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ORIGINAL ARTICLE

THE CARDIORESPIRATORY RESPONSES TO INHALATION AND PENTOBARBITAL ANESTHESIA IN THE MOUSE

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Abstract Transgenic mice experiments have become increasingly popular to research human inherited disease. However, a number of Japanese researchers have difficulty with the selection of anesthesia, after the classification of ketamine, probably the most used anesthesia, as a narcotic drug in 2006. Therefore, we compared the effects of inhalation anesthesia (2% of isoflurane, sevoflurane and enflurane) and intraperitoneal pentobarbital anesthesia (50 mg/kg) on the electrocardiogram (ECG) and blood oxygen saturation (SPO_2) of mice. With inhalation anesthesia, the heart rate (HR) and SPO_2 were within an acceptable range. In contrast, the HR significantly decreased after initiation of pentobarbital anesthesia, and gradually returned to a low rate. Importantly, pentobarbital anesthesia significantly lowered SPO_2, and heart rate variability analysis showed unstable beat-to-beat intervals during pentobarbital anesthesia, suggesting that inhalation anesthesia is more suitable for evaluation of cardiorespiratory responses than pentobarbital anesthesia. During anesthesia, propranolol, a β-adrenergic blocker, significantly decreased heart rate. Atropine, a parasympathetic blocker, also significantly increased heart rate. Our data suggest that inhalation anesthesia is suitable for cardiorespiratory analysis in mice.

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Key words: Inhalation isoflurane anesthesia; pentobarbital; hemodynamic status; blood gas.

Introduction

Pharmacological animal studies require reliable general anesthesia methods. Transgenic mice have become increasingly popular for human inherited cardiac disease research and electrophysiological studies have been performed in transgenic mice to characterize the electrical phenotype of the heart. However, little is known regarding the impact of experimental conditions or model selection on the outcome of electrophysiological studies in mice.

The type of anesthetic used varies depending on the type of experiment. Some anesthetics have cardioprotective effects, which may be relevant in designing ischemia/perfusion protocols[1, 2)]. Several studies have examined the influence of commonly used anesthetics on the short-term and non-invasive assessment of cardiac function with echocardiography[3, 4)]. Zuurbier *et al.* reported that at similar surgical levels of anesthesia, the preferred anesthetic (isoflurane or ketamine–medetomidine–atropine) depends on the mouse strain used and whether

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monitoring of blood pressure or heart rate (HR) is the focus of the study[5].

Sodium pentobarbital and ketamine/xylazine have been widely used in mice[6], but inhalation anesthetic agents such as halothane have significant advantages[7]. Isoflurane anesthesia has a slight effect on the hemodynamic status of mice when compared with other injectable anesthetics, such as pentobarbital and ketamine/xylazine[7, 8]. Therefore, in animal experiments, it is important to consider the use of inhalation anesthesia.

The use of isoflurane and injectable anesthetic combinations such as ketamine/xylazine has increased[9]; however, since ketamine was classified as a narcotic drug in 2006, its use in Japan has decreased significantly. Therefore, it is necessary to evaluate cardiorespiratory responses to inhalation anesthesia and other injectable anesthesia, such as pentobarbital, which has been widely used after 2006 in mice in Japan.

In this study, we evaluated the cardiorespiratory responses of mice during intraperitoneal pentobarbital anesthesia or inhalation anesthesia (isoflurane, sevoflurane and enflurane). The data obtained in this study could provide a basis for anesthetic regimens in future pharmacological studies.

Materials And Methods

This study was performed in accordance with the institutional guidelines of Hirosaki University (Hirosaki, Japan) and was approved by the Animal Care and Use Committee. Institute of Cancer Research (ICR) mice, one of the most commonly used mouse lines in Japan, were purchased from Japan SLC Inc. (Hamamatsu, Japan) and housed under standard laboratory conditions. Ten male ICR mice (12–16 weeks-old) weighing 32 ± 1 g were used.

