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Diluted Isoflurane as a Suitable Alternative for Diethyl ether for Rat Anaesthesia in Regular Toxicology Studies

Toshiaki NAGATE[1)]*, Tomonobu CHINO[1)], Chizuru NISHIYAMA[1)], Daisuke OKUHARA[1)], Toru TAHARA[1)], Yoshimasa MARUYAMA[1)], Hiroko KASAHARA[1)], Kayoko TAKASHIMA[1)], Sayaka KOBAYASHI[1)], Yoshiyuki MOTOKAWA[1)], Shin-ichi MUTO[1)] and Junji KURODA[1)]

[1)]*Toxicology Research Laboratory, R&D, Kissei Pharmaceutical Co., Ltd., 2320–1 Maki, Hotaka, Azumino-City, Nagano-Pref. 399–8305, Japan*

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ABSTRACT. Despite its explosive properties and toxicity to both animals and humans, diethyl ether is an agent long used in Japan in the anaesthesia jar method of rat anaesthetises. However, in response to a recent report from the Science Council of Japan condemning diethyl ether as acceptable practice, we searched for an alternative rat anaesthesia method that provided data continuous with pre-existing regular toxicology studies already conducted under diethyl ether anaesthesia. For this, we examined two candidates; 30% isoflurane diluted with propylene glycol and pentobarbitone. Whereas isoflurane is considered to be one of the representatives of modern volatile anaesthetics, the method of propylene glycol-diluted 30% isoflurane used in this study was our modification of a recently reported method revealed to have several advantages as an inhalation anaesthesia. Intraperitoneal pentobarbitone has long been accepted as a humane method in laboratory animal anaesthesiology. These 2 modalities were scrutinized in terms of consistency of haematology and blood chemistry with previous results using ether. We found that pentobarbitone required a much longer induction time than diethyl ether, which is suspected to be the cause of fluctuations in several haematological and blood chemical results. Conversely, only calcium ion concentration showed a slight difference from traditional results in the case of 30% isoflurane. Additionally, serum prolactin and corticosterone levels indicated that 30% isoflurane induced less stress than ether, confirming that 30% isoflurane can both provide results consistent with diethyl ether, while at the same time remove its disadvantages. As such 30% isoflurane appears to be a strong alternative anaesthetic agent for future regular toxicology studies in Japan.

KEY WORDS: anaesthesia, diethyl ether, euthanasia, isoflurane, pentobarbitone.

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On June 1, 2006, the Science Council of Japan submitted its "Guideline for the Appropriate Implementation of Animal Testing", which was intended to provide a standard of voluntary regulation about the domestic care and use of laboratory animals. This included for the first time a series of initiatives to halt the use of widespread diethyl ether (ether) in Japanese laboratories. Ether has long been phased out in other countries for its use in animal experiments due to its explosive properties and toxicity to both animals and humans, and carcasses of animals euthanized by ether require special storage, handling, and disposal, because ether fumes retained in their bodies may lead to unexpected disasters [2]. Stimulation of the airway leads to coughing and hyper-secretion of mucus [11], and electron microscopic studies have revealed that the permeability of tight junctions in the tracheal epithelia increases following ether exposure [14]. Ether alters various endocrinological parameters [7, 12], which indicates severe stress in the animals. In fact, recent reports and literature are in agreement that ether anaesthesia/euthanasia should be kept away from animal testing [2, 11]. Beyond these physical matters, what should be discussed more is the ethical concern of applying such a possibly unsafe and non-pharmaceutical agent on laboratory animals. Criticism is inevitable if agents like ether, which are not used on humans, are used in animals.

In spite of these data, ether is still used in animal anaesthesia and euthanasia in Japanese laboratories [10, 15, 21, 23]. There is a huge amount of historical data from toxicology studies that were conducted under ether anaesthesia. Collectively, these findings have had tremendous benefit by revealing toxic properties in numerous compounds, many of which are still present today in drugs, food additives, and cosmetics. Thus, it is required that when shifting to a new method of anaesthesia/euthanasia in similar studies, data migration should be mitigated for the least impact possible to maintain continuity throughout these studies. However, ether itself is known to affect the physiological parameters of laboratory animals to an unignorable extent [3, 13]. As such, departure from ether is embracing the contradiction that if animal stress is tightly related to the fluctuation of parameters, it might be difficult to remove only the stress while keeping continuous data.

