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Blood biochemistry and hematological changes in rats after administration of a mixture of three anesthetic agents

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ABSTRACT. Currently, given the concerns regarding animal welfare, it is required that anesthesia or analgesia be used during surgery in experimental animals. Therefore, it is important to understand how anesthesia affects the health conditions of experimental animals. In this study, rat blood biochemistry and hematological changes were examined following administration of a mixture of three anesthetic agents—medetomidine, midazolam and butorphanol (MMB). One of three MMB dose combinations was subcutaneously administered to rats. After 1 hr, rats were treated with atipamezole, to reverse the anesthetic effects. Blood biochemistry and hematological parameters were assessed at 1, 4 and 24 hr post-MMB treatment. We also recorded body weight and food intake at 0, 2, 4, 6 and 24 hr post-MMB administration. Following MMB administration, transient increases were observed in glucose (GLUC) levels, hematocrit (HCT) values and hemoglobin (HGB) levels, whereas transient decreases were observed in total protein (TP) content and white blood cell (WBC) counts. Most of these parameters returned to control values 24 hr following MMB administration. Additionally, body weight and food intake decreased in MMB-treated rats. In conclusion, intermediate and high doses of MMB changed some blood biochemistry and hematological parameters, body weight and food intake. In contrast, low-dose MMB did not cause these effects. Therefore, depending on the experimental design, MMB may influence the results of studies that use laboratory animals. Consequently, anesthetic agents used in laboratory animals should be chosen based on detailed knowledge of their pharmacological effects.

KEY WORDS: anesthesia, atipamezole, butorphanol, medetomidine, midazolam

Prevention of pain and distress is essential in experimental animals used in biomedical research. This can be achieved by using appropriate anesthetics and analgesics [24]. Most general anesthetic methods consist of a combination of injected and inhaled anesthetics. Intraperitoneal (i.p.) or subcutaneous (s.c.) administration is commonly used in laboratory rats [1, 6, 26]. Compared to inhaled anesthesia, injected anesthesia presents greater challenges with respect to control of anesthesia time and depth control following the injection [9, 13, 18, 19, 30]. Therefore, despite prior dosage testing, it is often difficult to maintain injected anesthesia. In addition, individual differences in anesthetic effects can lead to overdosing in some animals [1, 6, 29]. Until recently, sodium pentobarbital or a mixture of ketamine and xylazine were widely used in laboratory animals. However, ketamine was labeled as a narcotic by the Japanese Narcotics and Psychotropic Control Law revision in 2007, and the use of ketamine has become highly regulated. Therefore, the use of this compound requires the involvement of qualified personnel, such as a narcotics researcher, or the facility's drug application specialist. In addition, sodium pentobarbital is likely to cause a potentially fatal decrease in blood pressure, or inhibition and cessation of respiration at the depth of anesthesia required for surgery. Furthermore, when used as a single agent, the analgesic effect of pentobarbital is weak [11].

Hence, there is a strong need for a novel anesthesia injection method that controls the depth of anesthesia, and is not limited by strict government regulation. It is also important to understand the effects of anesthetics on blood biochemistry when

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selecting an agent for an experiment. The combination of three anesthetics, medetomidine, midazolam and butorphanol (MMB), has recently attracted attention due to the increased use of injected anesthesia in laboratory animals [15, 17]. We previously showed that temporary changes in blood biochemistry occur in mice following MMB administration [22]. Blood biochemistry and hematological parameters, body weight and food consumption are critical for the evaluation of new compounds. The health conditions of experimental animals is also important. Therefore, in the present study, we investigated blood biochemistry and hematological parameters, body weight, food consumption and health conditions following MMB administration in rats.

MATERIALS AND METHODS

Animals

We used 8-week-old male RccHan^{TM}: WIST rats obtained from Japan Laboratory Animals, Inc. (Tokyo, Japan). The animals were acclimated for at least 7 days before experimental use. Animals were pair-housed in polycarbonate cages with autoclaved bedding (Aspen Chips, TAPVEI, Ltd., Kortteinen, Finland), and had free access to tap water (in a polycarbonate bottle) and sterilized food (CLEA, Tokyo, Japan). Ambient temperature was controlled by a central temperature management system, and was maintained at 20 to 26°C. Relative humidity was maintained between 30 and 70%. The animals were maintained under a 12-hr light:dark cycle (lights on from 7 a.m. to 7 p.m.). All animal procedures were in accordance with the guidelines of VETE-301 (2015.4.1), EXPE-102 (2017.2.15), and CARE-001 (2016.3.17) and were approved by the Institutional Animal Care and Use Committee at Chugai Pharmaceutical Co., Ltd.