General anesthesia

In the inhalation group, anesthesia was induced by placing the mice in an anesthesia induction chamber (15 × 15 × 7 cm) containing 2% isoflurane (Forane; Abbott Japan Co., Ltd., Tokyo, Japan) and room air. Anesthesia was maintained for 45 min (anesthetic maintenance state) using 2% inhalation anesthesia. In a preliminary experiment, the dose of pentobarbital causing disappearance of the righting reflex for > 30 min was 50 mg/kg. Thus, for pentobarbital anesthesia, the ICR mice were anesthetized intraperitoneally with 50 mg/kg sodium pentobarbital (Nembutal; Dainippon-Sumitomo Seiyaku Co., Ltd., Tokyo, Japan). The time disappearance of the righting reflex for 50 mg/kg pentobarbital anesthesia was 40.2 ± 6.2 min in these animals. All experiments were conducted from 10:00 a.m. to 4:00 p.m.

Evaluation of heart rate and SPO_2

An electrocardiogram (ECG) recording (lead I) and calculation of HR, R-R interval, and SPO_2 were simultaneously measured (ML846 Power Lab system, and MLT SPO_2 sensor, AD Instruments, Dunedin, New Zealand). HR and other ECG parameters were evaluated with the manufacture's program. Standard deviation of the R-R interval (SDNN) was measured as HR variability, which was considered an indicator of cardiac vagal control[10]. For pharmacological analysis, mice were administered either atropine (1.0 mg/kg) for parasympathetic blockade or propranolol (1.0 mg/kg) for sympathetic blockade.

Statistical analysis

The results are expressed as means ± standard error (S.E.). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnet's *t*-test, and p values < 0.05 were considered to indicate

