

A comparison of the immunological effects of propofol and isoflurane for maintenance of anesthesia in healthy dogs

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ABSTRACT. Most anesthetics have an immuno-suppressive effect on cellular and neurohumoral immunity, and research shows that total intravenous anesthesia (TIVA) with propofol has a greater immuno-protective effect than inhalational anesthesia in human medicine. However, in veterinary clinics, these effects remain ambiguous. To clarify the details, we focused on propofol and isoflurane, investigating clinical blood hematology and immunological profiles drawn from healthy dogs under and after two anesthesia techniques. Twelve healthy adult beagles were included in this study, randomly assigned to the propofol anesthesia group (group P: n=6) or the isoflurane anesthesia group (group I: n=6). In both groups, the number of lymphocytes in peripheral blood decreased after 2 hr of anesthesia (2 hr), but group P showed significantly less decrease than group I. For T-lymphocyte subsets examined by flowcytometry, the ratio of CD3+, CD4+ and CD8+ lymphocytes in the peripheral blood mononuclear cell (PBMC) of group P at 2 hr also exhibited a high level compared to group I. Moreover, for mRNA expression of cytokines measured by real-time PCR, the IL2 (pro-inflammatory cytokine) of group P showed no decrease like group I. The IL10 (anti-inflammatory cytokine) of group P also showed no increase like group I, while both cytokines maintained nearly the same level until 2 hr. These results suggest that, compared to propofol, isoflurane had more strongly immuno-suppression caused by anesthesia, and propofol itself might have some immuno-protective effects. Thus, TIVA with propofol might benefit immunological support in the perioperative period of dogs.

KEY WORDS: anesthesia, canine, immuno-suppression, isoflurane, propofol

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Anesthesia is widely-used in veterinary medical clinics as a support for clinical treatments and surgery; however, it is also well-known that anesthesia itself suppresses the immune system. Influencing the functions of immunocompetent cells and inflammatory mediator gene expression and secretion, most anesthetics have a direct suppressive effect on cellular and neurohumoral immunity [7]. Every anesthetic has different immunosuppressive effects, and researchers generally agree that total intravenous anesthesia (TIVA) is less immunosuppressive than inhalational anesthesia [11]. Most commonly used in veterinary medicine, propofol also has shown immunosuppressive effects, but fewer than the other volatile anesthetics; moreover, it has some protective influences on the immune system. For example, while inhalation anesthetics induced apoptosis in normal peripheral lymphocytes *in vitro* [9, 12], propofol showed less induction of apoptosis and cytotoxicity [26]. Propofol also exhibits less suppressive characteristics for neutrophil phagocytosis

compared with isoflurane [3], more activated T-helper cells in human lung cancer patients under anesthesia [19] and more protective effects for alveolar inflammatory response compared with desflurane [21]. As a result of these studies, many researchers have reported that TIVA, especially with propofol, has more beneficial immunological effects than volatile anesthetics in human medicine.

In veterinary medicine, TIVA and propofol have come to be generally used in many cases; however, there remain only a few reports of such beneficial effects [27], and the immunological details remain unclear. To clarify the differences in anesthetics' immuno-suppressive effects in dogs, we focused on propofol and isoflurane, investigating clinical blood hematology and biochemistry profiles drawn from healthy dogs under and after two different anesthesia techniques. Furthermore, we examined T-lymphocyte subsets for the immunological profiles, determining mRNA expression levels of inflammatory cytokines in peripheral blood mononuclear cells (PBMC), such as IL2, IL10, IL12 and IFN γ .

MATERIALS AND METHODS

Dogs: Twelve healthy adult beagles (6 males and 6 females) were included in the study. All dogs were kept in the animal facilities of our university, and mean age was 1.5 years old (range 1.2 to 3.2). The protocol was approved by the Committee on the Ethics of Animal Experiments of the

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Table 1. Sequences of primer pairs used for real-time RT PCR