Drugs

MMB consisted of a combination of medetomidine (Domitol, Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan), midazolam (Dormicum, Astellas Pharma, Inc., Tokyo, Japan) and butorphanol (Vetorphale, Meiji Seika Pharma Co., Ltd., Tokyo, Japan). Atipamezole (Antisedan, Nippon Zenyaku Kogyo Co., Ltd.) was used as a medetomidine antagonist. All drugs were stored at ordinary temperature (15 to 25°C) until use.

Drug preparation

All drugs were diluted in saline (Otsuka Normal Saline, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) before administration. MMB was prepared in accordance with previous reports [5, 11, 16, 29]. MMB was administered at a volume of 5 m*l*/kg, and atipamezole was administered at a volume of 1 m*l*/kg. Preliminary experiments were performed to determine the optimal MMB dose with sufficient depth of anesthesia to perform the surgery. The "MMB Low" dose was the optimal minimum dose. The "MMB Mid" dose was double that of the "MMB Low" dose, and the "MMB High" dose was double the dose of the "MMB Mid" dose. In all experiments, rats were maintained on hot water mats to stabilize body temperature while under anesthesia.

Depth of anesthesia evaluation

We used 3 parameters to assess the depth and duration of anesthesia, including the righting reflex, the abdominal region pinch reflex and the pedal withdrawal reflex in the hind limbs. Recovery of the righting reflex was defined as the time when an inverted animal began to roll over. To test the pedal withdrawal reflex, the interdigital web of limbs on both sides was lightly pinched and pulled using atraumatic forceps [15, 17, 22]. To test the abdominal pinch reflex, a 1-cm region of the abdominal skin was lightly pinched using hooked forceps. Adequate depth of surgical anesthesia was defined by the absence of all reflexes. The depth of anesthesia was evaluated after the start of the experiment. All reflex tests were performed and assessed by the same operator.

Experimental design

Animals were assigned to one of the following groups: Control group (No administration), MMB Low group (0.15/2.0/2.5 mg/kg), MMB Mid group (0.3/4.0/5.0 mg/kg), MMB High group (0.6/8.0/10.0 mg/kg) and Atipamezole (Ati) group.

To verify the route of administration, animals from the MMB Low, MMB Mid and MMB High groups were assigned to subgroups of 3 and dosed by i.p. or s.c. administration. The depth of anesthesia was evaluated at 5, 10, 15, 30, 45 and 60 min post-injection to determine the time of anesthesia induction and duration of sustained anesthesia. To provide recovery from anesthesia, 1 hr after MMB treatment, atipamezole was administered at a dose double that of medetomidine. Under isoflurane anesthesia, blood samples were collected from the abdominal portion of the vena cava, 24 hr after the initial anesthetic injection. Animals were then euthanized by exsanguination while under anesthesia, and necropsy was performed to assess possible influences of MMB on organs.

Confirmation of the effect of MMB administration

Changes in hematological and biochemical parameters in the Control (N=8), MMB Low (N=8), MMB Mid (N=8) and MMB High groups (N=8) were compared to those in the Ati group (N=8). Depth of anesthesia was evaluated 10, 30, 45 and 60 min after injection to confirm anesthesia induction time and sustained time. All groups, excluding the Control group, were administered atipamezole 1 hr after the start of the experiment to provide recovery from anesthesia. Blood samples (0.3 m*l*) were collected from the jugular vein at −24, 1, 4 and 24 hr after the start of the experiment. Following the final blood sample collection, hematological and blood biochemistry parameters were compared among groups. Clinical observations of body weight and food intake were measured 0, 2, 4, 6 and 24 hr after the start of the experiment. Food intake was defined as a decrease in food weight for 4 rats per cage.

Table 1. Influence of i.p. and s.c. MMB administration

Administration route	Group	No.	Under anesthesia (min) Introduction point (heads/Group)	End point (heads/Group)	Clinical observation[a]	Necropsy observation
i.p.	MMB Low	1	10	60	Reacted at Atipamezole administration	Hydronephrosis of the left kidney
		2	No introduction		Noparticular	Noparticular
		3	10	60	Noparticular	Noparticular
	MMB Mid	4	5	60	Noparticular	Noparticular
		5	10	60	Rapid breathing	Noparticular
		6	10	60	Noparticular	Noparticular
	MMB High	7	5	60	· Rapid breathing · Irregular respiration · Dark skin	Noparticular
		8	10	60	· Dark skin	Noparticular
		9	5	60	· Piloerection · Irregular respiration · Dark skin	Noparticular
s.c.	MMB Low	1	10	60	Noparticular	Noparticular
		2	10	60	Noparticular	Noparticular
		3	5	60	Noparticular	Noparticular
	MMB Mid	4	5	60	Noparticular	Noparticular
		5	5	60	Noparticular	Noparticular
		6	5	60	Noparticular	Noparticular
	MMB High	7	5	60	· Dark skin and white ear	Noparticular
		8	5	60	· Rapid breathing · Dark skin	Dead body discovery in 24 hr
		9	5	60	· Rapid breathing · Dark skin	A dark red point lay scattered in the pulmonary whole

a) Sustained urination was seen in all MMB treated rats.

