In Vivo and In Vitro Studies of the Inhibitory Effect of Propofol on Human Platelet Aggregation

Hiroshi Aoki, M.D.,* Toshiki Mizobe, M.D., Ph.D.,† Shinji Nozuchi, M.D.,* Nonko Hiramatsu, M.D.*

Background: The inhibitory effects of propofol on platelet aggregation are controversial because the fat emulsion used as the solvent for propofol may affect platelet function. The effects of propofol on platelet intracellular calcium ion concentration and on aggregation were investigated.

Methods: Platelet aggregation was measured in 10 patients who received an intravenous infusion of propofol. Intralipos, the propofol solvent, was infused in 10 healthy volunteers and platelet aggregation were measured. The in vitro effects of propofol and Intralipos on platelets were also investigated. The inhibitory effects of various concentrations of propofol were studied. The effects of propofol on the changes in intracellular calcium level using a fluorescent dye, fura-2, were also observed. Template bleeding time was measured to determine the effect of propofol in clinical use.

Results: Platelet aggregation was significantly inhibited by infusion of propofol, although bleeding time was not prolonged. Intralipos did not inhibit platelets either in vitro or in vitro. Propofol significantly inhibited platelet aggregation in vitro and at 5.81 ± 2.73 μg/ml but not at 2.08 ± 1.14 μg/ml. The increase of intracellular calcium concentration was inhibited both in influx and discharge of calcium.

Conclusions: Propofol inhibited platelet aggregation both in vitro and in vivo. Inhibition of platelet aggregation appeared to be caused by propofol itself and not by the fat emulsion. This inhibitory effect was also supported by the suppressed influx and discharge of calcium. No change in the bleeding time suggests that this inhibitory effect does not impair hemostasis clinically. (Key words: Calcium ions; fluorescence, fura-2; high-performance liquid chromatography; intravenous anesthetics; triglyceride.)

BECAUSE platelets play an important role in hemostasis during surgery, the effect of drugs used to induce general anesthesia on platelets is an important clinical issue. Halothane has been found to inhibit platelet aggrega-

tion1–5 and to affect hemostasis, as other antiplatelet drugs do.6 Enflurane and isoflurane appear to have minimal or negligible effects on platelets and therefore are not believed to be associated with an increased risk of hemorrhage during general anesthesia.7–9 The effect of nitrous oxide on platelets is controversial.10–12 Intravenous anesthetics, opiates, and muscle relaxants are not believed to affect platelet function.6,9

Propofol, an intravenous anesthetic agent recently introduced into clinical use, has many advantages over inhalation anesthetics, including a rapid onset and a short duration of anesthesia and the absence of excitatory side effects, and therefore is widely used for general anesthesia.11 The effect of propofol on platelets is controversial.12–15 Because it is hydrophobic, propofol is administered in an aqueous emulsion (Diprivan; Zeneca Yakuhin K.K., Osaka, Japan). Because the fat emulsion itself may affect platelet function,16–19 it is not clear whether the inhibitory effects of Diprivan are related to propofol or to the fat emulsion.

We examined changes in platelet aggregation during general anesthesia by Diprivan infusion and investigated the effects of propofol, Diprivan, and the fat emulsion solvent on platelet aggregation in vitro. The effects of propofol on the intracellular calcium mobilization were also investigated.

Materials and Methods

This study was approved by our institutional committee on human research, and informed consent was obtained from all participants.

Platelet Aggregation Test

Blood samples collected from the antecubital vein were anticoagulated with 1:10 volume of 3.8% sodium citrate solution and then centrifuged at 800 rpm for 10 min. The upper layer was collected as platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained by centrifuging the residual blood sample at 3,000 rpm

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for 10 min. The platelets in sample solution were counted using a Coulter counter (model MD II; Coulter Electronics, Hialeah, FL) and adjusted to $2 \times 10^5$ cells/μL.