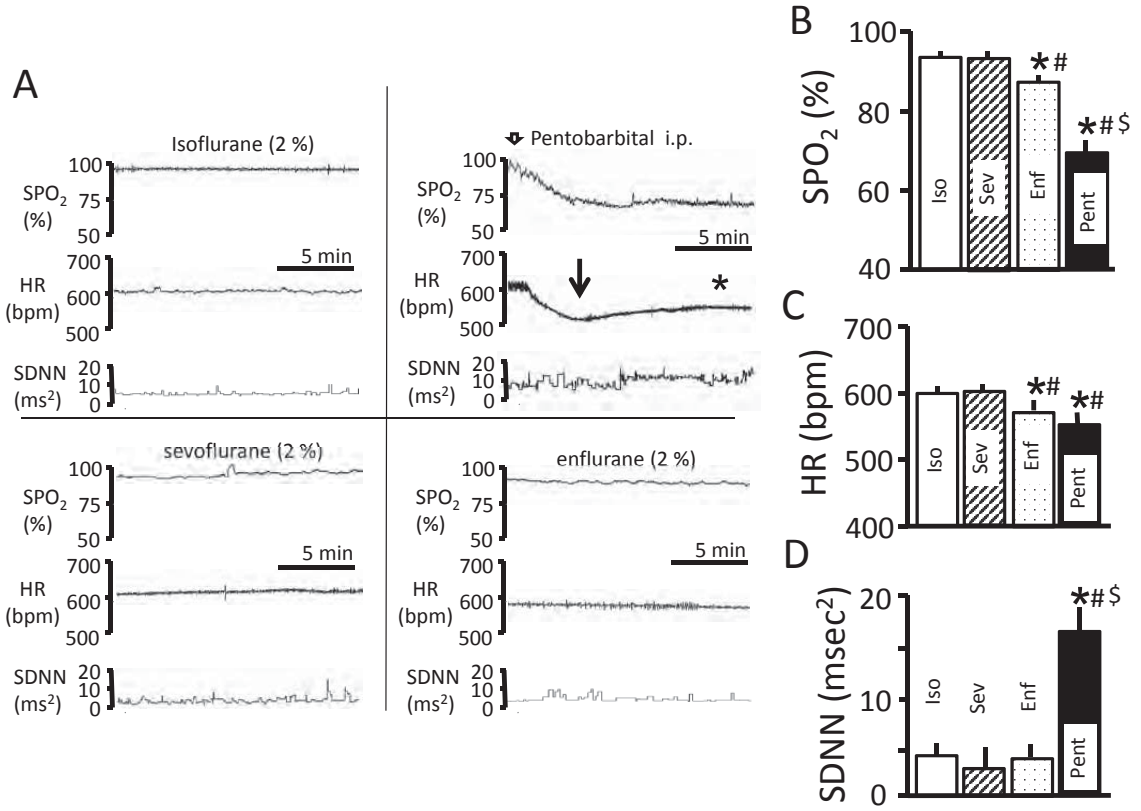

Figure 1 (A) Representative cardiorespiratory tracing during 2% isoflurane, sevoflurane, and enflurane, and pentobarbital administration. The anesthesias used for each recording are indicated. After intraperitoneal injection of pentobarbital (upper panel), SPO_2 and heart rate (HR) decreased significantly. The calculated R-R interval and SDNN (standard deviation of the R-R interval) are shown.
Statistical analysis of SPO_2 (B), heart rate (HR) (C), and SDNN (D) under gaseous isoflurane (Iso), gaseous sevoflurane (Sev), gaseous enflurane (Enf), or pentobarbital (Pent). Pentobarbital injection significantly decreased SPO_2 and HR, and significantly increased SDNN. $^{*}P < 0.05$ indicates significant differences between isoflurane and other modes of anesthesia. $^{\#}P < 0.05$ indicates significant differences between sevoflurane and enflurane or pentobarbital. $^{\$}P < 0.05$ indicates significant differences between enflurane and pentobarbital.

significant differences.

Results

Typical changes in SPO_2, HR, and standard deviation of R-R intervals (SDNN) during 2% isoflurane, sevoflurane, and enflurane, and intraperitoneal injection of pentobarbital (50 mg/kg body weight) are shown in Figure 1A. Both SPO_2 and heart rate remained stable during inhalation anesthesia (isoflurane, sevoflurane and enflurane). In contrast, pentobarbital injection resulted in an initial decrease in the HR (arrow head), followed by recovery (asterisk), but remained low. Pentobarbital significantly decreased SPO_2 saturation. Heart rate variability (SDNN) was also increased in response to pentobarbital, suggesting unstable pace-making.

Statistical analysis indicated a relatively high SPO_2 percentage with isoflurane or sevoflurane anesthesia, but enflurane decreased SPO_2 saturation (86.0 ± 1.3%, n = 7) and pentobarbital injection significantly decreased SPO_2 saturation (69.5 ± 2.4 %, n = 6) (Figure 1B). Isoflurane anesthesia resulted in higher respiratory rate (68.7 ± 1.7/min, n = 6) than that of pentobarbital anesthesia (33.5 ± 2.3/min*, n = 6, *p<0.05 vs. isoflurane anesthesia). Compared