Blood biochemistry and hematology

Separate portions of blood were transferred to tubes containing either lithium heparin or trisodium EDTA as anticoagulants. Heparinized samples were centrifuged 1,900 × g at 4°C for 10 min and resulting plasma supernatants were transferred to fresh tubes for blood biochemistry analysis using a TBA-120FR automatic chemical analyzer (Toshiba Medical Systems, Co., Ltd., Tochigi, Japan). EDTA blood was analyzed for hematology using XT-2000iV (SYSMEX, Co., Ltd., Kobe, Japan).

Histopathology

Hematoxylin and eosin (HE) staining was used to assess pathological pulmonary changes after MMB administration. Pulmonary tissue samples were fixed in 10% neutral buffered formalin. Preserved pulmonary samples then were trimmed, embedded in paraffin, sectioned at 3-μm thicknesses, mounted on glass slides, stained with HE, and cover-slipped using standard methodologies.

Data analysis

All results are expressed as the mean ± standard deviation. All data were analyzed with parametric Dunnett's multiple comparison tests using Win 8.1_JMP 11.2.1 (SAS Institute, Inc., Cary, NC, U.S.A.).

RESULTS

Verification of the route of administration, Influence of MMB route of administration on anesthesia induction and endpoint, and clinical observations

Anesthesia induction and endpoint were compared for i.p. and s.c. administration routes. After i.p. MMB injection, anesthesia was induced in all rats (except for 1 rat from the i.p. MMB Low group), and the induction time ranged from 5 to 10 min, regardless of dose, and the end point of anesthesia was 60 min (Table 1). In the s.c. MMB Low group, anesthesia induction time ranged from 5 to 10 min, whereas induction occurred at 5 min in all rats in the s.c. MMB Mid and High groups. The endpoint for all s.c. groups was 60 min (Table 1).

All rats, regardless of the administration route, exhibited sustained urination while under anesthesia. Clinical observations of animals under anesthesia revealed rapid breathing, irregular respiration, and darkening of the skin in the MMB High group (Table 1). Rapid breathing was observed in the i.p. MMB Mid group. Additionally, 1 rat from the s.c. MMB High group was found dead 24 hr after MMB administration, and at necropsy dark red spots were observed across both lungs (Table 1). One rat from the i.p. MMB Low group showed congenital hydronephrosis of the left kidney at necropsy (Table 1).

Table 2. Clinical observations of health condition at each time point after MMB administration

	Control (N=8)	MMB Low (N=8)	MMB Mid (N=8)	MMB High (N=8)	Ati (N=8)
0–1 hr (Under anesthesia)					
No abnormality	8	0	0	0	8
Rapid breathing	0	0	0	5	0
Irregular respiration	0	0	0	3	0
Deep respiration	0	0	0	2	0
Dark skin	0	0	0	2	0
Urination	0	8	8	8	0
2 hr					
No abnormality	8	8	0	0	8
Lying down but normal activity	0	0	4	7	0
Low activity	0	0	4	0	0
Lying down and immobility	0	0	0	1	0
4 hr					
No abnormality	8	8	0	0	8
Lying down but normal activity	0	0	8	1	0
Low activity	0	0	0	3	0
Lying down and immobility	0	0	0	4	0
Abnormal vocalization	0	0	0	1	0
6 hr					
No abnormality	8	8	3	0	8
Low activity	0	0	2	6	0
Abnormal vocalization	0	0	0	2	0
Low temperature	0	0	4	4	0
24 hr					
No abnormality	8	8	8	8	8
Necropsy					
Noparticular	8	8	8	6	8
A dark red point lies scattered in the pulmonary whole	0	0	0	2	0