Platelet aggregation was assessed by the turbidimetric technique described by Born and Cross using an aggregometer (Hema Tracer 601; Nikoh Bioscience, Tokyo, Japan). Platelet-rich plasma was placed in a silicon-coated glass cuvette and warmed to 37°C for 5 min. Because the fat emulsion contained in Diprivan or Intralipos (The Green Cross Company, Osaka, Japan) causes turbidity in both PRP and PPP, platelet aggregation cannot be assessed in terms of the increase in optical density. Thus, we determined the minimal adenosine diphosphate (ADP) concentration required to induce irreversible aggregation (the ADP threshold). Briefly, five distinct concentrations of ADP (1-5 μM, in 1-μM increments, final concentration) were prepared and added to the samples. The minimal concentration that induce irreversible aggregation was determined as the ADP threshold. An increase in the ADP threshold indicates inhibition of platelet aggregation.

**Measurements of Template Bleeding Time**

To investigate the effect of Diprivan on hemostasis, template bleeding time was measured in 0.5 min increment in 9 patients before and during the administration of Diprivan using Simplate (Organon Teknica, Durham, NC).

**Measurements of Propofol and Triglyceride Concentration**

The propofol concentration was measured based on the method of Pavan et al. with some modifications. After aggregation studies were completed, the residual blood samples were centrifuged at 3,500 rpm for 10 min and the supernatants were collected. The samples were mixed with acetonitrile—60% perchloric acid (2:1 vol/vol) and then centrifuged at 3,500 rpm for 10 min. The propofol concentration in the supernatants was measured by high-performance liquid chromatography, using a solvent delivery pump (Hitachi L-6000, Tokyo, Japan) equipped with an autosampler (Hitachi AS-200), an ultraviolet detector (Hitachi L-7400), and a chromatointegrator (Hitachi D-2500). Chromatographic separation was done on a 250 × 4.6-mm column packed with 5 μm Hypersil ODS (GL-Pack, GL Sciences, Tokyo, Japan). Acetonitrile-double distilled water (2:1) adjusted to a pH of 4.0 using acetic acid was eluted at a flow rate of 1.5 ml/min. A 20-μl sample of standard solution of propofol was injected, with thymol (10 μg/ml) used as the internal standard. Absorbance was measured at 270 nm.

Triglyceride is considered a reliable marker of fat emulsion based on the results of previous reports and our pilot study (data not shown). The triglyceride concentration was measured by the lipoprotein lipase-glycerol kinase-glycerol-3-phosphate oxidase method, with some modifications. The absorbance of the red quinone dye that is synthesized through these reactions was measured at 540 nm and 660 nm using the Hitachi Autoanalyzer 736.

**Study Protocol**

**In Vivo Study: Part 1.** The effect of propofol-induced anesthesia was investigated in 10 patients (five men and five women; ages 34 to 64 yr; weight, 56.5 ± 9.3 kg) scheduled for minor surgery of the face and neck region. All patients were classified as American Society of Anesthesiologists physical status I and had no hematologic abnormalities. Patients receiving medications that may affect platelet function were excluded. Patients were premedicated with intramuscular injections of atropine sulfate (0.01 mg/kg) and hydroxyzine (1 mg/kg). Thirty minutes later, 2 mg/kg propofol (0.2 ml/kg Diprivan) was administered intravenously to induce general anesthesia. Tracheal intubation was facilitated by administration of 0.1 mg/kg vecuronium bromide. Thereafter, anesthesia was maintained by a continuous infusion of propofol at a rate of 5-8 mg·kg⁻¹·h⁻¹, together with appropriate doses of vecuronium and fentanyl. No inhalation anesthetics, including nitrous oxide, were used during the procedure.

Blood samples were collected at three points: before the induction of anesthesia (control), 5 min after the bolus injection of Diprivan, and 2 h after the end of Diprivan infusion. The platelet aggregation test was performed and plasma concentrations of propofol and triglyceride were measured.

**In Vivo Study: Part 2.** The effect of the fat emulsion on platelet aggregation was examined in 10 healthy volunteers (five men and five women; ages 25 to 34 yr; weight, 59.7 ± 10.6 kg). After an overnight fast, 0.2 ml/kg of a 10% Intralipos solution, which is the solvent used in Diprivan, was injected intravenously. Blood samples were collected before and 5 min after the bolus injection, and platelet aggregation and the triglyceride concentration were measured.

