Exp. Anim. 66(4), 397–404, 2017

—Original—

Pharmacological properties of various anesthetic protocols in 10-day-old neonatal rats

Atsushi TSUKAMOTO[1)], Yui KONISHI[1)], Takako KAWAKAMI[1)], Chiharu KOIBUCHI[1)], Reiichiro SATO[2)], Eiichi KANAI[3)], and Tomo INOMATA[1)]

[1)]*Laboratory of Laboratory Animal Science, Faculty of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuou-ku, Sagamihara, Kanagawa 252-5201, Japan*
[2)]*Laboratory of Veterinary Internal Medicine 3, Faculty of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuou-ku, Sagamihara, Kanagawa 252-5201, Japan*
[3)]*Laboratory of Veterinary Radiology, Faculty of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuou-ku, Sagamihara, Kanagawa 252-5201, Japan*

Abstract: In general, the anesthesia in neonates involves high risk. Although hypothermic anesthesia is recommended in rats up to the age of 7 days, neonatal anesthesia for later periods has not been standardized. The present study investigated the pharmacological properties of conventional anesthetic protocols in 10-day-old SD rats. The rats were anesthetized with four anesthetics: a combination of ketamine and xylazine (K/X); a combination of medetomidine, midazolam, and butorphanol (M/M/B); isoflurane; and sevoflurane. Anesthetic depth was scored by reflex response to noxious stimuli. Induction and recovery times were recorded. Vital signs and mortality rate were evaluated for safety assessment. All rats died after administration of K/X at a dose of 60/6 mg/kg, whereas K/X at 40/4 mg/kg resulted in insufficient anesthetic depth, indicating inappropriate for neonatal anesthesia. Although M/M/B at the adult rat dose (0.15/2/2.5 mg/kg) did not provide surgical anesthetic depth, the mouse dose (0.3/4/5 mg/kg) showed sufficient anesthetic depth with relatively stable vital signs. Isoflurane required a long induction period, and caused remarkable respiratory depression and hypothermia, resulted in a 25% mortality rate. In contrast, sevoflurane provided consistent surgical anesthetic depth with rapid induction. Although respiratory rate decrease was markedly observed, all rats survived. Among the anesthetic protocols investigated in the present study, sevoflurane and M/M/B at the mouse dose were recommended for the neonatal anesthesia. Compared with adult rats, the required dose of both anesthetics in neonates was higher, possibly associated with their lower anesthetic sensitivity.

Key words: anesthesia, neonates, refinement, rodents

Introduction

When conducting animal experiments, it is necessary to comply with the basic principles of laboratory animal welfare, the "3Rs." In particularly, appropriate anesthesia is important for the compliance with the "refinement" of the animal experimentation; it also ensures that the experimental results are reliable and reproducible. An appropriate anesthetic must have adequate anesthetic action (i.e., sufficient analgesic, sedative, and hypnotic actions). In addition, because of the potential for systemic adverse reactions, safety must also be ensured. A

(Received 4 April 2017 / Accepted 12 June 2017 / Published online in J-STAGE 30 June 2017)
Address corresponding: A. Tsukamoto, Laboratory of Laboratory Animal Science, Faculty of veterinary medicine, Azabu University, 1-17-71 Fuchinobe, Chuou-ku, Sagamihara, Kanagawa 252-5201, Japan.
This study was presented at 63rd Annual Meeting of Japanese Association for Laboratory Animal Science.

typical side effect that causes anesthesia-related mortality is cardiorespiratory depression [14, 25], which may affect experimental procedures. Owing to recent concerns regarding animal welfare and third-party certification of experimental facilities, the standardization of laboratory animal anesthesia is now required.