	Forward (5' to 3')	Reverse (5' to 3')	Amplicon size (bp)	GenBank accession number
GAPDH	ATGATTCTACCCACGGCAAA	ATGGACTTCCCGTTGATGAC	76	NM_001003142
IL2	CAGAGCAACAGATGGAGCAA	TTATTAACCTCATTAAAAGCAACTG	61	NM_001003305
IL4	CAGATATCTCAGAGGACTCTACAGGA	TTCTTGATTTCATTCATAGAACAGGT	75	NM_001003159
IL6	CAGGACCCCAGCTATGAAC	GGGAAGCAGTAGGCCATCAC	78	XM_850499
IL10	TGGGAGAGAAGCTCAAGACC	TCTCACAGGGCAGAAATCG	66	NM_001003077.1
IL12p35	GGAAAGGCCTCTTTATGACG	CCATCTGGTACATCTCAAGTCC	70	NM_001003293
IL12p40	GCGTCTCCCTCATGACC	GGGTGCCAGTCCAACCTCTAC	68	NM_001003292
IFN γ	CGGTGGGTCTCTTCGTAG	TGGCTCTGAATGATTGTTTG	72	NM_001003174
TGF β	TGGCTGYCCTTGATGTCAC	CGAAAGCCCTCGACTCC	71	NM_001003309

Obihiro University of Agriculture and Veterinary Medicine (Permit number 24–89).

Anesthesia and instrumentation: The dogs were randomly assigned to the propofol anesthesia group (group P: n=6) or the isoflurane anesthesia group (group I: n=6). A catheter (surflo IV catheter, Terumo, Tokyo, Japan) was inserted into a cephalic vein before anesthesia. All dogs were allowed to breathe oxygen by face mask for 5 min prior to anesthesia.

Dogs in group P received midazolam (0.3 mg/kg, dormicum; Asteras, Tokyo, Japan) intravenously as a co-induction agent. Five min later, they were induced with propofol (6 mg/kg, Propofol 1%; Nichi-Iko, Toyama, Japan) administered by slow IV injection, at a rate of approximately 6 mg/kg/min. After endotracheal intubation (6–7 Fr induction tube, TOP, Tokyo, Japan), the dogs were connected to the small animal anesthesia machine (Acoma, Tokyo, Japan) with rebreathing circuit (MC Medical, Tokyo, Japan) using a 100% oxygen flow rate of 2 l/min. After induction, mechanical ventilation was started immediately using with pressure cycled respirator (COMPOS B-EV; Metran, Kawaguchi, Japan) at 13 mmH₂O of peak inspiratory pressure, 6/min of breathing frequency and 1.6 sec of inspiratory time. EtCO₂ was maintained within the reference range (35 to 45 mmHg). Anesthesia was maintained by continuous intravenous infusion of propofol (0.5 mg/kg/min) and fentanyl (0.2 μ g/kg/min). Both agents were diluted in normal saline (0.9% saline, Otsuka-Pharm, Tokyo, Japan) and administered by syringe pump (JMS syringe pump SP115, JMS co., Hiroshima, Japan) at 5 ml/kg/hr. While maintaining a state of anesthesia, monitoring occurred for heart rate, hemoglobin oxygen saturation (SpO₂), rectal temperature and EtCO₂ every 30 min by biological information monitor (Colin BP-608 Evolution, Omron, Tokyo, Japan). After 2 hr of maintenance, anesthesia was discontinued. Dogs were mechanically ventilated until signs of spontaneous ventilation were apparent, and extubated appropriately.

Dogs in group I received the same co-induction agent, induction, intubation, ventilation, monitoring and extubation. During maintenance of anesthesia for 2 hr, the dogs were maintained with isoflurane (Isoflu; Dainippon Sumitomo Pharm, Osaka, Japan) and fentanyl (0.2 μ g/kg/min, continuous intravenous infusion). The vaporizer's (Vaporizer MKIIIa, Acoma) setting was adjusted for end-tidal isoflurane concentration (EtISO) between 1.1 and 1.2%. Dogs were

transfused with normal saline at the same volume as group P. In both protocols, one anesthetist evaluated the adequate depth of anesthesia via muscle relaxation, eyeball position, palpebral reflex and pain sensation. In order to achieve a similar level of pain sensation, fentanyl was used in both groups at the same dose.

Blood sampling: Blood specimens were taken from the jugular vein of all dogs at the same time points, before anesthesia ('pre'), after anesthesia (2 hr), 1 day later (day 1), 3 days later (day 3) and 7 days later (day 7).

