Original Article

Effects of atipamezole and flumazenil on stress-related hormonal and metabolic responses in cats anesthetized with medetomidine, midazolam, ketamine and isoflurane undergoing ovariohysterectomy and castration

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Abstract

This study examined the post-operative effects of atipamezole (Ati) and flumazenil (Flu) on stress-related neurohormonal and metabolic responses in isoflurane-anesthetized cats premedicated with medetomidine, midazolam and ketamine and undergoing ovariohysterectomy or castration. Client-owned mixed-breed cats (n = 108) were treated with 50-µg/kg medetomidine and 0.5-mg/kg midazolam, followed by 5-mg/kg ketamine (MMK) intramuscularly (IM) and maintained under isoflurane anesthesia. The cats were divided into nine groups (six cats/group): control (saline IM), 50-µg/kg Flu IM or intravenously (IV), 100-µg/kg Ati IM or IV, 200-µg/kg Ati IM, 100-µg/kg Ati + 50-µg/kg Flu IM or IV and 200-µg/kg Ati + 50-µg/kg Flu IM. Five blood samples were taken: before pretreatment, post-operatively during anesthesia and 10, 60, and 120 mins after antagonist administration. MMK-isoflurane anesthesia decreased adrenaline, noradrenaline, cortisol and non-esterified fatty acid (NEFA) levels. It also caused hyperglycemia. Compared to the controls, Ati IV rapidly reversed the decreased catecholamine and cortisol concentrations in ovariohysterectomized cats. Ati IV and/or Ati + Flu IV tended to induce rapid recovery from hyperglycemia and reverse the NEFA decreases. Compared to IM, the IV administration of Ati, alone and in combination with Flu, induced a more rapid recovery from anesthesia. Compared to Ati alone, the Ati–Flu combination improved the quality of recovery. Thus, Ati IV is effective for rapidly reversing stress-related neurohormonal and metabolic effects in MMK-isoflurane anesthetized cats. Ati–Flu IM aids in rapid recovery without altering post-operative neurohormonal and metabolic changes.

Keywords: Cats, medetomidine, midazolam, atipamezole, flumazenil, stress hormone

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Introduction

In feline veterinary medicine, the alpha(α)$_2$-adrenoceptor agonist medetomidine is an effective analgesic and muscle relaxant. However, it induces undesirable effects, such as bradyarrhythmia, hyperglycemia and emesis in cats (Cullen, 1996; Kanda and Hikasa, 2008[a]; Murahata and Hikasa, 2012). A combination of medetomidine with midazolam and ketamine (MMK) produces good anesthesia in cats, with fewer adverse effects or better analgesia potentiation (Ueoka and Hikasa, 2008; Polson *et al*., 2012; Shah *et al*., 2019; Bruniges and Yates, 2020). Antagonism may be required when anesthetized animals show a profound depression of vital signs, adverse effects and/or delayed recovery from anesthesia. Atipamezole (Ati) and flumazenil (Flu) either completely or partially antagonize the effects of MMK in cats (Savola, 1989; Vähä-Vahe, 1990; Ilkiw *et al*., 2002; Granholm *et al*., 2006; Ebner *et al*., 2007; Ueoka and Hikasa, 2008; Ueoka and Hikasa, 2015). After surgery and when combined with MMK, these antagonists can accelerate awakening from anesthesia and are useful in anesthesia-associated emergency and critical care. In cats, Ati, both alone and in combination with Flu administered intravenously (IV), effectively antagonizes the anesthetic and neurohormonal effects induced by MMK (Ueoka and Hikasa, 2008; Ueoka and Hikasa, 2015). Compared to Ati alone, Ati in combination with Flu leads to a similar recovery time from MMK anesthesia but improves the quality of recovery by reducing excitation and hyperaesthesia (Ebner *et al*., 2007). The antagonistic effects of Ati and Ati–Flu also differ depending on the route and timing of administration in MMK-anesthetized normal and castrated cats (Ebner *et al*., 2007; Bruniges and Yates, 2020).

Stressors such as anxiety, excitement, pain, anesthesia and surgery induce neurohormonal and metabolic changes in animals; these changes are characterized by elevated blood cortisol levels, catecholamines, glucose and non-esterified fatty acids (NEFA) (Desborough, 2000). Actions mediated by α_2-adrenoceptors are closely coordinated with these events (Ambrisko and Hikasa, 2002 ; Kanda and Hikasa, 2008[a]). MMK anesthesia suppresses the release of catecholamine and cortisol, suppresses lipolysis and induces hyperglycemia in cats (Ueoka and Hikasa, 2015). It is important to evaluate cats' stress-related hormonal and metabolic responses due to the post-operative administration of potential antagonists in clinical practice. In terms of the overall effect of these antagonists on general anesthesia and post-surgical recovery, the use of potential antagonists may be advantageous to recovery from anesthesia if the sympathoadrenal system is adequately but not excessively activated. However, to the best of our knowledge, there are no published reports on the effects of Ati and Flu, both alone and in combination, on the stress responses of anesthetized cats undergoing surgery. Therefore, this study aimed to evaluate the effects of the intramuscular (IM) and IV administrations of Ati and Flu, both alone and in combination and at different administration times, on key stress-related neurohormonal and metabolic changes in MMK–isoflurane-anesthetized cats undergoing ovariohysterectomy and castration.

Materials and Methods

Animals: We prospectively recruited 108 client-owned mixed-breed cats (54 males, 54 females) at the Kamohara Animal Hospital for ovariohysterectomy or castration. They were clinically healthy and ranged in age from 6 months to 1 year. On average, the males weighed 4.1 ± 0.7 kg (mean ± standard deviation [SD]) and the females weighed 3.0 ± 0.4 kg. Each cat owner provided informed consent for data collection. Physical and routine hematological examinations before the study revealed that all values were within normal physiological ranges. All cats fasted for 12 h but had *ad libitum* access to water. Owners brought their cats to our hospital early in the morning on the day of surgery. After preparation for surgery and anesthesia, each cat rested in a darkened cage for 2–3 h before anesthesia. After complete recovery from anesthesia, all cats received water *ad libitum* and food.