To date, several general anesthetic protocols have been applied to rodents [5, 11, 14, 26]. General anesthesia is mainly classified into injection anesthesia and inhalation anesthesia. A typical injection anesthetic is ketamine-based anesthesia or a combination of medetomidine, midazolam, butorphanol [4, 17]. These injectable anesthetic protocols are versatile and are widely used in rodents, but care must be taken to manage cardiorespiratory adverse reactions. Typical inhalation anesthetics include isoflurane and sevoflurane [11]. These anesthetics have a comparatively broad margin of safety, with rapid induction and wakening. Furthermore, the depth of anesthesia can be also adjusted in real time, and it is possible to manage the anesthesia for short or long periods of time [14]. Following the commercial availability of ready-to-use inhalation anesthesia devices for small rodents, the use of inhalation anesthesia has increased [8]. In this way, there are some anesthetic options for adult rodent surgery. When choosing the appropriate anesthesia for animal experimentation, it is necessary to understand the characteristics of the anesthesia and assess the risks in each animal. Although recent studies have provided the pharmacological properties of various anesthetic protocols in adult rodents [4, 19, 25], the effect individual factors, such as age, has not been fully investigated.

Immediately after birth, neonates adapt to life outside the mother's body by rapidly developing each organ through major cardiorespiratory changes; hence, various organs, including the nervous system, are underdeveloped in neonates, which increases the anesthetic risk compared with that of adults [1, 15]. In rats, hypothermic anesthesia has been recommended for anesthesia in neonates that are up to 7 days old [21]. However, there are many unclear points with regard to the validity of the anesthetic methods in neonates in the latter period. In the present study, the characteristics of various anesthetic protocols that are conventionally used in adult rats were investigated in 10-day-old neonatal rats.

Materials and Methods

Animals

All experiments in the present study were approved by the Animal Research Committee of Azabu University. Pregnant-Sprague Dawley (Slc:SD) rats were purchased from Japan SLC Inc. (Shizuoka) and housed in individual plastic cages exposed to a 12h light cycle with free access to water. The room was air-conditioned at a temperature of 22 ± 1°C and a humidity of 55 ± 5%. The rats were fed a pelleted rodent diet (Lab diet, Japan SLC, Inc.). After birth, the pups were kept in cages with their littermates and mothers until just prior to anesthesia. Ten-day-old rat pups were used in anesthetic experiments. After the experiment was completed, the animals were euthanized by the intraperitoneal administration of 150 mg/kg sodium pentobarbital (Somnopentyl, Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan).

Anesthetic agents and protocols

The clinical agents investigated in the present study include medetomidine hydrochloride (Domitol, Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan), midazolam (Dormicum, Astellas Pharma Inc., Tokyo, Japan), butorphanol (Vetorphale, Meiji Seika Pharma Co., Ltd., Tokyo, Japan), ketamine hydrochloride (Ketalar, Sankyo Lifetech Co., Ltd., Tokyo, Japan), xylazine (Celactar, Bayer Yakuhin Ltd., Tokyo, Japan), isoflurane (Isoflu, DS Pharma Animal Health Co., Ltd., Osaka, Japan), and sevoflurane (Sevoflo, DS Pharma Animal Health Co., Ltd.).

Ten-day-old rats were assigned to six groups and anesthetized with one of the following: a combination of ketamine and xylazine (K/X); a combination of medetomidine, midazolam, and butorphanol (M/M/B); isoflurane, and sevoflurane. The dose of K/X was set at 60/6 mg/kg and 40/4 mg/kg, according to dose determination study. M/M/B was also investigated at two doses, which have been used commonly in mature rats (M/M/B: 0.15/2/2.5 mg/kg) [18] and mice (M/M/B: 0.3/4/5 mg/kg) [17]. The volume (ml) of K/X and M/M/B was adjusted to 0.05 ml.

The concentration of isoflurane and sevoflurane is set to 4.0 and 8.0%, respectively. The concentration of isoflurane and sevoflurane during maintenance was adjusted to the minimum alveolar concentration (MAC) × 1.5 (isoflurane: 3.0%, sevoflurane: 3.8%) [20]. Inhalation anesthesia was performed using a rodent inhalation an-

esthesia apparatus (SomnoSuite Small Animal Anesthesia System, Kent Scientific Corporation, Connecticut). The vaporized inhalant anesthetics are delivered with room air at the flow rate of 32 ml/min. During anesthesia, rat pups were placed on a nylon pad to maintain a constant surface temperature beneath them. After administration of anesthetics, anesthetic depth and vital signs were evaluated in each rat pup every 5 min for 60 min.