Hematology and biochemistry profiles: Five hundred μ l of blood specimens with EDTA were analyzed using an automatic cell counter (Celltac alpha, Nihon-Koden, Tokyo, Japan) to count RBC and WBC. Blood smears of each specimen were made and evaluated for a manual WBC differential. Hct and total protein (TP) were measured by hematocrit tube (Terumo) and refractometers (Master3 alpha, Terumo). Plasma was collected from 1 ml of heparinized blood specimens and measured for ALB, ALT, ALKP, BUN and CRE using the Vet Test 8008 chemistry analyzer (IDEXX, Tokyo, Japan). For Na, K and Cl, a Fuji DRI-CHEM 7000 (Fuji-Film, Tokyo, Japan) was used. Also, calculated was a decreasing rate compared to 'pre' (defined as 100) for RBC, WBC, TP and lymphocytes.

PBMC: Five ml of heparinized whole blood specimen was mixed with the same volume of phosphate buffered saline (PBS) and layered on 3 ml of lymphocyte separation medium (Nakarai, Kyoto, Japan). The sample was centrifuged at 2,000 rpm for 50 min at RT. PBMC from the upper part of the Ficoll layer were collected and washed with PBS before Lysing Buffer (BD Biosciences, San Jose, CA, U.S.A.) was added. After incubation for 5 min at RT, the sample was washed twice with PBS and counted with a hemocytometer (Improved Neubauer; Elma, Tokyo, Japan).

Flowcytometry: PBMC were adjusted at 1×10^6 cells /50 μ l before addition in each tube of a 5 μ l isotype control antibody, FITC-anti canine CD3 antibody and FITC-anti canine CD4/RPE-anti canine CD8 antibody (AbD serotec, Oxford, U.K.) (Table 1). After incubation, it was washed twice with PBS and analyzed for T-lymphocyte subsets using flowcytometry (EPICS XL, Beckman Coulter, Brea, CA, U.S.A.).

Total RNA and cDMA: To the PBMC, ISOGEN2 (Nippon gene, Toyama, Japan) was added and incubated for 5 min at RT. Samples were then homogenized with QIA-shredder-

TM250 (Qiagen, Venlo, The Netherlands), and the total RNA was collected according to the manufacturer's protocol. One μ g of total RNA was reverse transcribed using an oligo (dT) 12–18 (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) and ReverTra Ace (Toyobo, Osaka, Japan) for 10 min at 30°C, 60 min at 42°C and 5 min at 99°C. The final volume of cDNA was 50 μ l.

Real-time PCR: Real-time PCR was performed on a LightCyclerTM instrument (Roche, Mannheim, Germany) using the Universal Library Probes (#91 (IFNgamma), #120 (IL2), #146 (IL4), #133 (IL6), #98 (IL12p35), #154 (IL12p40), #111 (IL10, GAPDH), #91 (IFN γ) and #102 (TGF β), Roche). All primers were designed with an assay design center (Roche), according to the canine mRNA sequence of each cytokine (Table 1). PCR conditions were 1 cycle of 95°C for 10 min, 45 cycles at 95°C for 10 sec, 60°C for 30 sec and 1 cycle at 40°C for 30 sec. GAPDH was used as an internal standard. To examine each expression level, the 'pre' value was defined as 1. All samples were examined independently in two separate experiments.

Statistical analysis: A comparison between 2 groups in the study was performed with the Mann-Whitney *U* test, and statistical significance was set at $P<0.05$.

RESULTS

Monitoring: The heart rate of group P decreased significantly less than that of group I. It was 91 ± 13.4 at 0 min, decreased to 66 ± 10.0 after 30 min and maintained this low rate until 120 min. On the other hand, the heart rate of group I didn't change significantly during anesthesia (Fig. 1a). There was no significant difference in SpO₂, EtCO₂ or temperature during anesthesia between the two groups. The time of awakening was 21 ± 5.8 min in group P and 22 ± 5.1 min in group I, also showing no difference.