Study protocol: We randomly assigned the cats to one of nine treatment groups (six cats/group) for both ovariohysterectomy and castration. Each cat was intramuscularly (IM) administered a mixture of 50-µg/kg medetomidine hydrochloride (Dorbene, Syva Laboratorios, S.A., Spain) and 0.5-mg/kg midazolam (Sandoz, Tokyo, Japan), followed by an IM administration of 5-mg/kg ketamine (Fujita Pharmaceutical, Tokyo, Japan) 10 mins later. Medetomidine and midazolam were mixed in the same syringe immediately before injection. A further 10 mins after the ketamine injection, anesthesia was induced with 4% isoflurane in oxygen at a total gas flow rate of 1.5 l/min using a face mask attached to the ADS 1000 veterinary anesthesia delivery system (Engler, Hialeah, FL). Then, a cuffed endotracheal tube was inserted. Cats were placed supine and maintained at a surgical depth of isoflurane anesthesia through a non-rebreathing system under controlled ventilation. During isoflurane anesthesia, the cats underwent castration or ovariohysterectomy using standard methods. Pre-operatively, an analgesic (0.3-mg/kg meloxicam; Inflacam, Chanelle Pharmaceuticals Manufacturing Ltd, Ireland) was injected subcutaneously in all cats, followed by once daily for several days after surgery if necessary. Lactated Ringer's solution was infused IV at 10 ml/kg/hr during anesthesia and surgery. The duration of isoflurane anesthesia was 30 and 60 mins in castrated and ovariohysterectomized cats, respectively.

When the inhalation of isoflurane was halted, an antagonist was administered as follows, depending on the treatment group: 0.1-ml/kg physiological saline solution IM (control group), 50-µg/kg Flu (Anexate; Astellas Pharmaceutical, Tokyo, Japan) IM, 100-µg/kg Ati (atipamezole hydrochloride; Orion corporation, Pharmaceutical, Espoo, Finland) IM, 200-µg/kg Ati IM, 100-µg/kg Ati + 50-µg/kg Flu IM, 200-µg/kg Ati + 50-µg/kg Flu IM, 50-µg/kg Flu IV, 100-µg/kg Ati IV, or 100-µg/kg Ati + 50-µg/kg Flu IV; groups will be referred to as control, Flu IM, Ati100 IM, Ati200 IM, Ati100 + Flu IM, Ati200 + Flu IM, Flu IV, Ati100 IV, and

Ati100 + Flu IV, respectively. Antagonists were mixed in the same syringe immediately before injection and injected into the cephalic vein. The endotracheal tube was extubated once a laryngeal reflex was visible. During the recovery process, the cats remained in separate cages in a room with an air temperature of 25 °C. General postoperative management and care were performed in all cats. More than 120 mins after the administration of the potential antagonists, another analgesic, butorphanol (0.1–0.4 mg/kg; Vetorphale, Meiji Seika, Tokyo, Japan) was injected IM to cats with signs of pain such as vocalization, anorexia and posture. There were no issues with surgery or anesthesia in any of the cats.

Anesthesia and intraoperative monitoring: An agent-specific precision vaporizer was used to administer isoflurane. Gas samples were drawn from the breathing circuit through a tube attached to an adapter positioned at the oral end of an endotracheal tube. During anesthesia, we assessed the expired end-tidal isoflurane (EtIso) and carbon dioxide ($EtCO_2$) concentrations, arterial oxygen saturation of pulse oximetry (SpO_2), heart rate (HR), respiration rate (RR), rectal temperature (RT) and mean blood pressure (MBP) using the oscillometric method, either continuously or intermittently, with a multi-parameter monitor (BSM-5192; Nihon Kohden, Tokyo). During controlled ventilation, RR was adjusted to a range of 25–40 mmHg $EtCO_2$ and ranged from 8 to 15 breaths/min in all groups. SpO_2 was >98% in all cases. Mean EtIso, HR, MBP, RR, $EtCO_2$ and RT during isoflurane anesthesia in ovariohysterectomy or castration groups are shown in Table 1. There were no significant differences in EtIso, HR, MBP, RR, EtCO2 and RT values across groups in both surgeries (Table 1).

Table 1 Mean expired end-tidal isoflurane concentration (EtIso), heart rate (HR), mean blood pressure (MBP), respiration rate (RR), expired end-tidal carbon dioxide ($EtCO_2$) and rectal temperature (RT) during isoflurane anesthesia in cats premedicated with medetomidine (50 µg/kg), midazolam (0.5 mg/kg), and ketamine (5 mg/kg) undergoing ovariohysterectomy or castration.