Depth and times of anesthesia

Anesthetic depth was assessed with reaction to nociceptive stimulus, partly modified from a previously reported method [17, 25]. By using forceps, three reflexes were evaluated: the pedal withdrawal reflex in the forelimbs and hind limbs, and the tail pinch reflex. The anesthetic depth score was determined based on the number of reflex reactions (0–3); when all three reflexes were lost, the score was 3. All stimuli were induced by the same investigator. Surgical depth was defined as≥2. Time course of anesthetic score during 60 min was evaluated in each anesthetic group, and area under the curve (AUC) of anesthetic score-min was calculated from them. We also evaluated the sex difference of anesthetic score in each group. Induction was evaluated in each rat pups and was considered complete when the righting reflex was lost after the administration of anesthesia. The recovery time was measured in the inhalation anesthesia group and was defined as the time span between cessation of the gas supply and recovery of the righting reflex.

Vital signs recording and mortality rate

For the safety assessment, rectal temperature, heart rate, respiratory rate, and saturation O_2 (SPO_2) were recorded during the period of anesthesia. Rectal temperature was measured with a commercial rodent thermometer (Right Temp, Kent Scientific Corp.). In addition, a rodent pulse oximeter (MouseSTAT, Kent Scientific Corp.) was placed on tail base to measure the heart rate and SPO_2. At each time point, we recorded the maximum value during 1 min as the value of rectal temperature, heart rate, and SPO_2. Respiratory rate was assessed according to the number of movements of the thorax wall each min. After anesthesia, the rats were observed for 24 h and mortality rate was calculated in each anesthetic group.

Statistical analysis

Statistical analyses were performed using a commercial statistical software program (StatMate IV ATMS, Japan). Repeated analysis of variance (ANOVA) was used for the analysis of each parameter. When the data were significant, Dunnett's multiple comparison method was performed to compare each parameter between the baseline and other time points. The difference of the mean parameter in each group was assessed by one-way ANOVA followed by Tukey's test. In each group, sex differences of the anesthetic score were analyzed by Student's *t*-test. For all analyses, the level of statistical significance was defined as $P<0.05$.

Table 1. The mortality rate and the rate of surgical anesthetic depth (%) in each anesthetic group

	Mortality rate	The rate of reaching surgical anesthetic depth
K/X (40/4)	0	0
K/X (60/6)	100	-
M/M/B (0.15/2/2.5)	0	50
M/M/B (0.3/4/5)	0	100
Isoflurane	25	75
Sevoflurane	0	100

Results

Mortality

At first, mortality rate was compared in each anesthetic group (Table 1). The administration of K/X at 60/6 mg/kg caused sudden death immediately after administration. Therefore, anesthetic action and vital sign monitoring could not be assessed in the K/X (60/6) group. The mortality rate in the isoflurane group was 25%. In contrast, in the groups treated with K/X (40/4), both doses of M/M/B, and sevoflurane, no rats died after anesthesia.

Time and depth of anesthesia

Next, the time and depth of anesthesia were assessed in each group. Injectable anesthetic groups such as K/X and M/M/B experienced rapid induction compared with inhalation anesthesia, such as isoflurane and sevoflurane (K/X 40/4: 1.3 ± 0.2 min, M/M/B 0.15/2/2.5: 1.1 ± 0.1 min, M/M/B 0.3/4/5: 1.1 ± 0.2 min, isoflurane: 3.6 ± 0.8 min, and sevoflurane: 3.4 ± 0.8 min, $P<0.05$). The recovery time of sevoflurane was shorter than that of isoflurane (sevoflurane: 13.0 ± 4.6 min, isoflurane: 20.0 ± 5.7 min, $P<0.05$). The time course and AUC of the anesthetic score is shown Fig. 1. The sevoflurane group