Hematology and Biochemistry profile: In both groups, the RBC significantly decreased at 2 hr compared with the value at 'pre'. It then recovered at day 1 (Fig. 1b). RBC also showed a similar tendency in TP and ALB (Fig. 1c and 1d). While the WBC of group P slightly decreased at 2 hr compared with 'pre', that of group I decreased more significantly at 2 hr (Fig. 1e). The number of lymphocytes in group P was significantly higher than that in group I at 2 hr, still maintained at day 1 (Fig. 1f). There was no significant difference in ALT, ALKP, BUN, CRE, Na, K or Cl (data not shown). From the above results, we focused on the decrease at 2 hr in some parameters, calculating the decreasing ratio from 'pre' to 2 hr. There were no significant differences between the two groups in the ratios of TP and RBC; however, the ratio of lymphocytes in group P was significantly lower than in group I (P: 28.3%, I: 55.7%, Fig. 1g).

T-lymphocytes subsets in PBMC: According to the comparison of lymphocytes between groups P and I, we hypothesized that propofol would show some protective effects on lymphocytes in dogs compared with isoflurane. To clarify the details, we examined the T-lymphocyte subsets in PBMC. For group P, the number of CD3+ lymphocytes in PBMC was slightly decreased at 2 hr compared with

'pre'. For group I, it remarkably decreased at 2 hr (Fig. 2a). Moreover, CD3+ lymphocytes of group P at 2 hr exhibited significantly higher than that of group I ($P<0.05$), which was still maintained at day 1. This tendency was also exhibited in the number of CD4+ and CD8+ lymphocytes (Fig. 2b and 2c).

Quantitative analysis of cytokine mRNA by real-time RT PCR: The expression levels of cytokines in PBMC are shown in Fig. 3. In IL2, one of the stimulatory cytokines, group P maintained the same level compared to 'pre' at 2 hr; however, group I showed a marked decrease from 'pre' to 2 hr. In addition, at day 1, IL2 of both groups exhibited still decreased levels compared to 'pre' (Fig. 3a). In IL10, while group P at 2 hr also maintained the same level compared to at 'pre', group I showed an increase from 'pre' to 2 hr (Fig. 3b). Moreover, in IL12p35, IL12p40 and IFN γ , both groups showed no change at 2 hr, but group I exhibited a marked increase at day 3 compared with group P (Fig. 3c–3e). At the IL4, IL6 and TGF β mRNA expression level, there were no significant differences between the groups (data not shown).

DISCUSSION

Within the monitoring profiles, heart rates in group P showed a greater decrease than in group I. One of the cause for this observation was the medicinal action of the drug; propofol itself attenuates inotropic response to beta-adrenoceptor stimulation, and propofol, combined with fentanyl, reduced heart rates in humans [1]. However, one study reported that no bradycardia occurred in dogs under TIVA with propofol and fentanyl, with heart rates between 80 to 120 beat/min [30]. In that report, propofol was used at 0.2–0.4 mg/kg/min, less than in our study (0.5 mg/kg/min), therefore explaining this difference and why bradycardia occurred in group P. On the other hand, fentanyl may cause bradycardia, due to opioid-induced medullary vagal stimulation [14], in contrast, isoflurane increased heart rates in anesthetized dogs by decreasing cardiac vagal activity [16]. Therefore, isoflurane may be associated with the smaller decrease in heart rate seen in group I, in spite of using fentanyl simultaneously.

In both groups, all hematological parameters (TP, RBC and lymphocytes) decreased at 2 hr compared with 'pre'. This finding could result from blood dilution. It has already been reported that both propofol and isoflurane cause vaso-dilation during anesthesia in human patients [20, 24] and in dogs [15]. Moreover, the transfusion of crystalloid solution itself has been shown to cause blood dilution during anesthesia [18]. However, in this study, blood pressure was not monitored under anesthesia. Intraoperative hypotension, caused by anesthesia, may suppress the immune system in humans [31] and in rats [4]. Thus, this hypotension may have affected our results. This measurement would be important and helpful for future research in clarifying the details of this issue.