The purpose of this study was to investigate, select and propose to the public a suitable alternative for ether anaesthesia so as to give scientific evidence that ether is inconspicuously replaceable in regular toxicology studies without facility investment, such as for a precision vaporizer. For this, 2 candidates were selected: isoflurane inhalation by the jar method and intraperitoneal pentobarbitone. Isoflurane is one of the representatives of volatile modern clinical anaes-

* CORRESPONDENCE TO: NAGATE, T., Toxicology Research Laboratories, R&D, Kissei Pharmaceutical Co., Ltd., 2320–1, Maki, Hotaka, Azumino-City, Nagano-Pref. 399–8305, Japan.
e-mail: toshiaki_nagate@pharm.kissei.co.jp

thetics, and recently Itah and colleagues have reported that isoflurane diluted in propylene glycol lowers its partial vapour pressure and eases control of the anaesthetic state of the ICR mouse in an open-circuit anaesthesia system [16]. They used a one-litre glass beaker covered with an aluminium foil lid, and attached to that lid was a piece of cotton gauze soaked by the reagent. This process was modified in the present study for the Sprague-Dawley rat. The other ether-substituting candidate was pentobarbitone, which used to be a well-known, useful, and commonly recommended agent for laboratory animal anaesthesia/euthanasia [2]. Indeed, various comparison studies have been done to point out the fundamental differences and diverse outcomes between pentobarbitone and ether in many aspects. For example, ether, but not pentobarbitone, increases fasting plasma insulin [1] and also elevates hypothalamic LH-RH [20]. The two agents produced opposite effects on hypothalamus monoamine synthesis [18], however, evaluation of discrepancies in terms of regular toxicology studies has yet to be done.

In choosing a substitute for ether, we sought to minimize the differences in application and effect, as well as the handling-induced stress response. For this purpose, we compared a) the transition of the anaesthetic state, particularly in terms of handling efficiency; b) the stress-causing nature; and c) the continuity of haematological and blood chemical data, which can be compared to the results of regular ether toxicology studies.

MATERIALS AND METHODS

Animals and husbandry: Sixty Male Sprague-Dawley rats (Crj:CD(SD)IGS), aged 5 weeks and weighing between 100 and 160 grams , were obtained from Charles River (Kanagawa, Japan) in specific pathogen-free status. All animals were housed in pairs in flat bottom cages (300 × 355 × 175 mm; CLEA, Tokyo, Japan), and nested in sterile spruce chip beddings (Charles River). Room temperature was maintained at 22.5–23.5°C, humidity 42.5–65.2%, lighting schedule 8:00–20:00, and ventilation cycles 10–25 (HEPA-filtered air). Animals were fed γ-ray irradiated commercial food (CE-2; CLEA) and given filtered and UV-disinfected municipal tap water *ad libitum*. After one week of quarantine, the 6-week old animals underwent experiments. Every experimental procedure was inspected and approved by the institutional animal care and use committee of Toxicology Research Laboratories of Kissei Pharmaceutical Co., Ltd.

Anaesthesia: Animals were anaesthetized with the following reagents: isoflurane from Abbott (Tokyo, Japan); diethyl ether from Wako Pure Chemical (Osaka, Japan); pentobarbitone from Dainippon Pharmaceutical (Osaka, Japan); and propylene glycol (PG) from Nakalai Tesque (Kyoto, Japan). Preliminary studies were conducted to investigate optimal isoflurane dilution (see results). The isoflurane solution was prepared in an opaque bottle by adding designated amounts of isoflurane to PG, followed by thorough mixing by gentle shaking of the bottle. Animals were anaesthetized in transparent glass jars. As a nose cone, 50 m*l* centrifuge tubes inserted with cotton were used. For isoflurane, 1 m*l* volume of mixture was used for every 200 m*l* volume of jar (also determined in preliminary studies). For ether, 1 m*l* volume of mixture was used for every 20 m*l* volume of jar. Both dosages were doubled by the use of the nose cone. Pentobarbitone was injected intraperitoneally at 50 mg per kilogram of body weight.