Surgery	Group	EtIso (%)	HR (beats/min)	MBP (mmHg)	RR (breaths/min)	$EtCO_2$ (mmHg)	RT (°C) pre-operation	post-operation
Castration	Control	0.41 ± 0.16	97 ± 17	114 ± 7	11.3 ± 1.0	37.2 ± 3.6	38.2 ± 1.0	37.2 ± 0.8
	Flu IM	0.46 ± 0.04	98 ± 16	109 ± 12	11.2 ± 2.3	33.4 ± 1.0	33.2 ± 4.5	36.8 ± 0.3
	Ati100 IM	0.65 ± 0.13	99 ± 24	93 ± 25	11.0 ± 2.7	35.3 ± 3.2	38.2 ± 0.4	37.2 ± 0.5
	Ati200 IM	0.70 ± 0.23	108 ± 8	114 ± 18	12.2 ± 2.6	35.3 ± 3.2	37.9 ± 1.2	37.2 ± 1.5
	Ati100+Flu IM	0.43 ± 0.03	100 ± 18	87 ± 13	10.8 ± 1.7	33.5 ± 3.3	37.8 ± 0.2	36.8 ± 0.4
	Ati200+Flu IM	0.40 ± 0.05	99 ± 18	110 ± 14	11.2 ± 2.6	34.7 ± 4.6	38.3 ± 0.9	36.8 ± 1.1
	Flu IV	0.41 ± 0.02	102 ± 13	121 ± 25	10.8 ± 2.4	33.6 ± 5.3	37.6 ± 0.7	36.9 ± 0.6
	Ati100 IV	0.46 ± 0.06	99 ± 18	111 ± 23	9.8 ± 1.8	35.4 ± 4.0	38.2 ± 0.9	37.4 ± 0.9
	Ati100+Flu IV	0.44 ± 0.04	91 ± 29	106 ± 23	10.2 ± 1.5	33.2 ± 4.5	38.0 ± 0.5	37.2 ± 0.4
OHE	Control	0.69 ± 0.31	109 ± 28	104 ± 13	9.2 ± 1.6	30.1 ± 2.7	38.5 ± 0.7	36.6 ± 1.0
	Flu IM	0.43 ± 0.17	98 ± 14	99 ± 16	8.8 ± 0.8	28.3 ± 2.8	37.9 ± 0.7	36.0 ± 0.9
	Ati100 IM	0.68 ± 0.24	91 ± 15	92 ± 16	8.2 ± 0.4	27.6 ± 3.6	37.9 ± 0.4	36.3 ± 0.4
	Ati200 IM	0.70 ± 0.20	111 ± 21	90 ± 14	9.8 ± 1.2	26.3 ± 1.3	37.8 ± 0.4	36.3 ± 0.5
	Ati100+Flu IM	0.50 ± 0.08	102 ± 24	103 ± 10	10.3 ± 2.5	27.3 ± 2.6	37.4 ± 0.5	35.9 ± 0.9
	Ati200+Flu IM	0.43 ± 0.03	97 ± 8	90 ± 11	10.0 ± 1.4	27.5 ± 2.1	37.7 ± 0.4	36.1 ± 0.6
	Flu IV	0.43 ± 0.03	101 ± 16	118 ± 13	8.8 ± 1.0	26.0 ± 1.6	37.2 ± 0.5	36.0 ± 0.4
	Ati100 IV	0.43 ± 0.05	116 ± 20	119 ± 12	10.3 ± 1.9	27.2 ± 1.9	38.3 ± 0.6	36.5 ± 0.5
	Ati100+Flu IV	0.44 ± 0.06	93 ± 26	117 ± 17	9.7 ± 1.4	29.0 ± 1.8	37.8 ± 0.6	35.9 ± 1.0

Values represent mean ± SD of six cats; OHE = ovariohysterectomy; IM = intramuscularly; IV = intravenously; Ati = atipamezole; Flu = flumazenil.

Behavioral recovery scoring: We assessed the overall quality of recovery from anesthesia using a previously published scoring method (Ueoka and Hikasa, 2015), as follows: score 1 = excellent; score 2 = good; score 3 = moderate; score 4 = poor; score 5 = extremely poor. The observer was blind to treatment. Time to extubation and head-up motion after administering these potential antagonists were also assessed in all groups.

Blood sample collection: Blood samples (2 mL) were collected from the jugular or cephalic vein on five occasions: before pretreatment (baseline), after completing the surgical procedure while under anesthesia (post-operatively) and 10, 60, and 120 mins after the administration of the potential antagonists (recovery phases after the discontinuation of anesthesia). Post-operative blood samples were collected 25 mins after isoflurane anesthesia in the castration groups and 55 mins after isoflurane anesthesia in the ovariohysterectomy groups.

Sample processing and analysis: Blood was mixed with ethylenediaminetetraacetic acid to prevent clotting. Samples were immediately centrifuged to separate the plasma, which was frozen at −76 °C until analysis. We assessed glucose, NEFA, cortisol, adrenaline and noradrenaline levels according to previously published methods (Kanda and Hikasa 2008[a]; Ueoka and Hikasa, 2015). In brief, glucose and NEFA levels were determined by an enzyme assay and a spectrophotometer. A solid phase-antibody radioimmunoassay measured cortisol levels. Catecholamines were extracted on activated alumina and measured using high-performance liquid chromatography and an electrochemical detector.

Statistical analysis: All data was analyzed using Prism 7.0 (GraphPad, CA). All data is presented as mean ± SD. All data (other than score data) was tested for normality using the Shapiro-Wilk test. A repeated-measures one-way analysis of variance (ANOVA) was

used to examine differences across variables within each group. *Post hoc* Dunnett's multiple comparisons tests were used to identify differences from the baseline within each group. One-way ANOVA and *post hoc* Tukey's multiple comparisons tests were used to determine differences across groups. In all tests, the significance level was set at $P < 0.05$. Score data was analyzed using the Wilcoxon–Mann–Whitney test for treatment comparisons; $P < 0.00556$ was considered significant using a Bonferroni correction.

Results

Adrenaline: In all groups of castrated cats, the concentration of adrenaline either decreased or tended to decrease post-operatively compared with the baseline levels (Table 2). In the control group, adrenaline was lower 10 mins post-saline injection than at the baseline and after that increased gradually at 120 mins compared to post-operation. In the groups that received Ati and/or Flu, adrenaline concentrations 10 mins post-antagonist injection did not differ from the baseline values. In combined groups with Ati100 or Ati200, adrenaline was higher at 60 mins compared to post-operation. There were no differences in adrenaline between the groups at any time.

In all groups of ovariohysterectomized cats, adrenaline either decreased or tended to decrease post-operatively compared with the baseline levels (Table 2). In the control group, adrenaline was lower 10 mins post-saline injection than at the baseline but higher at 60 mins than post-operation. In groups that received Ati and/or Flu, adrenaline concentrations 10 mins after antagonist administration were not different from baseline values. In the Ati100 IV group, adrenaline was higher 10 mins after antagonist administration than post-operation and higher than that of the control group. There were no other differences between the groups at any time.

Noradrenaline: In all groups of castrated cats, noradrenaline decreased or tended to decrease post-operatively compared with baseline levels (Table 3). In the control group, noradrenaline was lower 10 mins after saline injection than at the baseline, whereas in groups injected with potential antagonists, noradrenaline did not differ from the baseline. There were no differences in noradrenaline between the groups at any time.

Table 2 Effects of atipamezole (Ati) and flumazenil (Flu), both alone and in combination, on plasma adrenaline concentrations (ng/ml) in isoflurane-anesthetized cats premedicated with medetomidine (50 µg/kg), midazolam (0.5 mg/kg), and ketamine (5 mg/kg) undergoing ovariohysterectomy or castration.