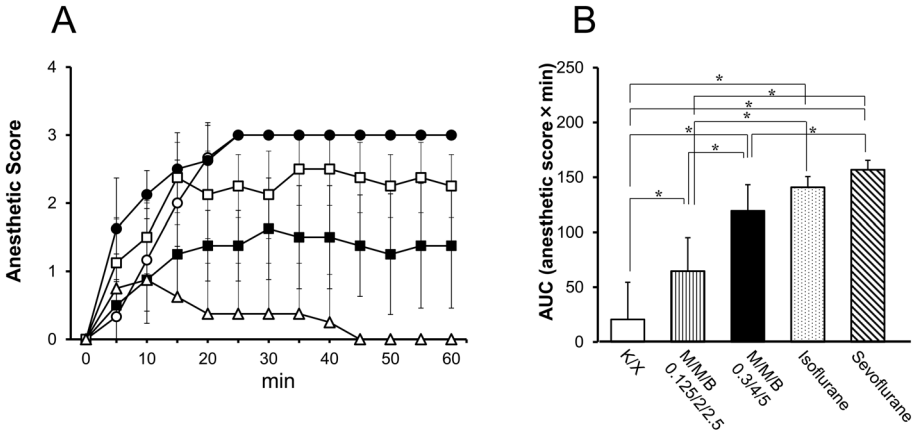

Fig. 1. Time course (A) and AUC (B) of anesthetic score in each group (n=8). (white triangle): K/X (40/4). (black square): M/M/B (0.125/2/2.5). (white square): M/M/B (0.3/4/5). (white circle): Isoflurane. (black circle): Sevoflurane.

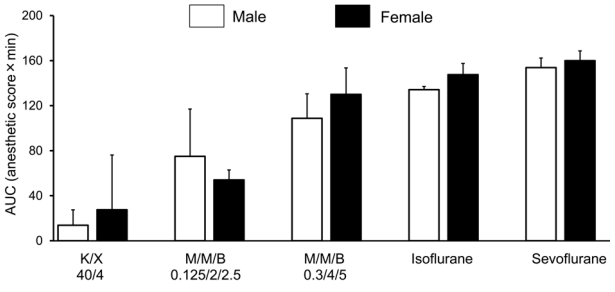

Fig. 2. Comparisons of AUC (anesthetic score × min) in male and female (n=4, each). No significant difference in anesthetic score was observed.

showed highest anesthetic score among groups. In contrast, K/X (40/4) showed significantly lower anesthetic score than other anesthetic groups. As shown in the graph, isoflurane required a longer period to achieve surgical anesthesia compared with sevoflurane (isoflurane: 15.0 ± 3.2 min, sevoflurane: 5.7 ± 1.7 min, $P<0.05$). M/M/B (0.3/4/5), isoflurane, and sevoflurane anesthesia reached surgical anesthetic depth in whole rats, whereas M/M/B (0.15/2/2.5) and K/X (40/4) showed inadequate anesthetic depth (Table 1). Sex-related differences of anesthetic sensitivity were not observed in either anesthetic group (Fig. 2).

Vital signs

Finally, vital signs were compared in five of the anesthetic groups, except for the group of K/X (60/6). The results of vital signs in each group are shown in Figs. 3–6. Baseline differences between the groups in each vital sign were not statistically significant. The isoflurane groups showed significantly lower rectal temperature compared with M/M/B group in both dose at 5 min, and with K/X (40/4) group at 35–60 min (Fig. 3). Significant decreases in heart rate were observed in every group (Fig. 4). M/M/B groups at either dose mediated significantly lower heart rate, compared with K/X (40/4) groups (5–60 min), and with both inhalant anesthetic groups (5–20 min). Respiratory rate was also significantly decreased immediately after anesthesia in all groups (Fig. 5). The respiratory rate of K/X (40/4) was higher than other anesthetic groups during whole anesthetic period. Inhalant anesthesia with isoflurane and sevoflurane showed significantly lower respiratory rate value, compared to M/M/B at either dose (M/M/B:0.15/2/2.5: 15–60 min, M/M/B:0.3/4/5: 25–60 min). The measured SPO_2 (%) over time is shown in Fig. 6. Although K/X (40/4) group showed remarkable SPO_2 decrease at 5 min, the value gradually recovered, and no significant difference was observed with baseline value at 20–60 min. Other anesthetic groups showed significant decrease of SPO_2 at entire anesthetic period. Inhalant anesthesia with isoflurane and sevoflurane caused significant decrease of SPO_2 value, compared to that in M/M/B. Isoflurane showed lower SPO_2 value, compared to sevoflurane at 5 min.