Time course: Induction and recovery times were measured by two experiments (Fig. 1). In the single-dose anaesthesia study, animals were observed for their transition to just enough anaesthesia to reach the surgical plane without making any efforts to maintain the anaesthetic state. In the duration anaesthesia study, which was done only with ether and isoflurane, a 15 min anaesthetic state was maintained by the nose cone. Disappearance of pedal reflex and reappearance of righting reflex were considered to represent achievement of surgical plane and recovery to conscious state, respectively.

Blood sampling and necropsy: Ten millilitres of blood were collected from the abdominal aorta under deep anaesthesia for the three reagents, followed by euthanasia by exsanguination. Every organ underwent careful inspection.

Haematology and blood chemistry: The following haematological items were measured by an automated haematology analyser ADVIA120 system and software suitable for rat specimens (Bayer-Medical, Tokyo, Japan): erythrocytes (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelets, leucocytes (WBC), neutrophils (NEUT), lymphocytes (LYMP), monocytes (MONO), eosinophils (EOS), and basophils (BASO). Reticulocytes (RETIC) were counted microscopically. Prothrombin times (PT), activated partial thromboplastin times (APTT), and fibrinogen levels (FBG) were measured by an AMAX blood coagulation analyser (Heinrich Amelung, Lemgo, Germany).

Blood chemical items were measured by an automatic analyser Model 7150 (Hitachi, Tokyo, Japan) for each method: total protein (TP) by the Biuret method; albumin (ALB) by the bromocresol green method; aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) enzyme activities by the JSCC method; creatine phosphokinase (CPK) activities by the GSCC method; glucose (GLU) by the hexokinase method; total cholesterol (CHO) by the enzyme method; triglycerides (TG) by the free glycerol elimination method; urea nitrogen (UN) by the urease-GLDH method; creatinine (CRE) by Jaffé's method; calcium (CA) by the *o*-cresolphthalein-complexion method; and inorganic phosphorus (IP) by the Fiske-Subbarow method. Sodium (NA) and potassium (K) ions were measured by the Ion-selective electrode method using a SERA-720 electrolyte analyser (Horiba, Kyoto, Japan), and chloride (CL) ions were measured by the coulometric titration method using a CL-6 chloride counter (Hiranuma, Ibaraki, Japan).

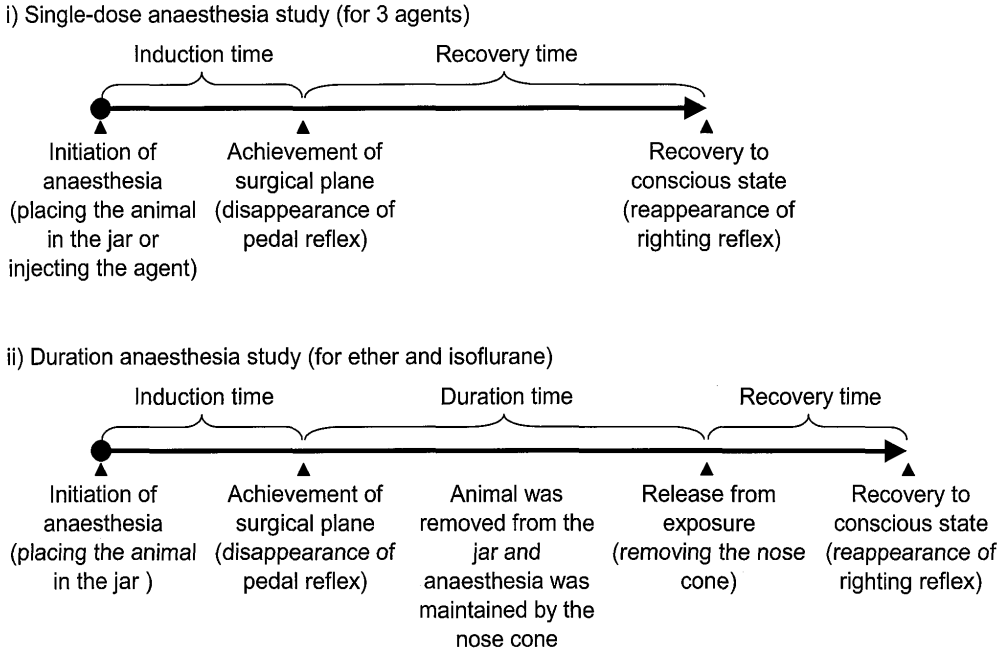

Fig. 1. Schematics of time course experiments.