Blood biochemistry analysis 24 hr after MMB administration revealed increased aspartate aminotransferase (AST) levels in the s.c. MMB Mid group (74.0 ± 5.3 U/*l*) compared to those of the i.p. MMB Mid group (57.0 ± 6.1 U/*l*, *P*=0.0225). Increased AST levels were also observed in the s.c. MMB High group (100.0 ± 1.4 U/*l*) compared to those of the i.p. MMB High group (61.7 ± 6.4 U/*l*, *P*=0.0061). Alanine aminotransferase (ALT) levels in the s.c. MMB High group (42.5 ± 0.7 U/*l*) were higher than those of the i.p. MMB High group (29.0 ± 2.0 U/*l*, *P*=0.0030) (Supplementary Table 1). In contrast, no significant differences between groups were observed in hematological parameters (Supplementary table 2). We found statistically significant changes in other parameters including triglyceride (TG), total bilirubin (TBIL), GLUC, potassium (K) and reticulocyte (RET); however, these changes were not considered relevant because they were not dependent on anesthetic dose.

Changes in clinical observation, body weight and food intake at 0, 2, 4, 6 and 24 hr after MMB administration

The s.c. route of administration was chosen for all subsequent experiments because of the rapid breathing observed in the i.p. MMB Mid group and the unstable anesthesia induction in the i.p. MMB Low group. Adequate depth of surgical anesthesia was observed at all relevant time points after s.c. administration. Rapid breathing, irregular respiration, and darkening of the skin was observed in all rats in the MMB High group (Table 2). No abnormalities were observed in the MMB Low group 2 hr after MMB administration. Conversely, low activity was observed 6 hr after MMB administration, in 2 rats from the MMB Mid group rats and 6 from the MMB High group (Table 2). MMB dosing resulted in dose-dependent effects on body weight (Supplementary Table 3), with significant decreases at 24 hr in the MMB Low, Mid and High groups (*P*=0.0254, *P*<0.0001 and *P*<0.0001, respectively), compared to the Ati group (Fig. 1). Similarly, food intake significantly decreased in MMB-dosed animals at 24 hr in the MMB Low, Mid and High dose groups, (*P*=0.0337, *P*<0.0001 and *P*<0.0001, respectively), compared to the Ati group (Fig. 2). Necropsy observations revealed dark red spots across both lungs in 2 of the 8 MMB High dose animals (Table 2). Histopathological analysis of tissue from one of these animals revealed focal inflammatory cell permeation around blood vessels and focal inflammatory cell infiltration into the alveoli (Fig. 3).

Changes in blood biochemistry at −24, 1, 4 and 24 hr after MMB administration

In the MMB Low group, GLUC (*P*<0.0001) and blood urea nitrogen (BUN) (*P*=0.0406) values were elevated 1 hr after MMB administration (Table 3, Supplementary Table 4). GLUC (*P*<0.0001) levels remained significantly elevated 4 hr after
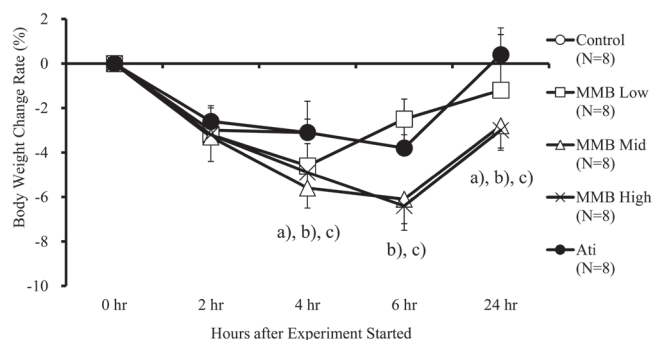
Fig. 1. Rate of change in body weight post-MMB administration. Values represent the mean ± standard deviation. Dunnett's multiple comparison tests were used to determine statistically significant differences between groups, [a] *P*<0.05 MMB Low vs. Ati, [b] *P*<0.01 MMB Mid vs. Ati, [c] *P*<0.01 MMB High vs. Ati.
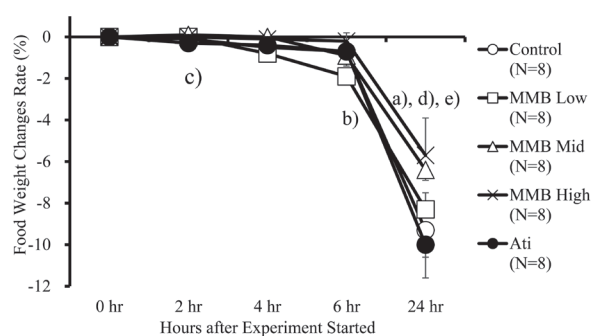