Discussion

The present study investigated the pharmacological properties of conventional anesthetic protocols in 10-day-old neonatal rats. Each anesthetic protocol re-

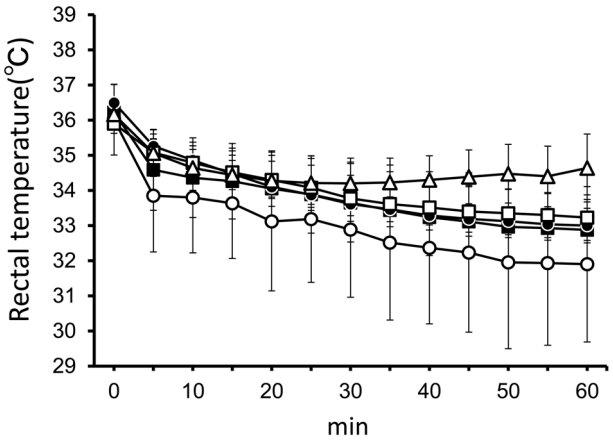

Fig. 3. Time course of rectal temperature in each group (n=8). (white triangle): K/X (40/4). (black square): M/M/B (0.125/2/2.5). (white square): M/M/B (0.3/4/5). (white circle): Isoflurane. (black circle): Sevoflurane.

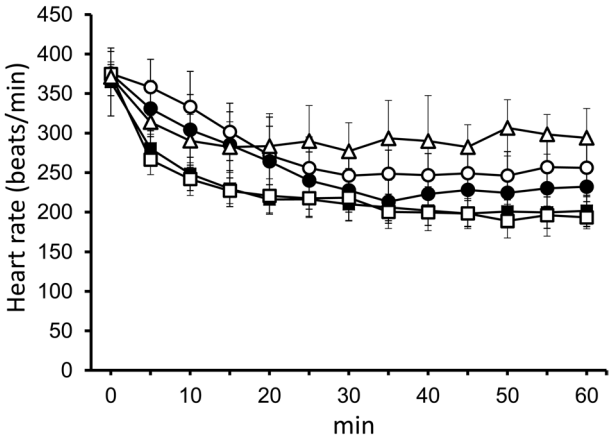

Fig. 4. Time course of heart rate in each group (n=8). (white triangle): K/X (40/4). (black square): M/M/B (0.125/2/2.5). (white square): M/M/B (0.3/4/5). (white circle): Isoflurane. (black circle): Sevoflurane.

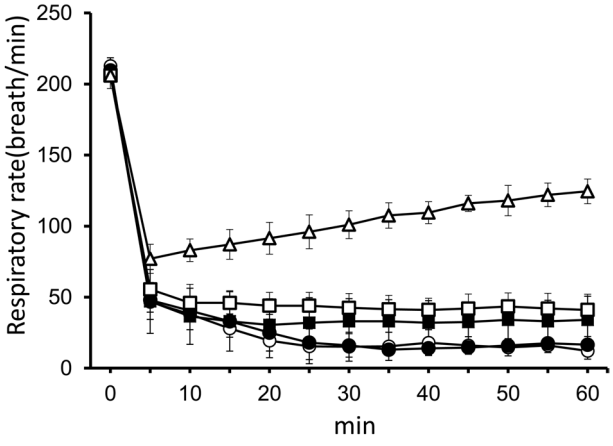

Fig. 5. Time course of respiratory rate in each group (n=8). (white triangle): K/X (40/4). (black square): M/M/B (0.125/2/2.5). (white square): M/M/B (0.3/4/5). (white circle): Isoflurane. (black circle): Sevoflurane.