Endocrinology: Serum levels of adrenocorticotropic hormone (ACTH), corticosterone, and prolactin were measured using commercially available enzyme immunoassay (EIA) kits; Rat ACTH EIA KIT (Phoenix Pharmaceuticals; CA, U.S.A.), Corticosterone Immunoassay (R&D Systems CA, U.S.A.), and Rat Prolactin EIA (American Laboratory Products Company, NH, U.S.A.).

Statistics: Data were first analyzed by the Bartlett test to evaluate variance homogeneity. If homogenous, a multiple-comparison test with Dunnett's method was applied. If variance was heterogeneous, the Kruskal-Wallis test with Scheffe's method was performed (differences between pentobarbitone and isoflurane were disregarded). A P value of less than 0.05 was considered statistically significant.

RESULTS

Preliminary dose-finding study: To adjust the methods from mice, as reported by Itah *et al.* [16], to rats, a preliminary dose-finding study was conducted. Rats were exposed to 10%, 20%, 30% or 40% isoflurane diluted in propylene glycol, which revealed that 30% was optimistic in terms of anaesthesia control and induction periods.

Time course: To evaluate the differences in transition to an anaesthetic state and procedure efficiency, induction and recovery times were measured for the 3 agents. For induction in the two time course studies, pentobarbitone and isoflurane showed elongations of approximately 4 min and 10 sec from ether, respectively (Fig. 2A). Recovery time in the single-dose anaesthesia study revealed no differences between ether and isoflurane, while pentobarbitone showed an elongated plateau of the surgical plane (Fig. 2B). In duration anaesthesia studies, recovery time with isoflurane was more than 8 min shorter than ether (Fig. 2C).

Haematology and blood chemistry: To evaluate the continuity of outcomes in regular toxicology studies between ether and the two candidates, haematological and blood chemical values, the two sets of items most likely to be influenced by differences in the means of anaesthesia, were measured. Haematologically, no statistical differences were observed between ether and either pentobarbitone or isoflurane (Table 1). Pentobarbitone showed significant differences from ether in 4 of 16 blood chemistry variables: total protein ($P<0.001$), glucose ($P<0.001$), inorganic phosphorus ($P<0.001$), and chloride ion levels ($P<0.01$). Isoflurane showed a difference in calcium level only ($P<0.05$) (Table 2).

Endocrinology: Endocrinological items were measured to explore the stress state of animals undergoing anaesthesia. In serum ACTH measurements, pentobarbitone showed significant suppression compared with ether ($P<0.05$) (Fig. 3A), but significantly increased serum corticosterone levels ($P<0.01$) (Fig. 3B). Isoflurane did not alter the outcome of either of these parameters. Serum prolactin findings revealed significant suppression for both pentobarbitone ($P<0.001$) and isoflurane ($P<0.005$) (Fig. 3C).

Necropsy: To detect any anatomical changes, necropsies were conducted on each animal, revealing no abnormalities in main organs or tissues, especially in the trachea and the lung, which would have been directly exposed to ether and isoflurane.

DISCUSSION

Outside of Japan, ether was long ago replaced by methoxyflurane, which became a common inhalation agent used in