Fig. 2. Rate of change in food intake (weight of food) post-MMB administration. Values represent the mean ± standard deviation. Dunnett's multiple comparison tests were used to determine statistically significant differences between groups, [a] *P*<0.05 MMB Low vs. Ati, [b] *P*<0.01 MMB Low vs. Ati, [c] *P*<0.05 MMB Mid vs. Ati, [d] *P*<0.01 MMB Mid vs. Ati, [e] *P*<0.01 MMB High vs. Ati.
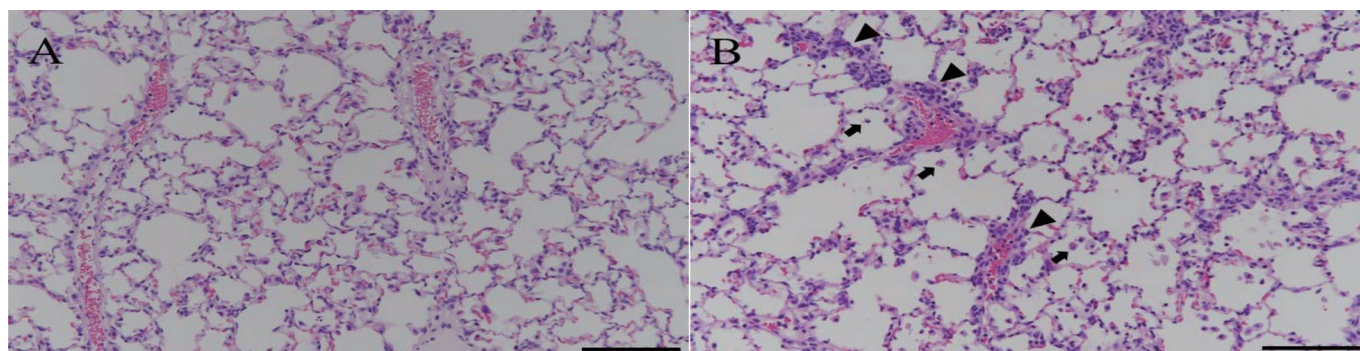

Fig. 3. Images of the pulmonary HE stains from the (A) Ati group and (B) MMB High group, in which dark red, scattered points were observed in the pulmonary hole. We observed focal inflammatory cell invasion around blood vessels (arrowheads), and dropout into the alveoli (arrows) in the MMB High group. Scale bars equal 10 μm.

MMB administration (Table 3, Supplementary Table 4). In the MMB Mid group, GLUC (*P*<0.0001) levels were elevated, whereas the total protein (TP) (*P*=0.0035) level was decreased 1 hr after MMB administration (Table 3, Supplementary Table 4). GLUC (*P*<0.0001) levels remained elevated 4 hr after MMB administration (Table 3, Supplementary Table 4). Furthermore, AST (*P*=0.0083) levels were elevated 24 hr after MMB administration (Table 3, Supplementary table 4). In the MMB High group, GLUC (*P*<0.0001) and BUN (*P*=0.0005) levels were elevated and TP (*P*=0.0035) levels were decreased 1 hr after MMB administration (Table 3, Supplementary Table 4). GLUC (*P*<0.0001) and BUN (*P*<0.0001) levels remained elevated 4 hr after MMB administration (Table 3, Supplementary Table 4). Furthermore, AST (*P*<0.0001) and ALT (*P*=0.0003) levels were elevated 24 hr after MMB administration (Table 3, Supplementary Table 4).

We found statistically significant changes in other blood biochemistry parameters (TBIL, IP, Ca, TG and K). However, these changes were not considered relevant because these effects appeared to be unrelated to either the anesthetic dose or the passage of time.

Changes in hematology −24, 1, 4 and 24 hr after MMB administration

In the MMB Low group, white blood cell (WBC) (*P*=0.0022) and lymphocyte (LYMP) (*P*=0.0001) counts decreased and hematocrit (HCT) (*P*=0.0286) values increased at 4 hr after MMB administration (Table 3, Supplementary Table 5). In the MMB Mid group, HCT values (*P*<0.0001) and hemoglobin (HGB) levels (*P*=0.0039) were elevated 1 hr after MMB administration (Table 3, Supplementary Table 5). HCT (*P*=0.0059) and HGB (*P*=0.0184) values remained elevated 4 hr after MMB administration, whereas WBC (*P*=0.0002) and LYMP (*P*<0.0001) counts decreased (Table 3, Supplementary Table 5). In the MMB High group, HCT (*P*<0.0001) and HGB (*P*<0.0001) values were elevated 1 hr after MMB administration (Table 3, Supplementary table 5). HCT (*P*<0.0001) and HGB (*P*<0.0001) values remained elevated at 4 hr after MMB administration (Table 3, Supplementary Table 5), whereas WBC (*P*=0.0001) and LYMP (*P*<0.0001) counts decreased (Table 3, Supplementary Table 5).

