat. *Exp. Anim.* 59, Supplement: 370.

37. Podhorna, J., McCabe, S., Brown, R. E. 2002. Male and female C57BL/6 mice respond differently to diazepam challenge in avoidance learning tasks. *Pharmacol. Biochem. Behav.* 72: 13-21.

38. Pypendop, B., Verstegen, J. 1999. Cardiorespiratory effects of a combination of medetomidine, midazolam, and butorphanol in dogs. *Am. J. Vet. Res.* 60: 1148-1154.

39. Raekallio, M., Ansah, O. B., Kuusela, E., Vainio, O. 2002. Some factors influencing the level of clinical sedation induced by medetomidine in rabbits. *J. Vet. Pharmacol. Ther.* 25: 39-42.

40. Salonen, M., Reid, K., Maze, M. 1992. Synergistic interaction between alpha 2-adrenergic agonists and benzodiazepines in rats. *Anesthesiology.* 76: 1004-1011.

41. Siller-Matula, J. M., Jilma, B. 2008. Strain differences in toxic effects of long-lasting isoflurane anaesthesia between Wistar rats and Sprague Dawley rats. *Food Chem. Toxicol.* 46: 3550-3552.

42. Tazima, Y. 1972. p. 12 In: Experimental Zoology Detailed Discussion. (Japanese) ASAKURA Publishing Co., Ltd., Tokyo.

43. Tazima, Y. 1989. p. 16-17 In: Biological Reference Data Book on Experimental Animals. (Japanese) SOFT SCIENCE, Inc., Tokyo.

44. Tsukada, H., Sato, K., Fukumoto, D., Kakiuchi, T. 2006. Evaluation of D-isomers of O-18F-fluoromethyl, O-18F-fluoroethyl and O-18F-fluoropropyl tyrosine as tumour imaging agents in mice. *Eur. J.*

Nucl. Med. Mol. Imaging 33: 1017-1024.

45. Verstegen, J., Petcho, A. 1993. Medetomidine-butorphanol-midazolam for anaesthesia in dogs and its reversal by atipamezole. *Vet. Rec.* 132: 353-357.

46. Voipio, H. M., Nevalainen, T. O., Virtanen, R. 1990. Evaluation of anaesthetic potency of medetomidine-ketamine combination in mice. *IXth ICLAS Symposium Proceedings*, 298-299.

47. Wang, X., Willén, R., Svensson, M., Ljungh, A., Wadström, T. 2003. Two-year follow-up of *Helicobacter pylori* infection in C57BL/6 and Balb/cA mice. *APMIS* 111: 514-522.

48. Watanabe, M., Tanaka, M., Tateishi, T., Nakura. H., Kumai, T., Kobayashi, S. 1997. Effects of the estrous cycle and the gender differences on hepatic drug-metabolising enzyme activities. *Pharmaco. Res.* 35: 477-480.

49. Wenger, S., Buss, P., Joubert, J., Steenkamp, J., Shikwambana, P., Hatt, J. M. 2010. Evaluation of butorphanol, medetomidine and midazolam as a reversible narcotic combination in free-ranging African lions (*Panthera leo*) *Vet. Anaesth. Analg.* 37: 491-500.

50. White, G. L., Holmes, D. D. 1976. A comparison of ketamine and the combination ketamine-xylazine for effective surgical anesthesia in the rabbit. *Lab. Anim. Sci.* 26: 804-806.

51. Xue, Y., Johnson, R., Desmet, M., Snyder, P. W., Fleet, J. C. 2010. Generation of a transgenic mouse for colorectal cancer research with

intestinal cre expression limited to the large intestine. *Mol. Cancer Res.* 8: 1095-1104.

52. Zambricki, E. A., Dalecy, L. G. 2004. Rat sex differences in anesthesia. *Com. Med.* 54: 49-53.

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