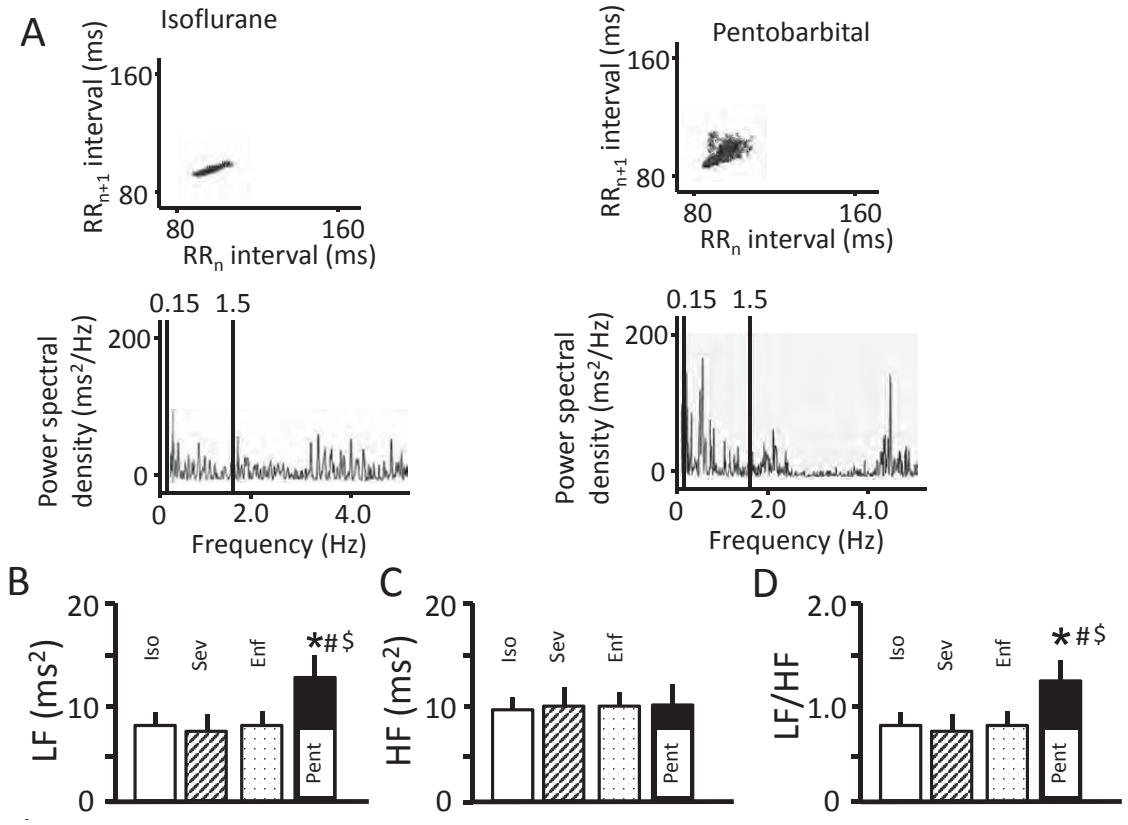

Figure 2 (A) Representative heart rate variability analysis during 2% isoflurane (left) and pentobarbital (right) administration. Poincaré plots (RR *n* vs. RR *n* +1) in which consecutive pairs of RR intervals during the control period are graphed with the *n* th+1 RR interval plotted against the *n* th RR period (upper panels). Representative power spectral densities (lower panels) during 2% isoflurane (left) and pentobarbital (right) administration.
Statistical comparison of LF (B), HF (C) and LF/HF ratio (D) under gaseous isoflurane (Iso), gaseous sevoflurane (Sev), gaseous enflurane (Enf), or pentobarbital (Pent). $^{*}P < 0.05$ indicates significant differences between isoflurane and other modes of anesthesia. $^{\#}P < 0.05$ indicates significant differences between sevoflurane and enflurane or pentobarbital. $^{\$}P < 0.05$ indicates significant differences between enflurane and pentobarbital.

with the SPO_2 under isoflurane or sevoflurane anesthesia, enflurane and pentobarbital anesthesia decreased SPO_2 significantly.

HR changes due to different anesthesia protocols showed similar results, whereas the differences were small. A relatively high HR resulted from isoflurane or sevoflurane anesthesia, but enflurane decreased HR (576 ± 5.2 bpm, n = 7). Pentobarbital injection decreased HR significantly (555 ± 7.0 bpm, n = 6) (Figure 1C). A dose of 50 mg/kg pentobarbital increased SDNN, while other inhalation anesthesia protocols resulted in limited changes (Figure 1D).

