

Effect of Propofol on Human Neutrophil Apoptosis *in vitro*

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Abstract

Purpose: Propofol is clinically used for anesthesia and sedation. It remains unclear whether propofol induces human neutrophil apoptosis. We sought to determine the effect of propofol on neutrophil apoptosis *in vitro*.

Methods: Peripheral neutrophils from healthy volunteers were treated with different concentrations of propofol (0, 5, and 50 µg/ml). Apoptosis and the mitochondrial trans membrane potential (MTP) were detected by fluorescence microscopy after Hoechst 33342 and JC-1 staining. Caspase activity was measured using luminescent substrates.

Results: A high concentration (50 µg/ml) of propofol significantly increased apoptosis, reduced MTP, and enhanced caspase-9 and caspase-3/7 activities. The addition of an MTP stabilizer and caspase-9 and -3 inhibitors decreased propofol-induced apoptosis. Caspase-8 activity was not affected by 50 µg/ml of propofol and propofol-induced apoptosis was not suppressed by the caspase-8 inhibitor. A clinically relevant concentration (5 µg/ml) of propofol did not affect apoptosis rate, MTP and caspase activities.

Conclusion: Propofol at the clinical relevant concentrations had no effect on neutrophil apoptosis, although a high dose of propofol increased rate of apoptosis. The mechanism of propofol-induced apoptosis is involved with the mitochondrial pathway.

Introduction

Neutrophils are cardinal cellular effectors of the innate host response to infection and injury. Neutrophils spontaneously die by apoptosis within approximately 1–2 days under normal conditions [1–3]. Delayed neutrophil apoptosis is associated with organ injury during sepsis [4,5], by contrast, accelerated apoptosis contributes to the immunodeficiency in acquired immunodeficiency syndrome [6,7]. Thus, the rate of neutrophil apoptosis is critical for efforts to resolve inflammations.

Propofol is an intravenous anesthetic agent widely used for the induction and maintenance of anesthesia and for sedation in intensive care units (ICUs) [8]. Although several reports have indicated that propofol anesthesia reduces the postoperative counts of neutrophils [9,10] and higher concentrations of propofol have pro-apoptotic effects on other leukocytic cells *in vitro* [11,12], the effect of propofol on neutrophil apoptosis remains unclear. If propofol has an anti or pro-apoptotic effect on neutrophil, it could influence the resolutions of inflammation or immune dysfunction.

The aims of this study were to evaluate whether propofol induces human peripheral neutrophil apoptosis *in vitro* and to confirm the changes in mitochondrial transmembrane potential (MTP) and the activity of caspases by propofol.

Materials and Methods

Human Peripheral Neutrophil Isolation and Culture

After obtaining approval from the Institutional Ethics Committee, informed consent was obtained from each healthy participant prior to the start of the study. Human heparinized peripheral venous blood was overlaid onto a Mono-Poly resolving medium (Dainippon Sumitomo Pharma, Osaka, Japan) and peripheral neutrophils were isolated by density gradient centrifugation [13]. The purity of neutrophils was assessed microscopically and viability was confirmed to be above 95% using the trypan blue exclusion test. Isolated neutrophils were cultured at a concentration of 1×10^6 cells/ml in 24-well plates with RPMI 1640 medium (Sigma, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum, 1% glutamine and 1% penicillin/streptomycin solution. The cells were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Neutrophils were treated with or without propofol (Diprivan, AstraZeneca, Osaka, Japan). To determine the mechanism underlying propofol-induced apoptosis, we preincubated neutrophils with a mitochondrial membrane stabilizer (bongkrekic acid: BA), a caspase-8 inhibitor (Z-IETD-FMK), a caspase-9 inhibitor (Z-LEHD-FMK), or a caspase-3 inhibitor (Z-DVED-FMK) (Merck Chemicals, Tokyo, Japan) before treatment with propofol.