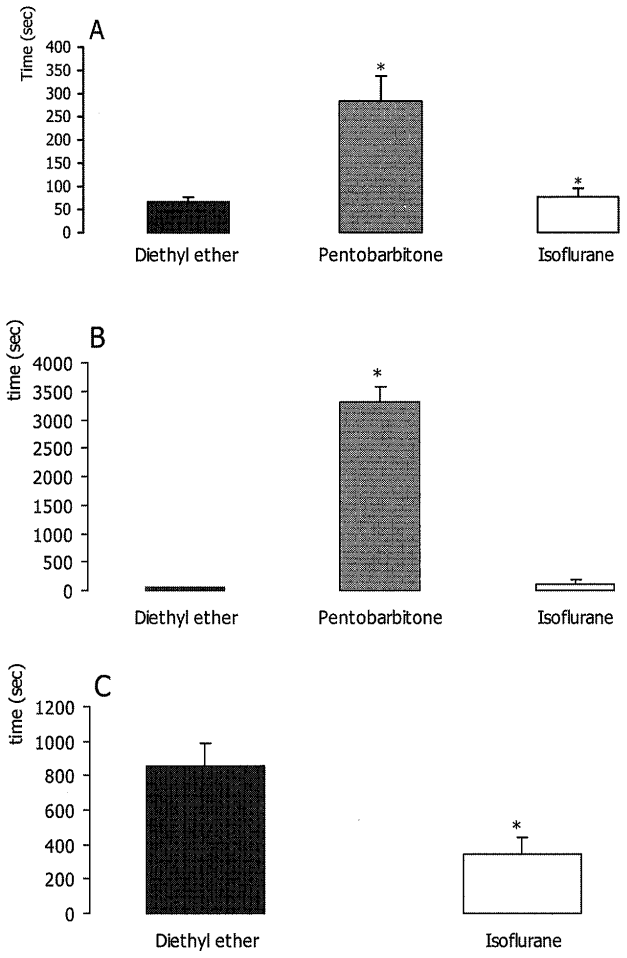

Fig. 2. Results of time course experiments. (A) Mean induction time in the two time course studies. (B) Recovery time in the duration study. (C) Recovery time in the single-dose study. Values are expressed as mean ± SD (n=6). * P<0.05; ** P<0.01.

rodents for many years, since the physical properties of this anaesthetic made its use possible with a minimum of equipment, for example with the anaesthesia jar [27]. However, the same properties found to be hazardous in animals were also found to be toxic to personnel, and the use of this agent was gradually replaced by new anaesthetics, such as halothane, isoflurane and sevoflurane. Still, the 2000 Report of the AVMA Panel on Euthanasia maintains that methoxyflurane (as well as ether) is conditionally acceptable as means of euthanasia for rodents and small mammals [2]. In Japan, domestic guidelines on the restriction of any anaesthetic agent had not been established until recently. Due to the difficulty in importing methoxyflurane, ether has continued to be the standard anaesthetic for laboratory rodents. For euthanasia, barbiturate overdose is the recommended course for almost all animals, which, depending on the administered dose, results in minimal discomfort [2]. Added to this its reasonable cost, and pentobarbitone represents the most likely candidates to replace ether. Isoflurane is an inhaled anaesthetic that is more inert and less toxic. As with other modern volatile anaesthetics, such as halothane or sevoflurane, isoflurane is physically stable and nonflammable because of its highly halogenated structure, and its lower solubility in blood and higher minimal alveolar concentration ensures rapid induction of the subject [8]. However, its relatively high vapour pressure and narrow therapeutic index requires precise control of concentration by refined equipment, meaning that only by modification of the agent to become applicable with minimum equipment may it become a legitimate candidate for ether replacement. Fortunately, a modified jar method was developed for easy administration of isoflurane, resulting in its inclusion in this study as an ether substitute [16]. Previous comparative toxicity studies between ether and modern clinical volatile anaesthetics, including isoflurane, in both human [25] and laboratory animals [26] have revealed that the potencies of the 2 agents are virtually analogous. Here, one of our goals was to extrapolate this consistency in precise haematological and blood chemical terms in *in situ* blood sampling in regular toxicology studies.

In this study, diluted isoflurane using a jar method showed more consistent results with ether in most experiments than intraperitoneal pentobarbitone. Although isoflurane showed a delay in induction time from ether in time course trials, 10 seconds may not significantly alter the handling effectiveness of the procedure. However, an extended lag, such as the 2 min exhibited by pentobarbitone, might cause technical challenges when the number of the animals is large. The differences in recovery times measured in this study agreed with previous literature [11].

Haematological and blood chemical results showed good continuity between ether and isoflurane, revealing only an increase in serum calcium ion concentrations. Part of the reason for this increase may be explained by the calcium-influx-inhibiting property of isoflurane; Simoneau *et al.* [24] and Tas *et al.* [28] showed that isoflurane inhibited bradykinin- or histamine-induced calcium influx in bovine aortic endothelial cells and primary human endothelial cells, respectively. Alternatively, Gärtner *et al.* [13] revealed that blood calcium ion concentration decreases after five minutes following one-minute of ether anaesthesia. Significantly higher prolactin levels were observed in ether-anaesthetized rats, similar to observations made by other authors [17]. Prolactin is a classic indicator of animal stress [22], and coupled to the lack of any other obvious stress, we were able to conclude that isoflurane is less stressful than ether as an anaesthetizing agent.