Since isoflurane anesthesia is the most commonly used, we further analyzed ECG parameters with isoflurane and pentobarbital anesthesia. Isoflurane anesthesia showed shorter PQ interval than that of pentobarbital anesthesia (35.4 ± 1.2 ms, n = 6, and 40.0 ± 0.8* ms, n = 6, isoflurane and pentobarbital, respectively, *p< 0.05 vs. isoflurane).There was no significant difference in QRS interval (10.7 ± 0.7 ms, n = 6, and 10.9 ± 0.6 ms, n = 6, isoflurane and pentobarbital, respectively). Pentobarbital anesthesia resulted in prolonged QT duration (21.8 ± 0.7 ms, n = 6, and 27.0 ± 1.0* ms, n = 6, isoflurane and pentobarbital, respectively, *p< 0.05 vs. isoflurane). No arrhythmia or ischemia

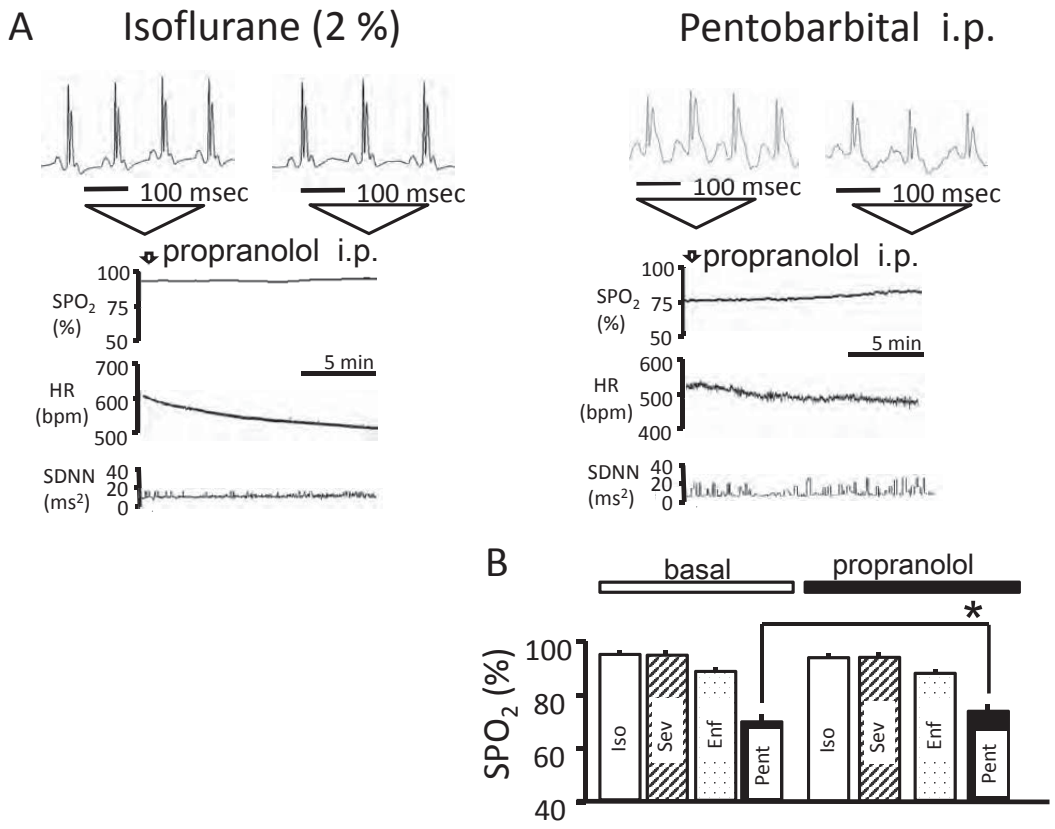

Figure 3 (A) Representative ECG recordings, SPO_2 values, and pharmacological effects of propranolol during isoflurane (left) and pentobarbital (right) administration. In both cases, intraperitoneal injection of propranolol (1 mg/kg body weight) significantly decreased heart rate (HR). The calculated SDNN (standard deviation of the R-R interval) values show that pentobarbital anesthesia increased SDNN more than did isoflurane anesthesia. ECG recordings before and 10 min after the administration of propranolol are shown (upper panels).
(B) Statistical analysis of SPO_2 after propranolol injection under gaseous isoflurane (Iso), gaseous sevoflurane (Sev), gaseous enflurane (Enf), or pentobarbital (Pent). The SPO_2 value with pentobarbital anesthesia resulted in lower SPO_2 values. $^*P < 0.05$ indicates significant differences between basal and propranolol injection.

was observed in either inhalation anesthesia (isoflurane, sevoflurane, or enflurane), or pentobarbital injection.