**In Vitro Study: Part 1.** Platelet-rich plasma obtained from 10 healthy volunteers (six men and four women;
aged 22-63 yr) was prepared as follows: (1) 5 µl autologous PPP was added to 1 ml PRP (control group); (2) 5 µl Diprivan was added to 1 ml PRP (Diprivan group); (3) 5 µl 10% Intralipos solution was added to 1 ml PRP (Intralipos group); and (4) 5 µl autologous PPP saturated with propofof was added to 1 ml PRP (propofol group). In the propofol group, 100 µl propofof was vortexed with autologous PPP and centrifuged at 3,500 rpm for 20 min. The clear lower layer was aspirated and added to PRP. After platelet aggregation tests were performed, the propofof concentration was measured by high-performance liquid chromatography. The propofof concentration was also measured in the samples from the Diprivan group. The triglyceride concentration was measured in the Intralipos group.

**In Vitro Study: Part 2.** To investigate the effects of different propofof concentrations on platelet aggregation, we obtained PRP from eight healthy volunteers and the samples were divided into four 1-ml aliquots (for the control and for three different concentrations of propofof). Autologous PPP (5 µl), which was adjusted to contain three distinct concentrations of propofof, respectively, was added to these samples of PRP except for the control. In the control samples, the same volume of autologous PPP was used. After the ADP thresholds were determined for each control sample, platelet aggregation of the sample was assessed and classified into “Inhibition” meaning that the increase of ADP threshold was recognized compared with the control value, and “No inhibition,” meaning no change in the value of the ADP threshold.

Propofof concentrations were determined by high-performance liquid chromatography.

**Measurement of Intracellular Calcium Concentration**
PRP obtained from six healthy volunteers and anticoagulated with 1:5 volume of acid-citrate-dextrose was incubated with 3 nmol fura-2 acetoxyethyl ester for 15 min at 37°C and then centrifuged at 1,200 rpm for 15 min in the presence of 0.3 U/ml apyrase. The washed platelets loaded with fura-2 were obtained by discarding the supernatant and resuspended in Tyrode-Hepes buffer, pH 7.4.28 Platelet counts in samples were adjusted to 10⁷ cells/µl using a Coulter Counter model MD II. Propofof was saturated in Tyrode-Hepes buffer and added to the samples, the concentration of which was determined by high-performance liquid chromatography after the procedure. The intracellular calcium concentration ([Ca²⁺]) and platelet aggregation were measured simultaneously using a calcium analyzer (CAF-110; JASCO, Tokyo, Japan) with repeated alternating excitation wavelengths of 340 and 380 nm and measuring emission at 505 nm.29 The extracellular calcium concentration was adjusted to 1 mm by adding CaCl₂ solution and was stirred for 5 min at 37°C. After the basal fluorescence intensity was recorded, the platelets were stimulated with 0.02 U/ml thrombin and the change of fluorescence intensity was monitored. The relative ratio of fluorescence intensity at 340 nm to that at 380 nm (R340:380)0.51 was recorded at the peak and at the plateau phases. The [Ca²⁺], at each point was calculated according to the equation of Tsien et al.,32 using the Kd of fura-2 and Ca²⁺ as 224 nm. Data are expressed as the rise in [Ca²⁺], from basal levels.

Ni²⁺ (final concentration of 1 mm) was added to the samples instead of CaCl₂ to block the influx of extracellular calcium and to demonstrate calcium release from the intracellular storage site.33,34

To investigate the influx of calcium into the cell, the entry of Mn²⁺ was evaluated using a quenching technique.33,34 MnCl₂ was added to the samples to a final concentration of 1 mm, and fura-2 fluorescence was monitored at the calcium insensitive excitation wavelength, 360 nm. Mn entry, which represents calcium influx, was measured as the rate of fluorescence decrease after stimulation by thrombin. Data were expressed as a percentage of the initial fluorescence.

**Materials**
Adenosine diphosphate and acetoniitrile were obtained from Wako Pure Chemical Industries, Osaka, Japan; propofof from Tokyo Kasei Kogyo, Tokyo, Japan; thymol from Sigma Chemical Company, St. Louis, MO; Apyrase from Sigma Chemical; and fura-2 AM from DOJINDO Laboratories, Kumamoto, Japan. Thrombin was donated by MOCHIDA Pharmaceutical, Tokyo, Japan. Diprivan and Intralipos were donated by Zeneka Yakuin K.K., Osaka, Japan and The Green Cross Company, Osaka, Japan, respectively. All other compounds were purchased from commercial sources.