Statistically significant changes observed in other hematological parameters (Eosinophil (EO), Monocyte (MONO) and Mean

Table 3. Changes in biochemistry, hematology and insulin levels after the anesthetic administration

Item	Control (N=8)				MMB Low (N=8)				MMB Mid (N=8)			
	−24 hr	1 hr	4 hr	24 hr	−24 hr	1 hr	4 hr	24 hr	−24 hr	1 hr	4 hr	24 hr
AST (U/l)	80.3 ± 20.4	75.8 ± 16.6	86.5 ± 21.3	78.5 ± 20.5	70.3 ± 11.7	61.3 ± 12.6	73.1 ± 12.3	72.8 ± 15.6	71.8 ± 14.9	64.3 ± 12.8	76.5 ± 10.1	89.0 ± 17.4[b]
ALT (U/l)	34.3 ± 2.7	31.0 ± 3.7	35.5 ± 8.7	34.8 ± 4.9	31.5 ± 2.1	28.5 ± 3.7	29.6 ± 5.2	30.8 ± 3.4	32.0 ± 4.8	27.3 ± 4.9	29.3 ± 4.3	34.8 ± 4.7
BUN (mg/dl)	19.2 ± 1.7	17.5 ± 1.9	15.3 ± 2.0	17.8 ± 1.7	19.1 ± 1.1	19.3 ± 1.8[a]	14.9 ± 1.8	19.2 ± 1.0	18.6 ± 2.6	18.0 ± 2.0	13.3 ± 2.4	17.9 ± 2.9
GLUC (mg/dl)	149.8 ± 7.0	140.5 ± 16.9	138.5 ± 13.7	140.3 ± 17.2	148.0 ± 12.0	347.5 ± 20.7[b]	186.6 ± 15.9[b]	133.3 ± 7.1	143.3 ± 8.9	358.3 ± 21.3[b]	184.0 ± 13.0[b]	127.3 ± 8.3
TP (mg/dl)	6.1 ± 0.2	6.0 ± 0.2	5.8 ± 0.3	6.1 ± 0.1	6.1 ± 0.4	5.8 ± 0.2	5.9 ± 0.3	5.9 ± 0.2	6.0 ± 0.3	5.5 ± 0.2[b]	5.8 ± 0.3	5.8 ± 0.2
Cl (mmol/l)	99.8 ± 1.3[a]	99.3 ± 2.1	98.5 ± 3.0	98.8 ± 2.1	100.5 ± 0.9	98.5 ± 1.8	97.0 ± 4.4	100.8 ± 1.5	101.5 ± 1.8	94.8 ± 2.8[b]	100.0 ± 1.9	100.8 ± 1.8
WBC (10³/μl)	7.3 ± 0.9	7.2 ± 1.5	7.7 ± 1.4	7.5 ± 2.0	8.6 ± 0.9	6.8 ± 0.7	5.3 ± 1.3[b]	8.3 ± 1.2	7.9 ± 1.8	6.5 ± 1.8	4.8 ± 1.6[b]	8.0 ± 1.5
HGB (g/dl)	14.4 ± 0.9	14.2 ± 0.4[a]	13.8 ± 0.5	13.4 ± 0.4	14.0 ± 0.8	13.8 ± 0.4	14.3 ± 0.4	13.1 ± 0.3	14.4 ± 1.1	14.4 ± 0.3[b]	14.4 ± 0.6[a]	13.5 ± 0.3
HCT (%)	41.7 ± 2.4	41.4 ± 1.3	40.2 ± 1.5	39.6 ± 1.2	40.9 ± 2.1	41.3 ± 1.3	42.3 ± 1.6[a]	38.9 ± 0.9	42.0 ± 2.7	43.5 ± 0.8[b]	42.7 ± 1.4[b]	39.8 ± 1.1
LYMP (10³/μl)	5.89 ± 0.98	5.95 ± 1.55	6.15 ± 1.40	5.99 ± 2.02	6.84 ± 0.74	5.41 ± 0.47	3.69 ± 0.45[b]	6.56 ± 0.88	6.54 ± 1.70	5.13 ± 1.14	3.00 ± 0.73[b]	6.27 ± 1.09
Insuline (ng/ml)	1.7 ± 0.4	1.8 ± 0.7	1.6 ± 0.3	2.0 ± 0.9	1.6 ± 0.6	0.8 ± 0.3[b]	2.5 ± 0.9[b]	1.8 ± 0.7	1.6 ± 0.7	0.7 ± 0.2[b]	1.7 ± 0.3	1.3 ± 0.4