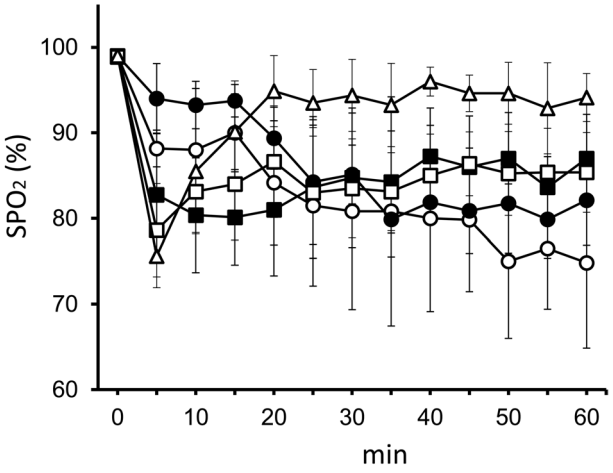

Fig. 6. Time course of SPO_2 rate in each group (n=8). (white triangle): K/X (40/4). (black square): M/M/B (0.125/2/2.5). (white square): M/M/B (0.3/4/5). (white circle): Isoflurane. (black circle): Sevoflurane.

sulted in markedly different pharmacological properties in neonates compared with adult rats.

The combination of ketamine and xylazine is common injectable anesthesia that has long been used for rodent surgery [14]. The major adverse reaction of K/X in rodents is cardiac depression, such as bradycardia and hypotension [4, 23, 25]. In adult rats, the standard dose of ketamine is reported to be 75–80 mg/kg when co-administered with xylazine [12, 14]. Currently, there are few reports that have investigated the anesthetic action and safety of K/X for neonatal rats. One study, which investigated the anesthetic action of K/X at 100/10 mg/kg, reported a high mortality rate [9]. In the present study, the doses of ketamine and xylazine were set at 40–60 mg/kg, and 4–6 mg/kg respectively, which were lower than the previous report. However, K/X at 60/6 mg/kg resulted in sudden death after injection in the neonatal rats. As the rats died immediately after administration, these deaths may be associated with cardiorespiratory depression. In contrast, the dose of 40/4 mg/kg did not reach surgical anesthetic depth. Therefore, K/X is not recommended for use as surgical anesthesia in neonatal rats because of its narrow margin of safety.

M/M/B is a balanced anesthesia that achieves surgical

anesthesia by the combination treatment of α_2 receptor agonist medetomidine, benzodiazepine sedative agent midazolam, and opioid analgesic butorphanol. In recent years, several studies have been conducted on M/M/B anesthesia in rodents, which has led to an increase in use for surgical purposes [17, 18, 25]. As this combination includes medetomidine, the risk of cardiac abnormalities, such as bradycardia, is relatively high compared with other general anesthetic protocols [25]. The dose of M/M/B in adult rats is reported to be 0.15/2/2.5 mg/kg [18], while the dose for mice was reported to be twice the recommended dose for rats (0.3/4/5 mg/kg) [17]. In the present study, we investigated M/M/B at the doses reported for adult mice and rats. M/M/B at 0.15/2/2.5 mg/kg resulted in lower cardiorespiratory depression compared with other anesthetic protocols. However, this dose did not provide sufficient anesthetic depth in neonates. In contrast, M/M/B at 0.3/4/5 mg/kg produced deep anesthesia with relatively stable vital signs. Based on these findings, the mouse dose was preferable for the use of M/M/B in neonatal rats. The drug sensitivity of M/M/B in neonatal rats may be lower in adults. The anesthetic action lasted more than 60 min in neonates. The reversal action of atipamezole in neonatal rats is an issue that will require further investigation.

Isoflurane is representative inhalation anesthetic agent that is widely used to provide surgical anesthesia in rats [14]. In the present study, the concentration of isoflurane during maintenance was set at 3%. This concentration corresponds to MAC × 1.5, which is commonly used for standard surgery. In adult rats, MAC was reported as 1.3–1.4% [11, 26], whereas it is reported as 2% in neonatal rats [20]; this is suggestive of a lower drug sensitivity in neonates. In adult rats, isoflurane mediated sufficient anesthetic depth with rapid induction and awakening [11], demonstrating a wide safety margin and accomplishing sufficient anesthetic depth with reduced cardiac and metabolic influence [7, 14, 19]. In contrast to adult rats, the administration of isoflurane in 10-day-old neonatal rats produced undesirable pharmacological effects. First, a long time was required to reach surgical anesthetic depth, which prolonged the anesthetic time. This may be associated with a high blood/gas partition coefficient [11, 22]. In addition, isoflurane in neonates caused remarkable hypothermia with a decrease in respiratory rate, compared with adults [26], which resulted in a 25% mortality rate. Therefore, the single use of isoflurane in neonatal rats is limited by the relatively long anesthetic induction time and its adverse reaction. Although warming pad was used for the maintenance of rat's temperature in the present study, the remarkable hypothermia was observed in some rat pups anesthetized with isoflurane. The warming level of the pad sensor was adjusted to that in adult rodents. As hypothermia may be major cause of isoflurane-induced death, special care may be required to keep the rat pups' temperature when using isoflurane in neonates.