Detection and Quantification of Neutrophil Apoptosis

To detect apoptosis, we stained the neutrophils with Hoechst nuclear stain as previously described [14,15]. After incubation, the cells were resuspended in 20 µl of PBS containing 5 µg/ml Hoechst 33342 (Sigma) and incubated for a further 20 min at 37°C. We analyzed the cells by fluorescence microscopy at 1000× magnification (BX 51; Olympus, Tokyo, Japan). Apoptosis was assessed on the basis of chromatin structure and condensation. For each condition, a minimum of 400 cells was counted and apoptosis was quantified as follows: apoptosis index (% apoptotic cells) = $100 \times (\text{number of apoptotic cells} / \text{total number of cells})$.

MTP Assay

To assess the MTP, we incubated neutrophils (1×10^6 /ml) with 7.7 µM JC-1 (Molecular Probes, Eugene, OR, USA) for 15 min at 37°C in the dark [15,16]. JC-1 fluorescence has two emission peaks, one of red fluorescence (JC-1 aggregates) indicating increased or normal MTP, and one of green fluorescence (JC-1 monomers) representing decreased MTP. After incubation with JC-1, the dye was removed and the neutrophils were analyzed by fluorescence microscopy (×1000 magnification; BX 51, Olympus). For each condition, a minimum of 400 cells were counted, and the proportion of cells with a decreased MTP was quantified as follows: neutrophils with a decreased MTP (%) = $100 \times (\text{number of cells with JC-1 monomers} / \text{total number of cells [cells with JC-1 aggregates and with JC-1 monomers]})$.

Caspase Activity Assay

Caspase-8, -9 and -3/7 activities were determined using the Caspase-GLO™ Assay kit (Promega, Madison, WI, USA) [15,17]. Caspase-GLO reagents were added directly to 1×10^4 neutrophils in white-walled 96-well plates, giving a final volume of 200 µl per well. The contents of the wells were gently mixed and incubated at room temperature for 30 min. We measured the luminescence of each sample in a plate-reading luminometer (GENios Plus; TECAN, Mannedorf/Zurich, Switzerland).

Statistical Analysis

All data are presented as means ± SD. Statistical analysis was carried out using two- or one-way ANOVA followed by a *post hoc* Tukey-Kramer test for multiple comparisons. Differences between the two groups were analyzed by unpaired *t*-tests. Statistically significant differences were defined as those with a $P < 0.05$.

Results

Effects of Propofol on the Neutrophil Apoptotic Index

Neutrophils were treated with propofol at concentrations of 5 and 50 µg/ml; concentrations that are 1- and 10-fold greater than the clinically relevant plasma concentrations [11,12]. The differences between untreated neutrophils and 50 µg/ml propofol-treated neutrophils after Hoechst 33342 staining are shown in Figure 1a and b. After incubation for 15 h, 50 µg/ml propofol increased the number of apoptotic cells as

compared with untreated cells. The apoptosis indices determined by Hoechst 33342 staining are shown in Figure 1c. The number of apoptotic neutrophils increased in a time dependent manner in all groups. After 15 hours-incubations, 50 $\mu\text{g/ml}$ propofol significantly increased apoptosis index compared with other two treatments (0

and 5 $\mu\text{g/ml}$ propofol). Propofol at a concentration of 5 $\mu\text{g/ml}$ did not significantly increase the apoptosis index compared with the untreated cells at any incubation periods