Pentobarbitone showed significant differences in several blood chemical and endocrine tests compared with ether. Serum glucose levels were prominently higher than ether, as well as than in resting values [19], which is similar to results published by Upton and Morgan [29]. However, ether is known to increase plasma glucose levels [4], and this change is more significant compared with pentobarbitone when it is measured after 15 min exposure in fasted rats [6]. This discrepancy may be explained by the time waited from initial exposure to sample collection, which directly reflects induction time. Glucose levels in ether-stressed rats begin

Table 1. Haematological values of anaesthetized rats

Parameter (unit)	Anaesthetic		
	Diethylether	Pentobarbitone	Isoflurane
RBC (10^{12}/l)	6.49 ±0.22	6.28 ±0.22	6.42 ±0.34
HGB (g/l)	14.1 ±0.4	13.7 ±0.3	14 ±0.5
HCT (l/l)	0.439 ±0.013	0.429 ±0.010	0.436 ±0.014
MCV (fl)	67.6 ±1.9	68.4 ±1.8	68.0 ±2.2
MCH (pg)	21.7 ±0.4	21.8 ±0.7	21.7 ±0.6
MCHC (g/l)	3.22 ±0.06	3.20 ±0.05	3.2 ±0.40
RETIC (%)	8.1 ±0.9	8.8 ±0.9	7.6 ±1.0
Platelets (10^9/l)	1402 ±134	1253 ±105	1295 ±150
WBC (10^9/l)	11.61 ±2.62	9.45 ±1.30	10.68 ±1.82
NEUT (%)	13.8 ±4.0	15.0 ±4.5	12.5 ±2.8
LYMP (%)	80.9 ±5.1	79.7 ±4.8	82.1 ±3.5
MONO (%)	3.7 ±1.1	3.7 ±0.6	3.6 ±1.5
EOS (%)	0.6 ±0.2	0.8 ±0.3	0.8 ±0.2
BASO (%)	0.3 ±0.1	0.3 ±0.1	0.4 ±0.1
PT (sec)	13.7 ±0.5	13.6 ±0.6	13.3 ±0.5
APTT (sec)	16.8 ±0.9	19.4 ±2.4	17.0 ±1.2
FBG (mg/dl)	336 ±30	309 ±22	328 ±28

Values expressed as mean ± SD (n=10).

Table 2. Blood chemical values of anaesthetized rats

Parameter (unit)	Anaesthetic		
	Diethyl ether	Pentobarbitone	Isoflurane
TP (g/dl)	4.9 ±0.1	4.6 ±0.1**	4.9 ±0.1
ALB (g/dl)	2.1 ±0.1	2.0 ±0.1	2.1 ±0.1
AST (U/l)	70 ±5	70 ±6	64 ±5
ALT (U/l)	39 ±7	39 ±7	37 ±3
ALP (U/l)	1187 ±148	1134 ±179	1145 ±184
CPK (U/l)	224 ±55	253 ±50	281 ±71
GLU (mg/dl)	180 ±11	201 ±12**	181 ±16
CHO (mg/dl)	76 ±8	67 ±11	69 ±8
TG (mg/dl)	61 ±20	54 ±20	72 ±16
UN (mg/dl)	13.5 ±1.6	14.6 ±1.8	14.3 ±2.0
CRE (mg/dl)	0.4 ±0.1	0.3 ±0.1	0.3 ±0.1
IP (mg/dl)	10.9 ±0.3	9.9 ±0.6**	10.6 ±0.5
CA (mg/dl)	11.0 ±0.2	10.9 ±0.3	11.3 ±0.2*
NA (mEq/l)	138.8 ±1.1	137.5 ±1.5	138.2 ±1.4
K (mEq/l)	5.10 ±0.18	4.57 ±0.20	5.18 ±0.32
CL (mEq/l)	104 ±2	107 ±3**	104 ±1

Values expressed as mean ± SD (n=10).
**: P<0.01; *: P<0.05 compared with diethyl ether.

to show a significant increase after 3 min, and peak at 6 min [13], meaning pentobarbitone samples were collected near the glucose-peak, while ether and isoflurane were less affected because of the elongated induction time, and as result showed irregularly low levels. This may also support the notion that serum corticosterone levels increase linearly at least until 15 min after the first handling [9]. On the contrary, differences in total protein values were significantly lower in pentobarbitone treated mice, which agrees with Upton and Morgan [29], probably due to dilution of blood by the injected solution since all other erythrocytic parameters were also indicative of haemodilution.