Heart rate variability changes

We further analyzed heart rate variability during the aforementioned four anesthesias. Figure 2A shows typical results of beat-to-beat dynamics with Poincaré plots (RR_n vs. RR_{n+1}). Pentobarbital anesthesia showed fluctuated changes in beat-to-beat dynamics. In the frequency domain analysis, LF (0.15 – 1.5 Hz) and HF (1.5 – 5 Hz) components were resolved in power spectral density (lower panels). Statistical analysis of LF components resulted in significant increase in pentobarbital anesthesia (B). HF components showed no significant differences in any of the four groups (C). As expected from LF component, pentobarbital showed higher LF/HF ratio than inhalation anesthesias (D).

Sympathetic blockade

We analyzed the pharmacological response to sympathetic blockade with propranolol, a typical adrenergic β-blocker, under isoflurane or pentobarbital anesthesia. Propranolol (1.0 mg/kg body weight) resulted in decreased HR in

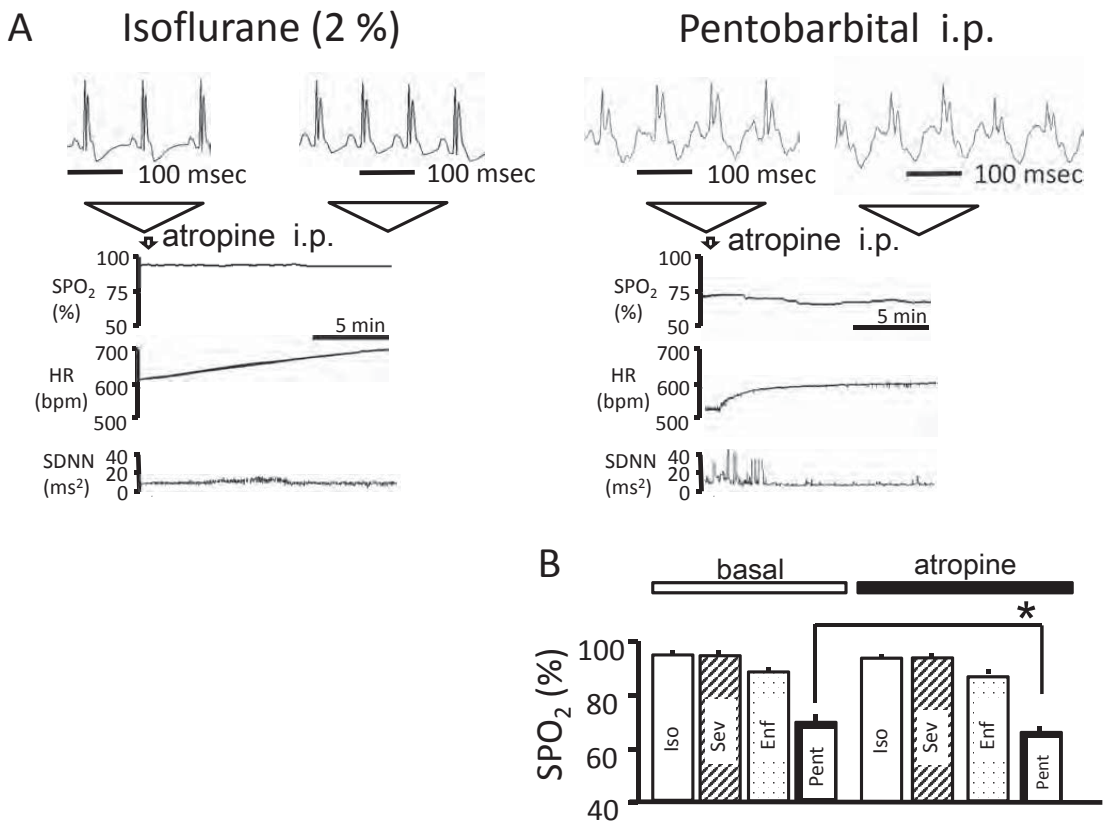

Figure 4 (A) Representative ECG recordings, SPO_2 values, and pharmacological effects of atropine during isoflurane (left) and pentobarbital (right) administration. In both cases, intraperitoneal injection of propranolol (1 mg/kg body weight) significantly decreased heart rate (HR). The calculated SDNN values show that pentobarbital anesthesia increased SDNN more than did isoflurane anesthesia. ECG recordings before and 10 min after the administration of atropine are shown (upper panels).
(B) Statistical analysis of SPO_2 after atropine injection under gaseous isoflurane (Iso), gaseous sevoflurane (Sev), gaseous enflurane (Enf), or pentobarbital (Pent). The SPO_2 value with pentobarbital anesthesia resulted in lower SPO_2 values. * < 0.05 indicates significant differences between basal and atropine injection.

an isoflurane-anesthetized mouse (Figure 3A left), while pentobarbital anesthesia resulted in decreased changes in HR (Figure 3A right), indicative of reduced responses to propranolol. Propranolol slightly but significantly increased SPO_2 in pentobarbital anesthesia from (69.5 ± 2.4 %, n = 6) to (73.2 ± 1.9%*, n = 6, *p<0.05 vs. basal stage), while no significant effect was observed with inhalation anesthesia (Figure 3B). Additionally, pentobarbital anesthesia resulted in a high SDNN, suggesting that the unstable HR under pentobarbital anesthesia may be related to cardiac vagal tone.