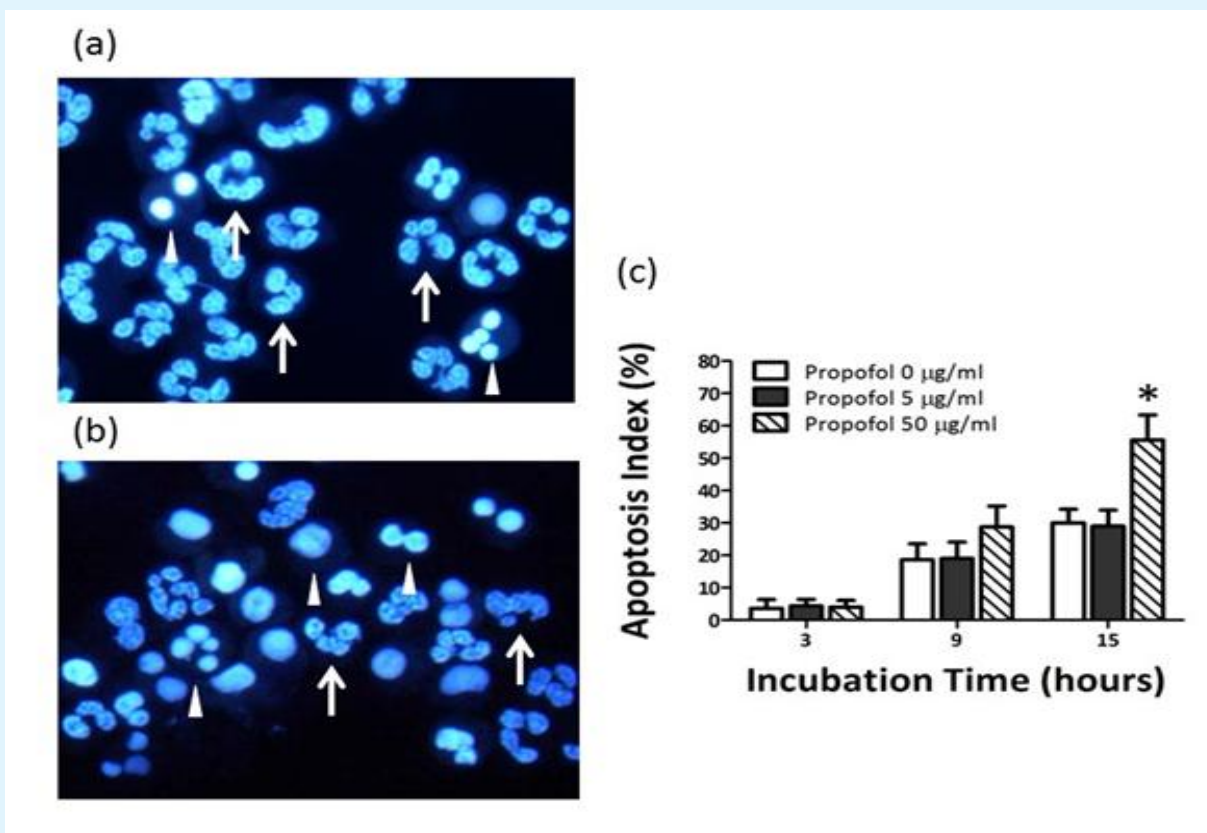


Figure 1: Effects of incubation with propofol for 15 h on human neutrophil apoptosis determined by Hoechst 33342 staining. Neutrophils were treated with 0 (a) or 50 $\mu\text{g/ml}$, (b) Propofol. Representative images of cells stained with Hoechst 33342 are shown. Arrows, normal cells; arrowheads, apoptotic cells, (c) Dose-dependent effects of propofol on the apoptosis index. Data are expressed as means \pm SD ($n=8$). * $P < 0.01$ vs. cells treated with 0 or 5 $\mu\text{g/ml}$ propofol.

Effects of Propofol on Neutrophil MTP and Caspase Activity

To determine the effect of propofol on the MTP and caspase activity, we treated neutrophils with 50 $\mu\text{g/ml}$ propofol. Figure 2a and b shows the effects of 0 and 50 $\mu\text{g/ml}$ propofol on neutrophil MTP upon JC-1 staining. After incubation for 5 h, neutrophils with a decreased

MTP were significantly increased in the presence of 50 $\mu\text{g/ml}$ propofol (Figure 2c). Although 50 $\mu\text{g/ml}$ propofol did not affect caspase-8 activity (Fig. 3(a)), it significantly increased caspase-9 and -3/7 activity after incubation for 8 h (Figure 3b & c).