Item	MMB High (N=8)				Ati (N=8)			
	−24 hr	1 hr	4 hr	24 hr	−24 hr	1 hr	4 hr	24 hr
AST (U/l)	72.5 ± 17.1	62.3 ± 5.9	84.4 ± 14.3	112.0 ± 6.7[b]	70.8 ± 7.3	67.8 ± 6.7	81.0 ± 8.5	65.0 ± 7.4
ALT (U/l)	30.5 ± 3.7	28.0 ± 4.1	38.4 ± 22.9	39.5 ± 5.0[b]	32.8 ± 4.9	30.3 ± 4.2	30.4 ± 3.8	29.5 ± 4.0
BUN (mg/dl)	20.0 ± 1.5	20.9 ± 1.2[b]	20.0 ± 4.5[b]	17.8 ± 2.1	18.6 ± 2.1	16.6 ± 2.9	13.3 ± 2.0	18.4 ± 2.3
GLUC (mg/dl)	142.5 ± 8.7	399.8 ± 13.8[b]	178.4 ± 10.6[b]	130.3 ± 7.5	138.8 ± 10.3	139.5 ± 5.8	126.0 ± 4.7	137.5 ± 5.8
TP (mg/dl)	6.1 ± 0.3	5.5 ± 0.3[b]	5.7 ± 0.1	5.7 ± 0.2	6.2 ± 0.3	5.9 ± 0.2	5.7 ± 0.2	5.9 ± 0.3
Cl (mmol/l)	100.8 ± 1.8	95.8 ± 2.0[b]	99.6 ± 1.7	101.5 ± 0.9	102.0 ± 1.9	100.8 ± 1.0	100.1 ± 1.4	100.0 ± 1.5
WBC (10³/μl)	7.4 ± 1.7	5.8 ± 1.1	4.6 ± 1.0[b]	7.7 ± 1.4	8.4 ± 1.8	6.2 ± 1.3	7.7 ± 1.2	7.4 ± 1.4
HGB (g/dl)	14.6 ± 0.6	14.8 ± 0.5[b]	15.2 ± 0.6[b]	13.2 ± 0.4	14.2 ± 0.6	13.7 ± 0.5	13.7 ± 0.3	13.2 ± 0.6
HCT (%)	42.6 ± 1.6	45.0 ± 1.4[b]	45.6 ± 2.1[b]	39.2 ± 1.4	41.3 ± 1.6	40.1 ± 1.1	40.1 ± 0.8	39.0 ± 2.0
LYMP (10³/μl)	6.24 ± 1.59	4.98 ± 0.90	3.08 ± 1.09[b]	5.84 ± 1.19	6.74 ± 1.45	4.89 ± 0.84	5.96 ± 0.82	5.85 ± 1.19
Insuline (ng/ml)	1.4 ± 0.6	0.7 ± 0.2[b]	1.8 ± 0.5	1.4 ± 0.2	1.3 ± 0.3	2.0 ± 0.6	1.5 ± 0.5	1.7 ± 0.7

The value is mean ± standard deviation. The statistics processing uses the parametric Dunnett's multiple comparison tests. a) $P<0.05$ vs. Ati, b) $P<0.01$ vs. Ati. All point of items were basically N=8 because all group had 8 rats. But some of samples were no data because of the fault of sampling. So insulin of MMB Low, Mid, High and Ati group was N=7.

corpuscular hemoglobin concentration (MCHC)) were not considered relevant because these effects appeared to be unrelated to either the anesthetic dose or the passage of time.

Changes in blood insulin levels at −24, 1, 4 and 24 hr after MMB administration

Temporary decreases in blood insulin levels were observed in the MMB Low (P=0.0003), Mid (P<0.0001) and High (P<0.0001) groups 1 hr after MMB administration (Table 3). Insulin levels were only elevated in the MMB Low group 4 hr after MMB administration (P=0.0089). In all MMB groups, 24 hr after MMB administration, insulin levels were no longer statistically different from those of the Ati group (Table 3).