Sevoflurane is the next-generation inhalation anesthetic of isoflurane [19]. Compared with isoflurane, sevoflurane has a lower blood/gas partition coefficient, which results in rapid induction of, and recovery from, anesthesia [13]. Furthermore, unlike isoflurane, sevoflurane gas is free from irritating properties and leads to stable induction [24]. Therefore, sevoflurane is most common inhalation anesthetic agent and is widely used in human medicine. In particular, sevoflurane is used for pediatric anesthesia in clinical practice [16]. To date, the superiority of sevoflurane over isoflurane has not been confirmed in rodents. In a mouse study, no significant differences between isoflurane and sevoflurane in terms of their anesthetic action and cardiorespiratory adverse reaction [6]. In this study, however, sevoflurane mediated rapid anesthetic action compared with isoflurane. Although there were no statistically significant differences, rectal temperature and SPO_2 were shifted stably in sevoflurane compared with isoflurane. As a result, all rats survived sevoflurane anesthesia. Based on these findings, sevoflurane has stable anesthetic action in neonatal rats, with fewer adverse reactions, which indicates it is an appropriate anesthetic agent for neonatal rats.

The present study aimed to determine the appropriate anesthetic protocol for anesthesia in neonatal rats. K/X should not be used as neonatal anesthetic owing to its narrow safety margin. When used at the mouse dose, M/M/B was applicable to neonates, whereas the adult rat dose resulted in insufficient anesthetic depth. Isoflurane was not recommended because of its slow anesthetic action and relatively high mortality rate. In contrast, sevoflurane provided sufficient anesthetic depth with rapid induction and recovery and did not cause severe cardiorespiratory depression. Therefore, M/M/B at an equivalent dose for adult mice and sevoflurane were recommended as potential surgical anesthetics for 10-day-old rats. Overall, 10-day-old neonatal rats showed lower drug sensitivity to anesthetic agents, except for K/X. Because neuronal systems develop even

after birth, neuronal function, such as pain perception, is immature in neonates, and requires a certain period of time to fully develop. Previous reports have indicated that the number and distribution of opioid receptors in neonates was quite different to that in adult rats [10]. Another report suggested that 2 weeks are required to achieve normal pain reaction in rats after birth [2]. Therefore, the development process of neuronal systems may have affected the anesthetic sensitivity in this study. Based on the present study, further studies are warranted to explore the appropriate anesthesia regimes for rats of different ages in the neonatal period. Notably, only K/X showed high anesthetic sensitivity with narrow range of margin. Ketamine is unique anesthetic agent, classified as dissociative anesthetic agent, which has fewer affinity on the cerebral cortex [3]. The difference in receptor affinity may be associated with this difference. Ketamine mainly functions as an antagonist of the N-methyl-D-aspartate (NMDA) receptor, while inhalation anesthesia and benzodiazepines mainly act on the gamma-aminobutyric acid (GABA) receptor [3, 27]. Including in human medicine, the exact mechanism of general anesthetics has not been fully clarified. Further studies are warranted to investigate these differences in anesthetic sensitivity and their underlying mechanism. In summary, the validity of anesthetics in 10-day-old rats was evaluated. The data demonstrated the appropriate anesthesia in neonatal rats, centered on laboratory animal welfare-based experimentation.

Acknowledgment

This work was partly supported by a Grant-in-Aid for Young Scientists (B).

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