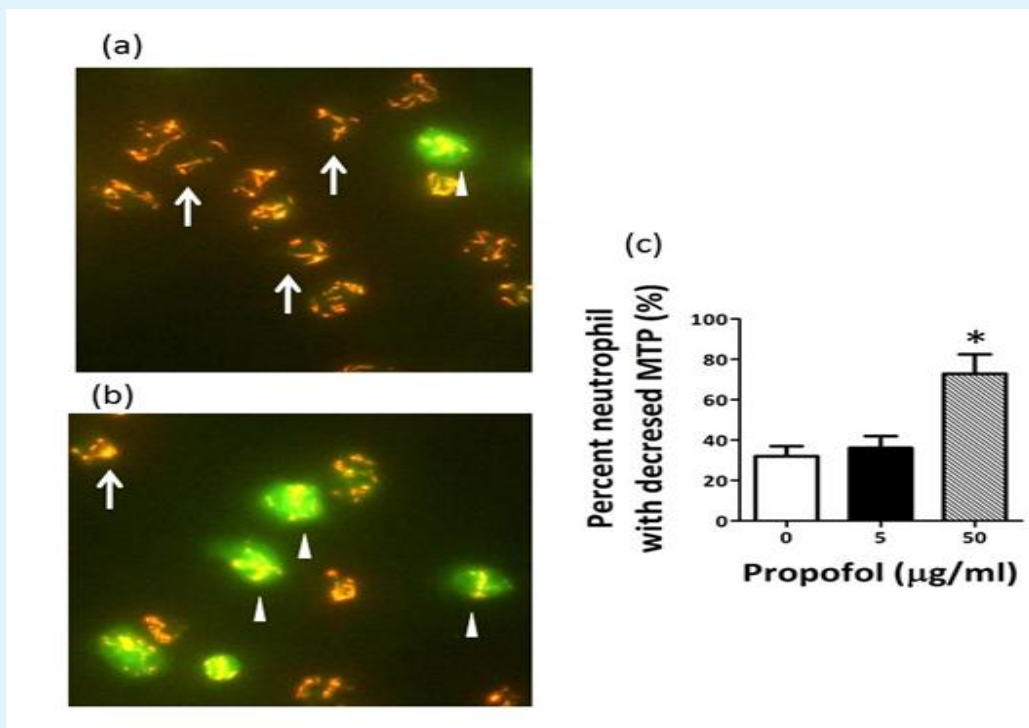


Figure 2: Effects of incubation with 50 µg/ml propofol for 5 h on neutrophil mitochondrial transmembrane potential (MTP) determined by JC-1 staining. Neutrophils were treated with 0 (a) or 50 µg/ml (b) propofol. Representative images of cells stained with JC-1 are shown. Arrows (red) indicate cells with JC-1 aggregates (high or normal MTP); Arrowheads (green) indicate cells with JC-1 monomers (decreased MTP). (c) Effects of propofol on the percentage of neutrophils with a decreased MTP. Data are expressed as means \pm SD (n=8). *P < 0.01 vs. 0 or 5 µg/ml propofol.