Parasympathetic blockade

A representative recording in response to atropine, a typical muscarinic receptor antagonist, is shown in Figure 3A. Intraperitoneal injection of atropine (1.0 mg/kg body weight) increased HR. Pentobarbital anesthesia resulted in decreased changes in HR (Figure 4A right), indicating reduced responses to parasympathetic blockade. Although the effect was small, atropine slightly but significantly decreased SPO_2 in pentobarbital anesthesia from (69.5 ± 2.4%, n = 6) to (67.4 ± 1.3%*, n = 6, *p<0.05 vs. basal stage), while no significant effect was observed with inhalation anesthesia (Figure 4B). Atropine injection

reduced SDNN in pentobarbital-anesthetized mice, suggesting reduced vagal tone.

Discussion

Pentobarbital is a commonly used injectable anesthesia in rodents[12], and isoflurane inhalation has been useful in mice[7]. We investigated the cardiorespiratory effects of inhalation (2% of isoflurane, sevoflurane and enflurane) and pentobarbital anesthesia. Our present data showed acceptable HR, SDNN and SPO_2 during inhalation anesthesia, while pentobarbital anesthesia decreased HR and SPO_2. These results are consistent with the report by Mastuda *et al.* that neither hypoxia nor hypercapnia occurred during isoflurane anesthesia with a ventilator system[13]. They also showed increased acidosis of the blood and a significant increase in $PaCO_2$ due to pentobarbital anesthesia. Further, the data obtained in isoflurane-anesthetized mice were sufficient for interpreting the results of pharmacological manipulation with an adrenergic β-blocker or a muscarinic antagonist. In the present study, we observed significant SPO_2 changes during pentobarbital anesthesia. Although significant changes were observed, we have to take into account that the experimental conditions during pentobarbital anesthesia was artificial, considering the low O_2 saturation. Therefore, our present results indicate that isoflurane anesthesia is suitable for pharmacological evaluation of the hemodynamic status of mice.