The change ratios of RBC between 'pre' and 2 hr in both groups were higher than that of TP, but not significantly. One study reported that the splenic size was enlarged in dogs un-

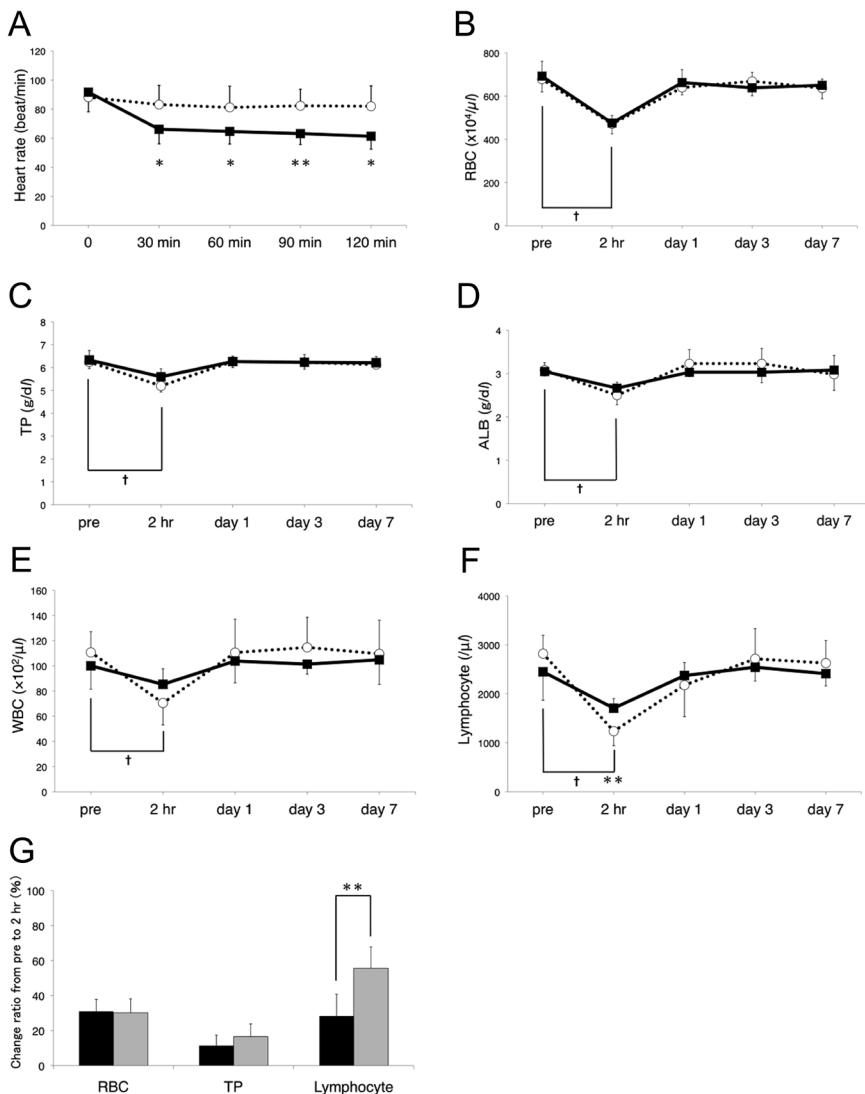


Fig. 1. Graphs illustrating monitoring and clinical hematatology/biochemistry profiles, (A) the heart rate during anesthesia, (B–E) RBC, TP, ALB, WBC and lymphocyte in peripheral blood before and after anesthesia. The Black square (■) with solid line represents group P, and the white circle (○) with dotted line represents group I. (G) The change ratio from ‘pre’ to 2 hr of RBC, TP and lymphocyte. The black and dark boxes represent groups P and I, respectively. * $P<0.05$, ** $P<0.01$ between groups P and I. † $P<0.01$ between ‘pre’ and 2 hr.

der anesthesia [29], indicating that RBC might be trapped in the spleen at 2 hr. However, there was a marked decrease in lymphocytes at 2 hr, the change ratio of group P was significantly lower than group I. It is impossible to account for this phenomenon simply with vasodilution; it could be related to some immunological protective effect of propofol.

In human medicine, both propofol and isoflurane have shown decreased lymphocytes in the immune response to hysterectomy, and even less in TIVA with propofol [17]. However, most of these reports were related to surgery, not only anesthesia. On the other hand, *in vitro*, isoflurane-induced apoptosis of lymphocytes [28] and propofol did not interfere with the proliferation of T-lymphocytes [2].