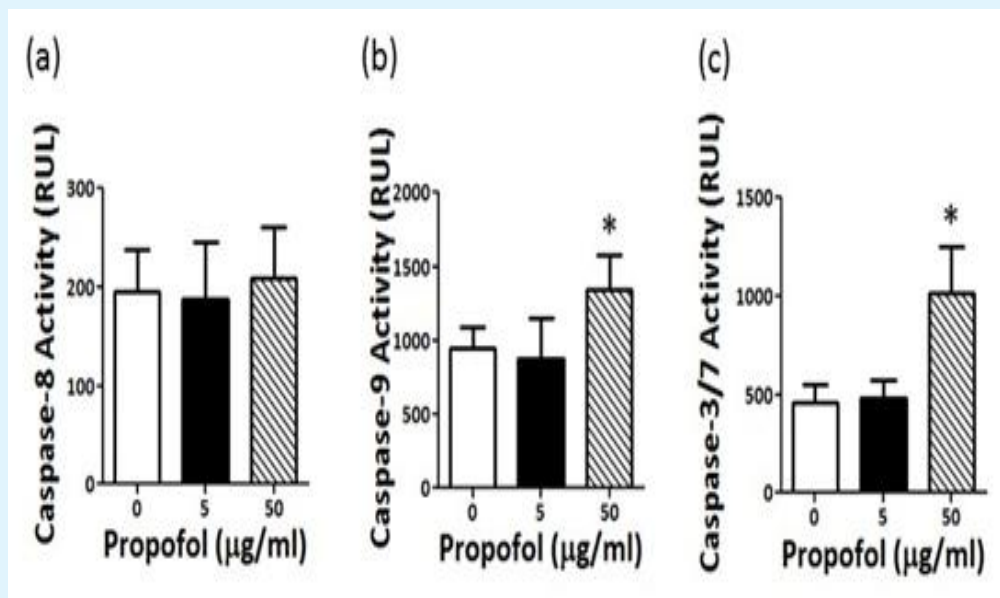


Figure 3: Effects of 50 µg/ml propofol on (a) caspase-8, (b) -9 and (c) -3/7 activities during neutrophil apoptosis. Activities were measured after incubation with propofol for 8 h. Data are expressed as means \pm SD (n=8). *P < 0.01 vs. 0 or 5 µg/ml propofol.

Effects of BA and caspase inhibitors on propofol-induced neutrophil apoptosis: To evaluate the role of changes in MTP and caspase activities in apoptosis induced by 50 $\mu\text{g/ml}$ propofol, we incubated neutrophils with 62 μM BA, Z-IETD-FMK, 100 nM Z-DVED-FMK or 100-nM Z-LEHD-FM for 1 h before adding 50 $\mu\text{g/ml}$

propofol, and then determined the apoptosis index. Apoptosis induced by incubation with 50 $\mu\text{g/ml}$ propofol for 15 h was significantly decreased by BA, Z-LEHD-FM and Z-DVED-FMK, but not by Z-IETD-FMK compared with the untreated cells (Figure 4).