Surgery	Group	Baseline	During anesthesia Post-operation	After antagonist administration (min)		
				10	60	120
Castration	Control	0.25 ± 0.16	0.11 ± 0.08*	0.12 ± 0.12*	0.27 ± 0.26	0.27 ± 0.19†
	Flu IM	0.25 ± 0.14	0.14 ± 0.13*	0.16 ± 0.11	0.37 ± 0.16	0.33 ± 0.27
	Ati100 IM	0.23 ± 0.13	0.08 ± 0.06*	0.15 ± 0.13	0.32 ± 0.18†	0.20 ± 0.10
	Ati200 IM	0.27 ± 0.09	0.13 ± 0.05*	0.17 ± 0.14	0.33 ± 0.15†	0.24 ± 0.10
	Ati100+Flu IM	0.21 ± 0.07	0.11 ± 0.06	0.19 ± 0.13	0.38 ± 0.56	0.51 ± 0.80
	Ati200+Flu IM	0.32 ± 0.13	0.15 ± 0.06*	0.25 ± 0.10	0.63 ± 0.78†	0.63 ± 0.44†
	Flu IV	0.30 ± 0.11	0.11 ± 0.08*	0.14 ± 0.09	0.31 ± 0.30	0.31 ± 0.27
	Ati100 IV	0.33 ± 0.19	0.11 ± 0.05*	0.24 ± 0.26	0.25 ± 0.22	0.23 ± 0.14
	Ati100+Flu IV	0.26 ± 0.08	0.13 ± 0.06*	0.22 ± 0.08	0.31 ± 0.11†	0.28 ± 0.23
Ovariohysterectomy	Control	0.27 ± 0.12	0.14 ± 0.10*	0.08 ± 0.13*	0.46 ± 0.12†	0.40 ± 0.22†
	Flu IM	0.26 ± 0.09	0.13 ± 0.09*	0.19 ± 0.15	0.41 ± 0.31	0.31 ± 0.23
	Ati100 IM	0.22 ± 0.09	0.12 ± 0.12	0.16 ± 0.16	0.64 ± 0.45†	0.71 ± 1.00†
	Ati200 IM	0.36 ± 0.15	0.22 ± 0.17	0.28 ± 0.23	0.37 ± 0.19	0.42 ± 0.28
	Ati100+Flu IM	0.22 ± 0.08	0.10 ± 0.05	0.30 ± 0.39	0.33 ± 0.19	0.21 ± 0.14
	Ati200+Flu IM	0.30 ± 0.14	0.08 ± 0.03*	0.22 ± 0.15	0.40 ± 0.26	0.52 ± 0.28†
	Flu IV	0.33 ± 0.11	0.17 ± 0.07	0.19 ± 0.21	0.41 ± 0.20	0.54 ± 0.32†
	Ati100 IV	0.27 ± 0.13	0.10± 0.08	0.51 ± 0.33†‡	0.34 ± 0.20	0.49 ± 0.38†
	Ati100 IV	0.30 ± 0.13	0.14 ± 0.07	0.21 ± 0.14	0.39 ± 0.33	0.40 ± 0.34

Values represent mean ± SD of six cats; IM = intramuscularly; IV = intravenously; * significantly different from baseline; † significantly different from post-operation; ‡ significantly different from control; the significance level is $P < 0.05$.

In all groups of ovariohysterectomized cats, noradrenaline decreased or tended to decrease post-operatively compared with the baseline (Table 3). In the control group, noradrenaline was lower 10 mins after saline injection than at the baseline. In the Flu IV group, noradrenaline was lower 10 mins after injection than at the baseline and higher at 120 mins than post-operation. In the Ati100 IM and Ati200 IM groups, noradrenaline was higher 60 mins after Ati injection than post-operation; similar changes were observed in the Ati100 + Flu IV groups. In the Ati100 IV group, noradrenaline was higher 10 mins after injection compared to post-operation and higher than in both the control and Flu IV groups. There were no other differences in noradrenaline between the groups at any time.

Cortisol: In all groups of castrated cats, cortisol decreased or tended to decrease post-operation and 10 mins after injection compared with the baseline levels (Table 4). Cortisol was higher 60 and/or 120 mins after injection than post-operation in the control, Flu IM, Ati200 IM, Ati200 + Flu IM, and Ati100 + Flu IV groups; however, there were no differences in cortisol between the groups at any time.

In all groups of ovariohysterectomized cats, cortisol decreased or tended to decrease post-operation compared to the baseline (Table 4). Cortisol was lower or tended to be lower 10 mins after injection compared

to the baseline levels in all groups, except for the Ati100 IV group, in which cortisol was higher at this time point compared to post-operation. In all groups, cortisol was higher or tended to be higher 60 and/or 120 mins after injection compared with the baseline and/or post-operation in all groups. At 10 mins after injection, cortisol was higher the Ati100 IV group than all other groups, including the control. There were no other differences in cortisol concentrations between the groups at any time.

Table 3 Effects of atipamezole (Ati) and flumazenil (Flu), both alone and in combination, on plasma adrenaline concentrations (ng/ml) in isoflurane-anesthetized cats premedicated with medetomidine (50 µg/kg), midazolam (0.5 mg/kg), and ketamine (5 mg/kg) undergoing ovariohysterectomy or castration.