**Statistical Analysis**
Values are the means ± SD. Friedman two-way analysis of variance by ranks was adopted for the analysis of the data of ADP thresholds in part 1 of the in vitro study. Two-way analysis of variance followed by Bonferroni correction with repeated measures was adopted to analyze the difference in the triglyceride concentration between the data in part 1 of the in vitro study. Unpaired
Table 1. Changes in the Adenosine Diphosphate (ADP) Threshold, the Triglyceride, and Propofol Concentration in In Vivo Study: Part 1

<table>
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<th>Control</th>
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<th>Postoperation</th>
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<td>Case 10</td>
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| Triglyceride (mg/dl) | 80.4 ± 30.9 | 125.0 ± 39.5* | 80.9 ± 37.6 |
| Propofol (μg/ml)    | —           | 2.91 ± 0.67   | 0.78 ± 0.35  |

Data are mean ± SD, assessed before (Control), 5 min after a bolus injection of Diprivan, and 12 h after the end of Diprivan infusion (Postoperation). *P < 0.05 versus control.

Tests were used to analyze differences in triglyceride concentrations in parts 1 and 2 of the in vivo studies. The Kruskal-Wallis test was used to analyze the ADP thresholds in part 1 of the in vitro study. One-way analysis of variance followed by Bonferroni correction was used to analyze the differences in triglyceride concentrations between the data of the in vivo studies and that in part 1 of the in vitro study. To analyze the results of template bleeding time, the Wilcoxon signed rank test was used. Paired t tests were used to analyze the data in the studies of intracellular calcium concentration. Probability values <0.05 were considered significant.

Results

In Vivo Study: Part 1

The average operative time and the average duration of the propofol infusion were 155 ± 35.7 and 160.7 ± 38.5 min, respectively. The total dose of infused propofol was 1,166.3 ± 279 mg, and the average rate of continuous infusion of propofol was 6.1 ± 1.8 mg·kg⁻¹·h⁻¹.

The ADP threshold for PRP increased significantly after the bolus injection of propofol (P < 0.05, n = 10), indicating inhibition of platelet aggregation. Two hours after surgery, it was not significantly different from the control value (table 1). Typical aggregation curves are shown in figure 1. The ADP threshold increased in nine of 10 patients after propofol injection.

The triglyceride concentration increased significantly after the induction of anesthesia (P < 0.05) but was not significantly different from the control value 2 h after surgery (table 1).

The propofol concentration decreased after operation compared with the concentration after the bolus injection of propofol (table 1). In the patient who showed no inhibition of platelet aggregation, the propofol concentration was 2.44 μg/ml.

In Vivo Study: Part 2

Injection of Intralipos did not alter the ADP threshold in any of the participants.

The triglyceride concentration before injection of Intralipos (63.4 ± 32.1 mg/dl) was not significantly different from the control value in part 1. After injection of Intralipos, the triglyceride concentration increased to 115.6 ± 56.2 mg/dl, which was not significantly different from the value after the bolus injection of Diprivan in part 1.

In Vitro Study: Part 1

The ADP threshold was significantly higher in the Diprivan group (P < 0.05) and in the propofol group (P < 0.01) compared with the control group. There was no significant difference in the ADP threshold between the control group and the Intralipos group (table 2).

The triglyceride concentrations in the control group were not significantly different from the control values in parts 1 and 2 of the in vitro study. The triglyceride concentration in the Intralipos group did not differ significantly from the in vivo values after injections of Diprivan and Intralipos.

In Vitro Study: Part 2

The propofol concentration was significantly higher in the “inhibition” group (5.81 ± 2.73 μg/ml) compared with the “no inhibition” group (2.08 ± 1.14 μg/ml, fig. 2). The propofol concentration at which platelet aggregation was inhibited in part 1 of the in vitro study (2.91 ± 0.67 μg/ml) was in the zone of overlap of the inhibition and no inhibition groups. The propofol concentration at which platelet aggregation was reversed 2 h after surgery (0.78 ± 0.35 μg/ml) corresponded to values in the no inhibition group.