Therefore, our results indicating a different change ratio in lymphocytes might be associated with the differences of immunological effect between propofol and isoflurane.

During the perioperative period, it is important to maintain a high number of lymphocytes in PBMC to prevent infectious diseases and early recovery from surgery, especially in cancer patients [5]. The immunosuppressive effects for lymphocytes have been associated with the production of pro- and anti-inflammatory cytokines [8]. To investigate the details of this issue, we focused on T-lymphocyte, which plays a critical role in cellular immune response during anesthesia; we also examined its subset and expression of cytokine.

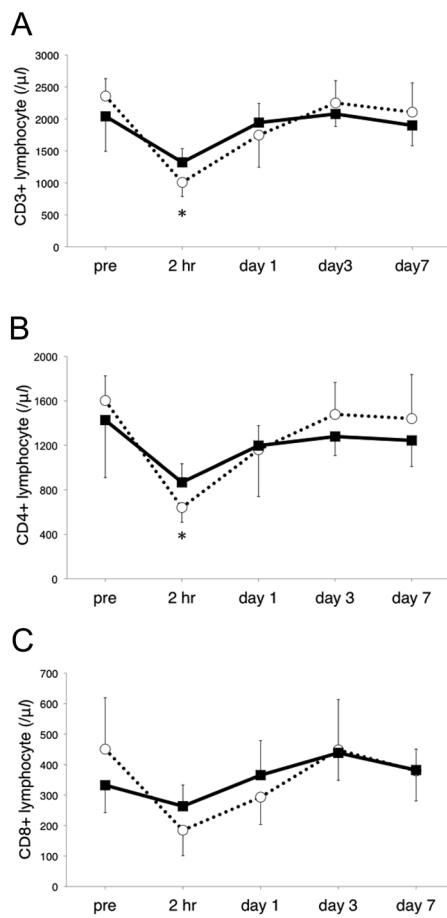


Fig. 2. Graphs illustrating the number of (A) CD3+, (B) CD4+ and (C) CD8+ lymphocytes in PBMC. The Black square (■) with solid line represents group P, and the white circle (○) with dotted line represents group I. * $P<0.05$ between groups P and I.

For T-lymphocyte subsets, studies have compared TIVA and sevoflurane in human patients, showing that the number of CD3+, CD4+ and CD8+ T-lymphocytes after TIVA showed less decrease than after sevoflurane anesthesia [22]. In our study, CD3+, CD4+ and CD8+ T-lymphocytes were also decreased at 2 hr in both groups, but group P showed a more moderate decrease than group I. Afterwards, group P recovered at day 1 at the same level as 'pre', while group I continued at a lower level until day 3. These results suggest that propofol had some immuno-protective effect in terms of the decrease of T-lymphocytes after anesthesia.

For mRNA expression of cytokines, the IL2 of group P showed no decrease like group I, maintaining the same level of 'pre' until 2 hr. IL2 is a pro-inflammatory cytokine, and in human medicine, researchers also observed no decrease after TIVA with propofol, compared to the decrease following sevoflurane anesthesia [23]. On the other hand, IL10 is thought to be an anti-inflammatory cytokine, and in our study, the IL10 of group P showed no increase like group I, also maintaining the same level of 'pre' until 2 hr. In human