Surgery	Group	Baseline	During anesthesia Post-operation	After antagonist administration (min)		
				10	60	120
Castration	Control	0.46 ± 0.23	0.42 ± 0.29	0.12 ± 0.08*	0.27 ± 0.11	0.29 ± 0.18
	Flu IM	0.43 ± 0.13	0.23 ± 0.19	0.29 ± 0.17	0.43 ± 0.50	0.42 ± 0.31
	Ati100 IM	0.48 ± 0.29	0.20 ± 0.12	0.23 ± 0.19	0.50 ± 0.26	0.43 ± 0.27
	Ati200 IM	0.51 ± 0.30	0.29 ± 0.22	0.34 ± 0.21	0.67 ± 0.38†	0.35 ± 0.24
	Ati100+Flu IM	0.49 ± 0.20	0.30 ± 0.10	0.35 ± 0.31	0.76 ± 0.76	0.61 ± 0.82
	Ati200+Flu IM	0.43 ± 0.17	0.20 ± 0.09*	0.39 ± 0.15	0.40 ± 0.12	0.66 ± 0.72
	Flu IV	0.43 ± 0.31	0.10 ± 0.06*	0.35 ± 0.31	0.42 ± 0.53	0.41 ± 0.18†
	Ati100 IV	0.49 ± 0.30	0.19 ± 0.17	0.30 ± 0.30	0.29 ± 0.33	0.24 ± 0.15
	Ati100+Flu IV	0.42 ± 0.29	0.13 ± 0.05	0.18 ± 0.09	0.31 ± 0.28	0.34 ± 0.44
Ovariohysterectomy	Control	0.49 ± 0.22	0.18 ± 0.12*	0.16 ± 0.07*	0.46 ± 0.38	0.47 ± 0.52
	Flu IM	0.44 ± 0.22	0.28 ± 0.13	0.37 ± 0.25	0.46 ± 0.36	0.34 ± 0.26
	Ati100 IM	0.51 ± 0.28	0.25 ± 0.25	0.57 ± 0.42	0.72 ± 0.58†	1.10 ± 1.49
	Ati200 IM	0.46 ± 0.28	0.21 ± 0.08*	0.34 ± 0.21	0.76 ± 0.51†	0.46 ± 0.22
	Ati100+Flu IM	0.44 ± 0.20	0.25 ± 0.10	0.35 ± 0.44	0.46 ± 0.34	0.49 ± 0.64
	Ati200+Flu IM	0.44 ± 0.25	0.21 ± 0.10	0.27 ± 0.23	0.21 ± 0.10	0.41 ± 0.12
	Flu IV	0.49 ± 0.28	0.14 ± 0.10*	0.16 ± 0.08*	0.18 ± 0.06	0.58 ± 0.34†
	Ati100 IV	0.51 ± 0.22	0.21 ± 0.16	1.55 ± 1.26†‡	0.61 ± 0.68	0.80 ± 0.90
	Ati100 IV	0.42 ± 0.13	0.27 ± 0.19	0.38 ± 0.26	0.71 ± 0.80	0.70 ± 0.51

Values represent mean ± SD of six cats; IM = intramuscularly; IV = intravenously; * significantly different from baseline; † significantly different from post-operation; ‡ significantly different from control or Flu IV; the significance level is $P < 0.05$.

Table 4 Effects of atipamezole (Ati) and flumazenil (Flu), both alone and in combination, on plasma cortisol concentrations (µg/dl) in isoflurane-anesthetized cats premedicated with medetomidine (50 µg/kg), midazolam (0.5 mg/kg), and ketamine (5 mg/kg) undergoing ovariohysterectomy or castration.

Surgery	Group	Baseline	During anesthesia Post-operation	After antagonist administration (min)		
				10	60	120
Castration	Control	4.9 ± 3.4	1.9 ± 1.2	1.2 ± 0.9*	5.5 ± 3.9	6.1 ± 2.1†
	Flu IM	5.2 ± 2.4	1.6 ± 0.8*	1.3 ± 0.5*	6.3 ± 2.6†	5.5 ± 2.5†
	Ati100 IM	4.8 ± 2.1	1.4 ± 0.6*	1.1 ± 0.4*	2.6 ± 2.8	3.2 ± 1.2
	Ati200 IM	4.6 ± 1.5	1.7 ± 1.1*	1.7 ± 0.7*	3.9 ± 1.0	4.8 ± 1.8†
	Ati100+Flu IM	5.9 ± 2.5	2.0 ± 0.8	1.7 ± 1.1*	4.4 ± 3.7	5.4 ± 4.0
	Ati200+Flu IM	4.6 ± 2.3	1.7 ± 0.7	1.6 ± 0.9	3.5 ± 0.7†	3.0 ± 0.6
	Flu IV	4.6 ± 1.0	1.8 ± 0.6*	1.5 ± 0.7*	4.8 ± 3.0	5.4 ± 2.1
	Ati100 IV	5.0 ± 2.9	1.8 ± 0.8	1.7 ± 1.1	3.1 ± 3.2	4.0 ± 2.9
	Ati100+Flu IV	4.6 ± 1.5	1.7 ± 0.9*	1.5 ± 0.6*	4.6 ± 1.8†	4.9 ± 1.5†
Ovariohysterectomy	Control	4.9 ± 1.4	2.0 ± 1.1*	2.0 ± 1.9*	7.9 ± 2.8†	9.4 ± 3.0*†
	Flu IM	4.9 ± 2.2	1.4 ± 1.3*	2.2 ± 2.5	9.7 ± 2.1†	8.7 ± 3.2†
	Ati100 IM	5.0 ± 2.2	2.1 ± 1.7	2.2 ± 1.6	8.6 ± 2.7†	8.9 ± 2.8*†
	Ati200 IM	4.7 ± 2.3	1.4 ± 0.8*	1.5 ± 0.8	9.8 ± 2.0*†	8.3 ± 2.9*†
	Ati100+Flu IM	4.2 ± 1.9	1.1 ± 0.5*	2.1 ± 1.2	8.2 ± 2.3*†	7.8 ± 2.9*†
	Ati200+Flu IM	4.3 ± 2.6	1.6 ± 1.0	2.1 ± 1.5	7.6 ± 2.6†	5.8 ± 2.7†
	Flu IV	4.3 ± 1.2	1.9 ± 0.8*	1.4 ± 0.8*†	7.7 ± 3.7	6.2 ± 3.8
	Ati100 IV	4.5 ± 0.9	1.7 ± 1.0*	5.9 ± 2.3†‡	5.8 ± 2.5	5.8 ± 2.3
	Ati100 IV	4.5 ± 1.7	1.2 ± 1.2*	1.0 ± 1.3*	6.8 ± 2.6†	5.7 ± 2.5†

Values represent mean ± SD of six cats; IM = intramuscularly; IV = intravenously; * significantly different from baseline; † significantly different from post-operation; ‡ significantly different from control, Flu IM, Ati100 IM, Ati200 IM, Ati100+Flu IM, Ati200+Flu IM, Flu IV or Ati100+Flu IV; the significance level is $P < 0.05$.

Glucose: In all groups of castrated cats, glucose concentration increased post-operation and 10 mins after injection compared with the baseline levels (Table 5). In the control group, glucose returned to the baseline levels 120 mins after saline injection, whereas in all other groups, it was lower 60 mins after injection than post-operation. There were no differences in glucose concentrations between the groups at any time.