The fact that pentobarbitone reduced serum ACTH agrees with Buckingham [5], who showed that it inhibited the morphine-induced secretion of corticotrophin-releasing-factor by directly affecting the hypothalamus. However, corticosterone levels for pentobarbitone were almost 4-times higher than ether, meaning more research is necessary to measure the relative stress caused by each chemical.

The significant difference seen in chloride ion levels between ether and pentobarbitone can be explained as the effect of using an anaesthesia jar; carbon dioxide expired from rats condensed within the jar, causing bicarbonate ion retention in body fluid and expulsion of chloride ions from the fluid by the mechanism of chloride-bicarbonate shifting. This hypothesis raises another idea that the acid-base status, though not included in this study, differs between ether and pentobarbitone. Lastly, while discrepancies in inorganic

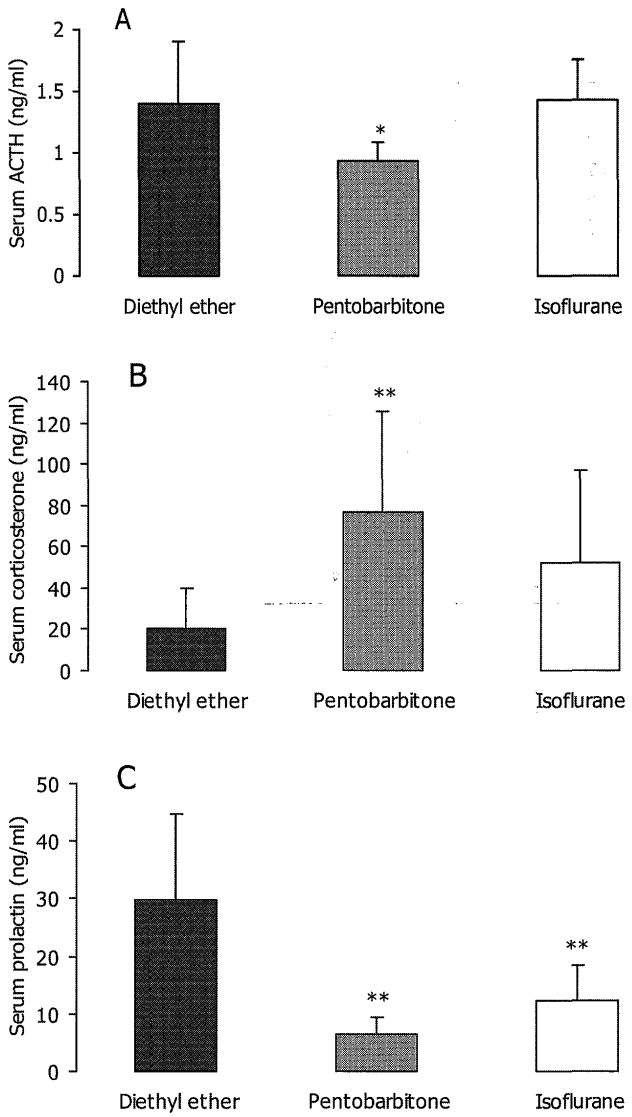

Fig. 3. Endocrinology. A=ACTH, B=corticosterone, and C=prolactin. Values are expressed as mean ± SD (n=6). Statistical analysis: for comparison between diethyl ether and other agents, * P<0.05; ** P<0.01.

phosphate levels are unexplainable at this point, it is obvious that this difference harms the continuity of the data.

Taken together, the results of this study as a whole indicate that anaesthesia-jar administered isoflurane yielded haematology and blood chemistry data consistent with ether, as well as reducing the animals' stress. Instead, pentobarbitone displayed an unignorable departure of anaesthetic outcomes, which was considerably different from the results from 30% isoflurane. Thus, we can now conclude that this study has provided a scientific basis that suggests, as far as available, that 30% isoflurane is one of the suitable alternatives to ether as an anaesthetic agent for upcoming regular toxicology studies in the Japanese laboratory. However, studies to extrapolate modern clinically used methods on laboratory animals are still limited, and further trials, such as those utilizing balanced anaesthesia or other inhaling anaesthetics, are needed.

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