Small animals, particularly mice with genetic alterations, are often used to evaluate the physiological importance of a single gene in models of human disease. Anesthesia is often required for experimental interventions and phenotypic evaluations in transgenic mice. A variety of anesthetic regimens has been used depending on strain differences, previous experience, and institutional regulations[14]. Inhalation anesthesia requires specialized apparatus and is used in only a limited number of laboratories. Experimentally, injection anesthesia, such as pentobarbital, is more commonly used even though controlling the depth of anesthesia is considerably easier with inhaled than with injected anesthetics. However, during pentobarbital anesthesia we found a significant decrease in HR, similar to a report by Janssen *et al.*, which could be related to the direct negative inotropic effects of pentobarbital[7]. It reported that isoflurane has less effect on systemic hemodynamics than pentobarbital anesthetics in mice[7]. Szczesny *et al.* also reported that isoflurane anesthesia is useful for experimental studies on mice due to simple administration, rapid anesthesia induction, easy control of anesthesia depth, low incidence of complications, and stable blood pressure and HR for a long period of time[15].

However, The well-known parasympatholytic effect of pentobarbital may also be related to the decrease in heart rate[16]. Nevertheless, comparative study including different kinds of inhalation anesthesia (isoflurane, sevoflurane and enflurane) and pentobarbital has never been well characterized concerning ECG and related heart rate variability.

In the present study, we used 2% for all three kinds of inhalation anesthesia, although minimum alveolar concentration (MAC), a standard estimate of anesthetic potency, is different. MAC in general anesthesia is ca.1.4% for isoflurane, ca.1.7% for sevoflurane, and ca.2.0% for enflurane in mice[17-19]. As Zuurbier *et al.*, used 2% isoflurane, which has the smallest MAC, we decided to use 2% for all inhalation anesthesias, which might affect heart rates and ECG recordings[5]. Nevertheless, all inhalation anesthesias resulted in higher heart rate and SPO_2 values than those of pentobarbital injection.

Appleton *et al.* reported that an anesthetic mixture of ketamine, xylazine, and acepromazine exerted greater effects on cardiac electrophysiological factors compared to pentobarbital or isoflurane[20]. In this study, we demonstrated that the cardiorespiratory state (HR, SDNN and SPO_2) was more stable with isoflurane anesthesia than with pentobarbital anesthesia. During the experiments, it was necessary to consider that the depth of anesthesia may also affect factors such as plasma cortisol and catecholamine levels. Thus, our results reveal the importance of selecting an appropriate anesthesia and its suitable dosage.

Zeller *et al.* reported that the anesthetic actions of barbiturates *in vivo* are mediated primarily by $GABA_A$ receptors[11]. Their results demonstrated that pentobarbital-induced increases in HR variability and prolongation of R-R intervals are not dependent on β3-containing $GABA_A$ receptors, suggesting that pentobarbital has several pharmacological targets. Pentobarbital anesthesia (60 mg/kg body weight) resulted in significantly reduced PaO_2 (53 ± 7 mmHg), which correlates with our present results. They also measured HR changes due to pentobarbital anesthesia and found that HR decreased from 620 ± 53 bpm to 220 ± 17 bpm (65% reduction). In the present study, the HR decrease induced by anesthesia was significantly less in inhalation-anesthetized mice than in pentobarbital-anesthetized mice (Figure 1). Furthermore, Zeller *et al.*, measured HR variability as the standard deviation of the interbeat interval and intraperitoneal injection of pentobarbital increased SDNN of the R-R interval from (conscious baseline state; 5.2 ± 0.2 $msec^2$) to (pentobarbital anesthesia; 40.4 ± 5.8 $msec^2$), while we observed a more limited effect in this study (pentobarbital anesthesia; 16.9 ± 4.9, $msec^2$). These differences might be due to differences in the doses of pentobarbital (60 or 50 mg/kg body weight) used or other experimental conditions. Nevertheless, isoflurane anesthesia resulted in acceptable HR variability (4.7 ± 1.2 $msec^2$), suggesting that inhalation anesthesia is suitable for evaluating cardiovascular parameters.

In summary, pentobarbital anesthesia decreased HR and SPO_2, while inhalation anesthesia resulted in acceptable HR, SDNN and SPO_2. These results indicate the importance of selecting an anesthesia methodology compatible with the objective of pharmacological studies and support the use of isoflurane inhalation for general anesthesia in mice.

Acknowledgement

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