In ovariohysterectomized cats, glucose was higher post-operation and 10 mins after injection than the baseline in all groups other than the Ati100 IV and Ati100 + Flu IV groups. It did not differ 10 mins after injection (Table 5). Elevated glucose concentrations returned to nearly baseline values 60 and 120 mins after injection in all groups. There were no differences in glucose concentrations between the groups at any time.

NEFA: In all castrated and ovariohysterectomized cats, NEFA concentration decreased or tended to decrease post-operation compared with the baseline levels (Table 6). NEFA concentration was lower 10 mins after discontinuing anesthesia, although sometimes insignificantly, in all groups other than the Ati100 IV group, in which it returned to baseline levels after 10 mins. In all groups, NEFA was greater 60 and/or 120 mins after injection compared to post-operation.

Table 5 Effects of atipamezole (Ati) and flumazenil (Flu), both alone and in combination, on plasma glucose concentrations (mg/dl) in isoflurane-anesthetized cats premedicated with medetomidine (50 µg/kg), midazolam (0.5 mg/kg), and ketamine (5 mg/kg) undergoing ovariohysterectomy or castration.

Surgery	Group	Baseline	During anesthesia Post-operation	After antagonist administration (min)		
				10	60	120
Castration	Control	86 ± 16	188 ± 66*	233 ± 78*	124 ± 64	69 ± 8†
	Flu IM	98 ± 23	270 ± 44*	281 ± 76*	93 ± 36†	81 ± 23†
	Ati100 IM	93 ± 11	229 ± 73*	221 ± 88*	93 ± 36†	81 ± 16†
	Ati200 IM	84 ± 22	238 ± 69*	236 ± 59*	107 ± 47†	82 ± 14†
	Ati100+Flu IM	99 ± 14	281 ± 67*	247 ± 62*	95 ± 32†	87 ± 21†
	Ati200+Flu IM	91 ± 18	327 ± 91*	266 ± 87*	89 ± 22†	93 ± 17†
	Flu IV	91 ± 26	233 ± 58*	273 ± 82*	87 ± 14†	75 ± 16†
	Ati100 IV	93 ± 31	270 ± 94*	279 ± 144*	109 ± 24†	85 ± 33†
	Ati100+Flu IV	81 ± 34	248 ± 106*	234 ± 98*	95 ± 36†	72 ± 10†
Ovariohysterectomy	Control	79 ± 9	160 ± 74*	177 ± 56*	109 ± 23	103 ± 28
	Flu IM	82 ± 19	226 ± 50*	228 ± 49*	119 ± 45†	114 ± 16†
	Ati100 IM	82 ± 26	175 ± 40*	172 ± 50*	120 ± 22†	118 ± 24†
	Ati200 IM	95 ± 26	190 ± 84*	199 ± 86	129 ± 58	107 ± 26
	Ati100+Flu IM	87 ± 11	232 ± 86*	232 ± 92*	127 ± 19†	114 ± 20†
	Ati200+Flu IM	85 ± 33	235 ± 44*	185 ± 55*	119 ± 51†	99 ± 18†
	Flu IV	85 ± 26	228 ± 55*	251 ± 39*	97 ± 61†	103 ± 42†
	Ati100 IV	81 ± 26	233± 73*	158 ± 117	120 ± 39	101 ± 27†
	Ati100 IV	81 ± 34	177 ± 84*	148 ± 59	120 ± 39	104 ± 22†

Values represent mean ± SD of six cats; IM = intramuscularly; IV = intravenously; * significantly different from baseline; † significantly different from post-operation; the significance level is $P < 0.05$.

Table 6 Effects of atipamezole (Ati) and flumazenil (Flu), both alone and in combination, on plasma non-esterified fatty acid concentrations (µEq/l) in isoflurane-anesthetized cats premedicated with medetomidine (50 µg/kg), midazolam (0.5 mg/kg), and ketamine (5 mg/kg) undergoing ovariohysterectomy or castration

Surgery	Group	Baseline	During anesthesia Post-operation	After antagonist administration (min)		
				10	60	120
Castration	Control	427 ± 153	156 ± 77	172 ± 103	535 ± 254	988 ± 422*†
	Flu IM	536 ± 243	230 ± 90	190 ± 52	615 ± 454	821 ± 500†
	Ati100 IM	416 ± 283	121 ± 77*	118 ± 100*	428 ± 196†	591 ± 274†
	Ati200 IM	401 ± 169	103 ± 54*	136 ± 58	415 ± 266†	668 ± 136†
	Ati100+Flu IM	524 ± 128	197 ± 57	317 ± 277	858 ± 393†	857 ± 361†
	Ati200+Flu IM	563 ± 199	213 ± 145*	259 ± 174*	859 ± 487†	677 ± 217†
	Flu IV	410 ± 218	197 ± 136	223 ± 126	685 ± 188†	852 ± 259*†
	Ati100 IV	497 ± 251	164 ± 92*	427 ± 444	643 ± 299†	763 ± 382†
	Ati100+Flu IV	411 ± 128	213 ± 85	256 ± 101	543 ± 385	762 ± 456†
Ovariohysterectomy	Control	440 ± 202	151 ± 106*	186 ± 82	688 ± 309†	871 ± 243*†
	Flu IM	501 ± 88	169 ± 91*	198 ± 96*	611 ± 156†	773 ± 319†
	Ati100 IM	439 ± 237	103 ± 67	242 ± 439	716 ± 396†	630 ± 461†
	Ati200 IM	406 ± 176	166 ± 67	178 ± 108	565 ± 558	661 ± 284†
	Ati100+Flu IM	535 ± 339	126 ± 71*	176 ± 112*	559 ± 122†	755 ± 165†
	Ati200+Flu IM	472 ± 85	180 ± 61	255 ± 280	790 ± 261*†	864 ± 211*†
	Flu IV	442 ± 197	145 ± 82	232 ± 252	490 ± 317†	633 ± 412†
	Ati100 IV	434 ± 249	197 ± 48	471 ± 338	858 ± 326†	856 ± 403†
	Ati100 IV	402 ± 259	175 ± 92	199 ± 106	469 ± 248	798 ± 327*†

Values represent mean ± SD of six cats; IM = intramuscularly; IV = intravenously; * significantly different from baseline; † significantly different from post-operation; the significance level is $P < 0.05$.