Template Bleeding Time

The values of template bleeding time were not significantly different before and during infusion of Diprivan (table 3).
Changes in Intracellular Calcium Concentration
The mean value of the change in [Ca$^{2+}$], at the peak induced by thrombin was $801.8 \pm 245.9$ nm in controls and $275.1 \pm 96.8$ nm in samples treated with propofol; these values were significantly different ($P < 0.01$). The mean value of the change in [Ca$^{2+}$], at the plateau was $166.7 \pm 43.4$ nm in controls and $87.6 \pm 26.7$ nm in samples treated with propofol; these values were also significantly different ($P < 0.05$). However, the simultaneously determined aggregation curves showed no significant difference in the maximum aggregation rate (82 $\pm$ 8.3% in control and 75.2 $\pm$ 18.1% in samples treated with propofol, respectively). When Ni$^{2+}$ was added to the sample, the mean value of the change in [Ca$^{2+}$], at the peak was $316.8 \pm 140.4$ nm in controls and $136.9 \pm 66.4$ nm in samples with propofol. These values were also significantly different ($P < 0.01$), suggesting the decreased discharge of [Ca$^{2+}$]. The mean concentration

Table 2. The Adenosine Diphosphate (ADP) Threshold, the Triglyceride, and Propofol Concentration in In Vitro Study: Part 1

<table>
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<th>Control</th>
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<td>ADP threshold (μM)</td>
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<tr>
<td>Triglyceride (mg/dl)</td>
<td>74.3 ± 35.0</td>
<td>95.2 ± 33.3</td>
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<td>Propofol (μg/ml)</td>
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<td>12.53 ± 4.90</td>
<td>13.40 ± 8.68</td>
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Data are mean ± SD.
**PROPOFOL INHIBITS PLATELET AGGREGATION**

**Propofol**

\[
\begin{align*}
\text{Propofol} &\quad (\mu g/ml) \\
10.0 &\quad \circ \circ \circ \\
5.0 &\quad \circ \circ \circ \\
0 &\quad \circ \circ \circ \\
\end{align*}
\]

**Inhibition**

**No inhibition**

Fig. 2. The relation between the concentration of propofol and platelet aggregation. Data are expressed as means ± SD. Inhibition: Platelet aggregation was inhibited in samples containing propofol when the same concentration of adenosine diphosphate as when the respective control sample was added, n = 14 (open circles). No inhibition: Platelet aggregation was not inhibited compared with the respective control sample, n = 10 (closed circles).

**Table 3. Template Bleeding Time before and during the Diprivan Infusion**

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**Fig. 3. Effects of propofol on the change in intracellular calcium concentration and aggregation when stimulated with thrombin (0.02 U/ml) in (A) 1 mM extracellular Ca^{2+}, and in (B) 1 mM extracellular Ni^{2+}. The profile of a representative case is shown. The upper traces in A and B show the change in the relative ratio of fluorescence intensity at 340 nm to that at 380 nm (R_{340/380}). The ordinate on the right side represents the corresponding intracellular calcium level (in nanomoles). The lower traces in A and B show the aggregation, represented in the optical density defined as 0% for washed platelet suspension and 100% for distilled water. The abscissa represents the time course of experiments (in min).**

of propofol in washed platelet samples was 17.36 ± 1.41 μg/ml. Figure 3 shows typical patterns of aggregation and change in the R_{340/380} value (indicating [Ca^{2+}]). Figure 4 shows the mean value of M_{2+} entry, expressed as the percentage decrease of fluorescence at 360 nm. After stimulation by thrombin, fluorescence decreased rapidly during 20-30 s and thereafter declined gradually during the procedure. The fast phase expresses the
Mn$^{2+}$ entry (which reflects Ca$^{2+}$ influx) caused by the stimulation of thrombin, and the second phase is attributed to the passive leakage into the cell. In both phases, the decrease of the fluorescence was significantly less with propofol, suggesting inhibition in Ca$^{2+}$ influx.