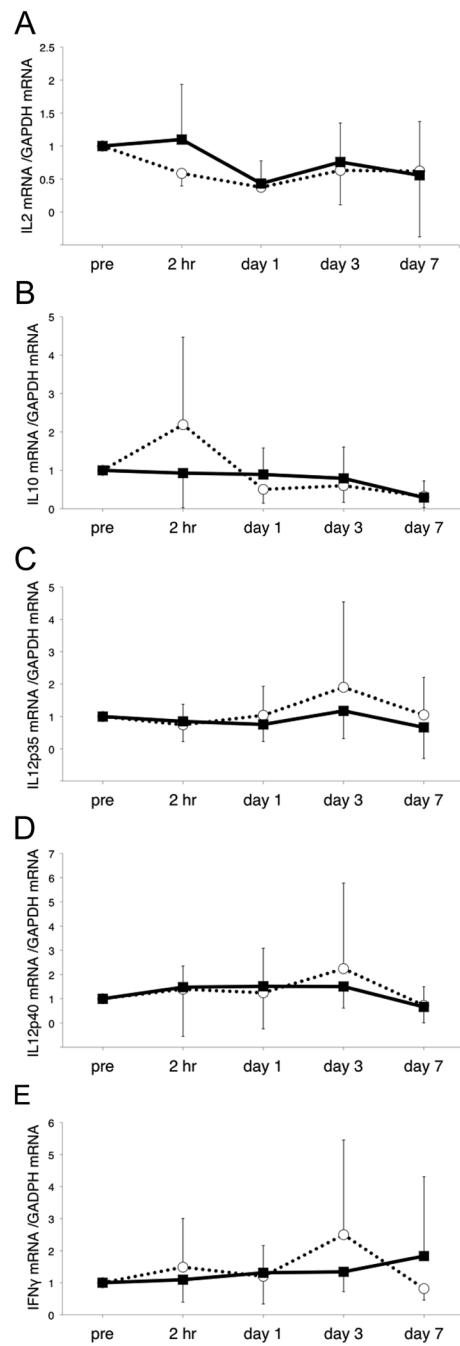


Fig. 3. Graphs illustrating the mRNA expression levels of (A) IL2, (B) IL10, (C) IL12p35, (D) IL12p40 and (E) IFN γ . The Black square (■) with solid line represents group P, and the white circle (○) with dotted line represents group I.

medicine, however, the IL10 of TIVA showed a higher level than that of inhalational anesthesia during the perioperative period [23]. This conflict might result from different study design, such as patients with or without surgery, IL10 measurement by real-time PCR or ELISA, and different usages and doses of anesthetic drugs. In our study, propofol showed

less IL2 decrease and less IL10 increase at 2 hr, indicating that propofol itself might have an immuno-protective effect on cytokine mRNA expression, countering the immunosuppression caused by anesthesia.

Group P's IL12 and IFN γ showed virtually no change, maintaining the same level until day 5, while that of group I showed an increase at day 3. This increase coincided with a recovery of CD8+ lymphocytes in group I at day 3, so it might be associated with the increase in CD8+ lymphocytes. IL12 and IFN γ are also made by CD8+ lymphocytes; they are also thought to stimulate the proliferation of Th1 cells, while inhibiting Th2 cells. In human cancer patients, the Th1/Th2 ratio in TIVA with propofol showed no change after anesthesia, while that in inhalational anesthesia with isoflurane showed significant decreases [25]. The Th1/Th2 ratio might be associated with cancer progression and metastasis, which also has been reported in dogs [6]. In our study, the Th1/Th2 ratio was not examined, but these results suggest that the Th1/Th2 ratio decreased on day 3 after isoflurane anesthesia, but propofol might prevent this kind of immunological deterioration.

In this experiment, we used fentanyl in both groups to imitate the current method commonly used in veterinary clinics. Future studies should investigate the immune effect of anesthetic agent alone, as well as its interaction with fentanyl. And, although we confirmed adequate anesthetic depth in both groups, we could not ensure that each group reached identical anesthetic depths. However, we used normal dosage of isoflurane [10] and a dosage of 0.5 mg/kg/min for propofol, which is higher than the common dosage (0.2–0.4 mg/kg/min) currently used in veterinary clinics [13, 30]. Nevertheless, immuno-suppression was higher in group I than in group P. Therefore, propofol itself may have had an immuno-protective effect unrelated to any differences in anesthetic depth.

These data show that isoflurane had more strongly suppression for immune system caused by anesthesia than propofol, and propofol might have some immuno-protective effects, particularly against the decrease of T-lymphocytes and changes in the pro- and anti-inflammatory cytokines. It also might be associated with the Th1/Th2 ratio during anesthesia in dogs. However, the number of samples in our study was inadequate, and the protein level of cytokine expression in PBMC should be examined. Furthermore, as we used only GAPDH as an internal standard for mRNA expression, it also should be examined the most suitable internal standard among all candidates. Hereafter, it becomes important to clarify the relationship between TIVA and cancer recurrence as well as the relationship between TIVA and the rate of incidence for infectious disease in the perioperative period. Further research may emphasize that TIVA with propofol in dogs has more immunological merit, making it useful for many purposes.

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