Recovery time and behavioral recovery scores: In both castrated and ovariohysterectomized cats, recovery times to extubation and head-up were rapid in the groups listed in decreasing order as follows: Ati100 + Flu IV, Ati100 IV, Ati200 + Flu IM, Ati100 + Flu IM, Ati200 IM, Ati100 IM, Flu IV, Flu IM, and the control groups (Table 7). However, there were no differences in the recovery times of the Ati100 IM and Ati200 IM groups, the Ati100 + Flu IM and Ati200 + Flu IM groups and the Ati100 IV and Ati100 + Flu IV groups.

Behavioral scores were lower in the Ati100 IV and Ati100 + Flu IV groups than in the Ati100 IM and Ati200 IM groups in castrated cats (Table 8). A similar pattern was observed in ovariohysterectomized cats but these differences were not statistically significant.

Table 7 Recovery time after the administration of atipamezole (Ati) and flumazenil (Flu), both alone and in combination, in isoflurane-anesthetized cats premedicated with medetomidine (50 µg/kg), midazolam (0.5 mg/kg), and ketamine (5 mg/kg) undergoing ovariohysterectomy or castration.

Group	Time to extubation (min)		Time to head-up (min)	
	Castration	Ovariohysterectomy	Castration	Ovariohysterectomy
Control	26.0 ± 4.7	31.0 ± 7.3	69.7 ± 8.7	65.2 ± 9.6
Flu IM	14.7 ± 1.9*	17.0 ± 2.4*	51.9 ± 6.9*	49.8 ± 9.5*
Ati100 IM	8.7 ± 0.5*†	8.7 ± 0.8*†	18.2 ± 1.5*†	19.7 ± 1.0*†
Ati200 IM	7.3 ± 1.0*†	9.0 ± 1.4*†	15.5 ± 1.6*†	18.8 ± 0.8*†
Ati100+Flu IM	5.7 ± 1.0*†	6.2 ± 1.2*†	17.3 ± 2.3*†	16.5 ± 3.3*†
Ati200+Flu IM	4.7 ± 0.8*†§	5.5 ± 1.1*†	14.5 ± 2.9*†	14.5 ± 2.8*†
Flu IV	8.3 ± 2.5*†	9.7 ± 1.8*†	34.7 ± 4.0*†§‡||¶	38.8 ± 3.7*†§‡||‡
Ati100 IV	4.0 ± 0.9*†§#	3.8 ± 1.5*†#	8.8 ± 2.5*†§||#	8.0 ± 2.0*†§‡#
Ati100+Flu IV	2.8 ± 0.8*†§‡#	2.8 ± 0.4*†§‡#	7.2 ± 1.7*†§‡||#	7.2 ± 2.1*†§‡#

Values represent mean ± SD of six cats; IM = intramuscularly; IV = intravenously; * significantly different from control; † significantly different from Flu IM; § significantly different from Ati100 IM; ‡ significantly different from Ati200 IM; || significantly different from Ati100 + Flu IM; ¶ significantly different from Ati200 + Flu IM; # significantly different from Flu IV; the significance level is $P < 0.05$.

Table 8 Behavioral recovery scores after the administration of atipamezole (Ati) and flumazenil (Flu), both alone and in combination, in isoflurane-anesthetized cats premedicated with medetomidine (50 µg/kg), midazolam (0.5 mg/kg), and ketamine (5 mg/kg) undergoing ovariohysterectomy or castration.

Group	Recovery score	
	Castration	Ovariohysterectomy
Control	2 (2–3)	2 (2–3)
Flu IM	3 (1–4)	2 (2–3)
Ati100 IM	3 (3–4)	3 (3–4)
Ati200 IM	4 (3–4)	4 (3–4)
Ati100+Flu IM	2 (1–3)	3 (2–3)
Ati200+Flu IM	3 (2–3)	3 (2–3)
Flu IV	3 (2–3)	2 (2–3)
Ati100 IV	2 (1–2) *†	2 (1–3)
Ati100+Flu IV	2 (1–2) *†	2 (2–3)

Values represent the median (range) of six cats; IM = intramuscularly; IV = intravenously; * significantly different from Ati100 IM; † significantly different from Ati200 IM; the significance level is $P < 0.00556$ by Mann-Whitney U test and Bonferroni correction.

Discussion

The present study demonstrated that MMK-isoflurane anesthesia reduced plasma concentrations of adrenaline, noradrenaline, cortisol and NEFA. It also increased glucose concentrations in both castrated and ovariohysterectomized cats. This study's neurohormonal and metabolic changes are similar to those noted in previous studies of MMK-anesthetized cats that did not undergo surgery (Ueoka and Hikasa, 2015); the only difference noted here is that cortisol concentration decreased with MMK-isoflurane anesthesia. The present findings indicate that premedication with MMK can be useful for perioperatively suppressing sympathetic-adrenal activity, other than hyperglycemic effects, in isoflurane-anesthetized cats.

The rationale for using fixed dosing of Ati and Flu as antagonists for MMK has been outlined in our earlier study (Ueoka and Hikasa, 2008; Ueoka and Hikasa, 2015). In the present study, the antagonists' intravenous and intramuscular routes of administration were selected based on the immediate onset of action that is most often used during emergency anesthetic situations and on slower action with accelerated awakening time, respectively. The present study revealed that in both castrated and ovariohysterectomized cats, recovery times from anesthesia were rapid in the following order; Ati 100 + Flu IV ≤ Ati 100 IV < Ati 200 + Flu IM ≤ Ati 100 + Flu IM < Ati 200 IM ≤ Ati 100 IM < Flu IV < Flu IM < control. These findings indicate that, compared to IM administration, the IV delivery of Ati alone and in combination with Flu induces more rapid recovery from anesthesia, regardless of the timing of antagonist administration (beyond 50 mins after medetomidine administration in castrated cats and 80 mins in ovariohysterectomized cats). On the other hand, the present study revealed that castrated cats had a better quality of recovery from anesthesia after the IV administration of Ati alone and combined with Flu than with the IM administration of Ati alone. However, even with IM administration, the addition of Flu to Ati compared with Ati alone improved the quality of recovery. These results agree with a previous finding that the use of a combination of Flu and Ati, in comparison to Ati alone, improves the quality of recovery by reducing excitation and hyperaesthesia (Ebner *et al*., 2007).