**Discussion**

A bolus injection of Diprivan inhibited platelet aggregation in vitro, as indicated by the increased ADP threshold. This inhibitory effect was reversed 2 h after operation. Concentrations of propofol and triglyceride increased after the bolus injection of Diprivan and returned to the control level 2 h after the end of surgery. Intralipos did not inhibit platelet aggregation but increased the triglyceride concentration to a similar extent as Diprivan did, suggesting that the inhibitory effect of Diprivan was induced by propofol and not by the fat emulsion. Bleeding time was not prolonged during anesthesia with Diprivan.

*In vitro* findings confirmed that platelet aggregation was inhibited by propofol and Diprivan but not by the fat emulsion. Propofol was saturated in autologous plasma and the concentration was confirmed by high-performance liquid chromatography, making it possible to examine the separate effects of propofol and its fat solvent on platelet aggregation. The concentration of propofol in part 1 was somewhat higher than the concentration just after the bolus injection of 2 mg/kg propofol (<10 μg/ml). The results presented in part 2 showed that propofol inhibited platelet aggregation even at concentrations <10 μg/ml.

Hansen et al. reported that Diprivan did not inhibit platelet aggregation in vitro. However, they did not measure triglyceride or propofol concentrations. Glen et al. observed no propofol-induced inhibition of platelet aggregation in rats, but their results cannot be extrapolated to humans because there is an interspecies difference in the response of platelets to ADP. Türkan et al. observed no inhibition of platelet aggregation during Diprivan-induced anesthesia in the presence of 20 μm ADP, but the lack of inhibition in their study may be due to the high dose of ADP. We determined the minimal concentration of ADP required to trigger aggregation in the present study. This method also made it possible to evaluate aggregation when conventional parameters of the aggregation rate were not useful because the fat emulsion caused turbidity in samples.

Fat emulsion has been found to inhibit platelets in studies using high-dose or prolonged infusion (>6 h) of Intralipos. Prolonged infusion of the fat emulsion was found to induce inhibition of platelet functions by the phagocytosis of lipid particles in platelet cytoplasm. Therefore, when Diprivan is infused for a prolonged period, the fat emulsion may cause marked platelet inhibition.

Although ADP was classified as a relatively weak agonist, a typical biphasic (primary and secondary) aggregation curve can be observed when platelets were stim-
ulated with ADP. In our study, the primary aggregation was inhibited by propofol. When the aggregation reaction begins, platelets initially show an elevation of [Ca^{2+}], and aggregation.\textsuperscript{59} Therefore, investigation of the change in [Ca^{2+}], was thought to be worthwhile.

The measurements of [Ca^{2+}], revealed that propofol inhibited the increase in [Ca^{2+}], induced by thrombin, a strong agonist for aggregating platelets.\textsuperscript{58} When Ni\textsuperscript{2+} was added to the extracellular fluid, it was reported to inhibit the calcium influx by blocking the plasma membrane divalent cation channel and making it possible to observe the release of calcium from the storage site (discharge).\textsuperscript{33,34} On the other hand, Mn\textsuperscript{2+} can pass through the calcium channel on the plasma membrane but binds to fura-2 and quenches its fluorescence. By using these divalent ions rather than Ca\textsuperscript{2+}, two distinct pathways, the influx and the discharge of calcium, can be estimated individually.\textsuperscript{34} From our results obtained using these divalent cations, propofol was found to inhibit both the influx and the discharge of calcium, supporting the inhibitory effect of propofol on platelets. Platelet aggregation induced by thrombin was not inhibited, even in the higher concentration of propofol, compared with our \textit{in vivo} and \textit{in vitro} studies using ADP.

In conclusion, during general anesthesia using Diprivan, platelet aggregation was inhibited, but the fat solvent did not inhibit platelet aggregation, suggesting that propofol itself was responsible for the inhibitory effect. The findings with intracellular calcium using a fluorescent dye, fura-2, also supported the inhibitory effect of propofol. When thrombin, a strong agonist, is used, platelet aggregation is not inhibited. Bleeding time is not prolonged. These findings support the fact that there is no clinical report referring to the hemorrhagic complications, although Diprivan is used in the anesthetic management of patients having surgery.

References

1. Ueda I: The effects of volatile general anesthetics on adenosine diphosphate-induced platelet aggregation. \textit{Anesthesiology} 1971; 34:405-8