DISCUSSION

Comparison of two routes of administration revealed that induction of anesthesia using an intermediate and high dose of MMB occurred 5–10 min after i.p. administration and 5 min after s.c. administration (Table 1). However, in the i.p. MMB Low group, MMB administration failed to induce anesthesia in one rat, as confirmed by a pain reaction at the time of Ati administration (Table 1). Additionally, one rat in the s.c. MMB High group was found dead 24 hr after MMB administration. Based on these results, we determined that s.c. administration provided more stable anesthesia induction than i.p. administration. Under anesthesia, respiratory problems and skin darkening were observed after administration by either route in the MMB High groups. In contrast, only one rat in the i.p. MMB Mid group exhibited such clinical observations (rapid breathing). At necropsy, no abnormalities were observed in i.p. dosed animals, apart from hydronephrosis of the left kidney, an event that was presumably congenital. Necropsy of s.c.-dosed animals revealed one abnormal pulmonary case. Based on these results, the MMB High dose appeared to be an overdose when administered by either route. Blood biochemistry and hematological parameters were compared 24 hr after MMB administration among groups. AST and ALT levels were elevated after s.c.-dosing (Supplementary Table 1); however, these effects were not supported by abnormalities at necropsy. The s.c. route was selected to confirm other changes induced by MMB administration, as it produced stable induction of anesthesia that did not differ based on dose. After MMB administration, dose-dependent decreases in body weight were observed in the MMB Mid and High groups. These effects were presumed to reflect differences at the time of awakening and recovery of activity following anesthesia, consistent with reduced food consumption. No clinical abnormalities were found in the MMB Low group 2 hr after MMB administration. However, low activity (lethargy) was observed in 2 of 8 MMB Mid group rats and 6 of 8 MMB High group rats 6 hr after MMB administration. As the effect of medetomidine is antagonized by Ati, low activity after awakening may be attributed to the influence of midazolam. Low activity is associated with decreased food and water consumption [14, 20, 21, 25]. The resulting food and water deprivation was presumed to influence blood biochemistry and hematological parameters. Consistent with previous studies, we found that fasting and dehydration resulting from decreased activity caused decreases in WBC and elevations in HCT and HGB values [4, 8, 14, 20, 21, 25, 27]. The WBC count decrease observed here was observed in all MMB administration groups, and values were restored to control values at similar time points, indicating that effects on WBC reflected the pharmacological action of MMB. We also detected a decrease in the number of WBC and LYMP counts; however, the basis for these effects is unclear. The observed decrease in TP and elevation in BUN levels were considered a result of fasting [7, 14, 20].

The presence of dark red spots in the lungs was only observed in the MMB High group. These focal lesions in the lungs were presumed to reflect injury to the pulmonary system caused by abnormal breathing during anesthesia, rather than to the pharmacological action of MMB. Clinical observations under anesthesia indicated that MMB influenced systemic circulation and respiration. MMB has been reported to decrease heart rate and blood oxygen saturation in rats [17]. Therefore, the abnormalities we observed in the respiratory lung tissue may be due to MMB overdosing.

A previous report indicated that surgical stress could induce hyperglycemia [10]. Thus, the increase in GLUC levels observed in the present study may reflect administration stress. In all MMB-dosed rats, GLUC values were elevated 1 and 4 hr after MMB administration (compared to those of controls at the respective time points), potentially due to the pharmacological action of MMB: specifically, due to inhibition of insulin secretion by pancreatic β-cells, and ATP-dependent potassium ion channel closing of the cell membrane by α2-adrenoreceptor agonists [5, 6, 10, 12, 16, 22, 23, 28, 31, 32]. Medetomidine, an α2-adrenoreceptor agonist, suppresses insulin secretion by stimulating α2-adrenoreceptors [3]. We previously found a similar change in mice ([22] and data not shown). Additionally, Ati has been reported to have α2-adrenoreceptor-blocking effects [2, 23]. Consistent with this, all MMB-dosed animals showed decreased blood insulin values 1 hr after MMB administration. By 4 hr after MMB administration, blood insulin levels were elevated in rats in the MMB Low group, and were restored to levels equivalent to those of the control (Ati) group in the MMB Mid and High groups. Notably, larger Ati doses have been reported to induce larger increases in blood insulin concentrations. Specifically, peak blood insulin levels increased to about 1.5-fold of the blood concentration prior to medetomidine administration [2]. Several hours later, blood insulin levels returned to levels indistinguishable from those before medetomidine administration, suggesting that there is no risk that the sudden insulin increase will cause hypoglycemia [2].

In this study, administration of MMB in rats caused a temporary change in the levels of blood biochemistry and hematological parameters. These changes were not observed for more than 24 hr after MMB administration. MMB administration in rats also attenuated body weight gain. However, these changes did not appear to adversely affect the health of rats, given that the clinical and necropsy observations were normal. The MMB High dose was considered an overdose based on clinical and necropsy observations.

Our study suggests that the anesthetic and analgesic agents usually used in animal experiments may cause changes in body weight, food intake, hematological and blood biochemistry parameters, which are common outcome measurements. Hence, it is important to be aware of the effects of these drugs before using them in experiments.

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