In ovariohysterectomized cats, the IV administration of Flu alone did not affect changes to noradrenaline concentrations in the non-medicated control, demonstrating Flu itself does not have a great influence on reversing the inhibition of catecholamine release caused by MMK-isoflurane anesthesia. This result is consistent with previous findings of the effect of intravenous Flu against MMK anesthesia in cats that are not undergoing surgery (Ueoka and Hikasa, 2015). Conversely, the IV administration of Ati rapidly and greatly reversed the decrease in adrenaline and noradrenaline induced by MMK-isoflurane anesthesia in ovariohysterectomized cats. Still, this effect was not significant in castrated cats, suggesting that the effect of Ati on catecholamine release depends on the timing

of antagonist administration and the degree of surgical injury. IM administrations of Ati alone and Ati–Flu also accelerated recovery from the decreased adrenaline and noradrenaline concentrations induced by MMK-isoflurane anesthesia. Noradrenaline levels in groups that received Ati alone were greater 10 and/or 60 mins post-injection than those who received Ati–Flu combinations. The increase may be due to the effect of un-antagonized residual midazolam since IM administration of midazolam alone increases plasma adrenaline and noradrenaline concentrations in cats (Kanda and Hikasa, 2008[b]). Therefore, IM administration of Ati effectively reverses the inhibition of catecholamine release induced by MMK-isoflurane anesthesia in ovariohysterectomized cats.

In the present study, MMK-isoflurane anesthesia decreased plasma cortisol concentrations, indicating that it inhibits adrenocortical activity in both castrated and ovariohysterectomized cats. This may be mainly due to medetomidine since premedication with medetomidine is reported to reduce or delay the increase in plasma cortisol concentrations induced by ovariohysterectomy in dogs (Benson *et al*., 2000; Ko *et al*., 2000). In castrated cats, both the IM and IV administrations of Flu alone, Ati alone and combinations of Ati–Flu did not affect the changes in cortisol release observed in the control. On the other hand, in ovariohysterectomized cats, Ati IV reversed the decreased cortisol concentration induced by MMK-isoflurane anesthesia 10 mins post-injection, demonstrating that intravenous Ati rapidly increases adrenocortical activity after anesthesia and surgery; this effect was not observed in groups that received Ati injections IM. These results suggest that, similar to the effect on catecholamine release, the effect of Ati IV on the release of cortisol depends on the timing and route of administration as well as the degree of surgical injury. However, compared to Ati alone, Ati–Flu combinations IV did not produce a rapid increase in cortisol concentration in the present study. Although the precise reason for this pattern is unknown, the Ati–Flu combination may help prevent excessive adrenocortical activity after administration in cats anesthetized with MMK-isoflurane.

In the current study, moderate hyperglycemia was induced by MMK-isoflurane anesthesia in both castrated and ovariohysterectomized cats. This hyperglycemia may be responsible for several effects: medetomidine-induced inhibition of insulin release mediated by α_2-adrenoceptors (Kanda and Hikasa 2008a), surgical injuries, decreased insulin and decreased peripheral insulin use of glucose associated with inhalant anesthesia (Desborough, 2000). In the present study, Ati IV, both alone and in combination with Flu, reduced post-operative hyperglycemia in ovariohysterectomized cats more quickly. This result suggested that the effect of Ati is due to the suppression of medetomidine-induced hyperglycemia through the blockade of α_2-adrenoceptors. Other than this result, the IM and IV administrations of Ati, both alone and combined with Flu, did not affect the changes in post-operative blood glucose levels observed in the control group of castrated and ovariohysterectomized cats. This may be due to the timing of antagonist administration (50 mins after medetomidine administration in castrated cats and at 80 mins in ovariohysterectomized cats) as well as the post-operative surgical injuries.

Changes in NEFA concentrations are clinically significant metabolic indicators of the stress response since NEFA is affected by hormones like cortisol and catecholamines (Desborough, 2000). Lipolytic activity is stimulated by cortisol and catecholamines (Desborough, 2000) and inhibited by medetomidine (Kanda and Hikasa, 2008[a]). In the present study, NEFA concentrations were reduced by MMK-isoflurane anesthesia in both castrated and ovariohysterectomized cats, which may be attributable to the decreased catecholamine and cortisol levels and the inhibited lipolysis by medetomidine. The present study showed that Ati IV hastened recovery from post-operatively decreased NEFA concentrations. This may be related to the antagonism for medetomidine-induced inhibition of lipolysis and increased catecholamine and cortisol levels, as previously mentioned. Other than this, however, the IM administration of Ati alone and in combination with Flu did not affect the post-operative changes in NEFA concentration after MMK-isoflurane anesthesia in both castrated and ovariohysterectomized cats. This result indicated that the IM route of administration does not largely influence post-operative lipolysis.

In conclusion, MMK-isoflurane anesthesia induced the inhibition of catecholamine and cortisol release, the inhibition of lipolysis and hyperglycemia in castrated and ovariohysterectomized cats. Compared with intramuscular administration, the intravenous administration of Ati alone and in combination with Flu induces more rapid recovery from anesthesia, regardless of the timing of administration. Compared to Ati alone, the addition of Flu improved the quality of recovery. The IV administration of Ati alone effectively reverses the post-operative neurohormonal and metabolic effects induced by MMK-isoflurane anesthesia in cats undergoing ovariohysterectomy. The IM administration of Ati–Flu combinations can induce rapid recovery from anesthesia without largely altering the stress-related neurohormonal and metabolic changes induced by MMK-isoflurane anesthesia and surgery. The IV administration of Ati reversed the decreases in catecholamine and cortisol concentrations induced by MMK-isoflurane anesthesia in ovariohysterectomized cats but these effects were not significant in castrated cats. In addition, Ati IV, both alone and in combination with Flu, reduced post-operative hyperglycemia in ovariohysterectomized cats more quickly but this effect was not found in castrated cats. These results indicate that the effect of Ati on the release of catecholamine and cortisol and hyperglycemia depends on the timing and route of administration as well as the degree of surgical injury. This study is the first to demonstrate that differences in the route and timing of the administration of Ati and Flu as antagonists for MMK-isoflurane anesthesia alter post-operative stress-related hormonal and metabolic responses.

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