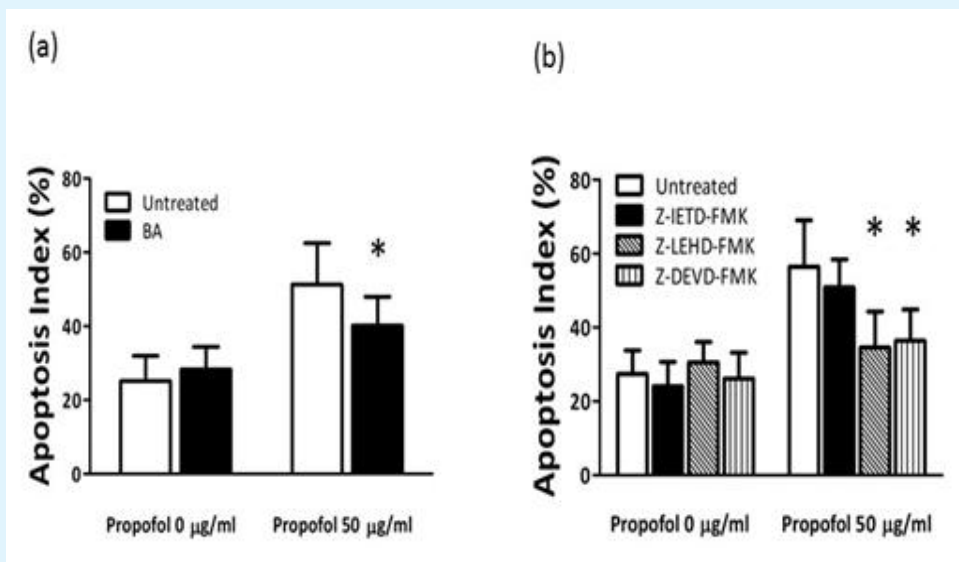


Figure 4: Effects of bongkreikic acid (BA) and caspase inhibitors on neutrophil apoptosis induced by 50 $\mu\text{g/ml}$ propofol. (a) Effects of BA on neutrophil apoptosis induced by 50 $\mu\text{g/ml}$ propofol. Neutrophils were pre-incubated with or without 62 μM BA and then treated with or without 50 $\mu\text{g/ml}$ propofol for 15 h. Apoptosis indices were assessed by Hoechst 33342 staining after 15 h incubation. Data are expressed as means \pm SD (n=8). *P< 0.05 vs. cells treated with 50 $\mu\text{g/ml}$ propofol alone. (b) Effects of inhibitors against caspase-8 (100 nM Z-IETD-FMK) caspase-9 (100 nM Z-LEHD-FMK) or caspase-3 (100 nM Z-DVED-FMK) on neutrophil apoptosis in cells treated with or without 50 $\mu\text{g/ml}$ propofol. Neutrophils were pre-incubated with or without the indicated inhibitor. The apoptosis index was assessed by Hoechst 33342 staining after incubation for 15 h. Data are expressed as means \pm SD (n=8). *P< 0.05 vs. cells treated 50 $\mu\text{g/ml}$ propofol alone.

Discussion

In this study, our results indicated that incubation of human neutrophils with a high concentration of propofol for 15 h increased the rate of apoptosis. On the other hand, a clinically relevant concentration of propofol did not affect rate of apoptosis after a long incubation period. We also found that apoptosis induced by 50 $\mu\text{g/ml}$ propofol was associated with decreased MTP and the activation of caspase-9 and -3/7 before chromatin structure and condensation of neutrophil. And our observations demonstrated that propofol-induced neutrophil apoptosis was suppressed by mitochondrial membrane stabilizer, caspase-9 and -3 inhibitors.

Our results indicate that propofol at concentrations above those normally used in clinical settings can accelerate neutrophil apoptosis. Our finding is consistent

with the reports documenting the pro-apoptotic effects of propofol on HL-60 cells, human peripheral mononuclear cells and lymphocytes [11,12]. Taken together, these findings indicate that a high concentration of propofol have a marked pro-apoptotic effect on the various human leukocytic cells.

We indicated that high doses of propofol accelerated MTP changes and caspase-9 and 3/7 activities before chromatin condensation in neutrophils were detected. The activation of the death receptor and mitochondrial pathways during spontaneous neutrophil apoptosis has been well documented [2,3,18]. Caspase-8 initiates the death receptor pathway [3], pro-apoptotic proteins released from mitochondria by reduced MTP activate caspase-9 in the mitochondrial pathway [16]. Caspase-3/7 activated by caspase-8 and -9 leads to DNA fragmentation and chromatin condensation [2,3,18]. Changes in the MTP and caspase activities increase in a

time-dependent manner [18] and decreases of MTP and activations of caspase-9 and -3/7 occur even before structural changes in the nucleus in this study. Therefore, our results suggest that an increase in the number of neutrophils with low MTP and activated caspase-9 and -3/7 is an early hallmark of apoptosis induced by propofol.

Activation of caspase-8 is also linked to the decreased MTP in spontaneous neutrophil apoptosis [1, 3]. By contrast, we confirmed that 50 µg/ml propofol accelerated the decreased MTP without increase of caspase-8 activity by propofol. In addition, apoptosis induced by propofol was depressed by mitochondrial membrane stabilizer, caspase-9 and -3 inhibitors in this study. We speculate that the mitochondrial pathway accelerates propofol-induced apoptosis to a greater extent than the death receptor pathway.

Several limitations of this study merit discussion. First, although spontaneous neutrophil apoptosis is initiated by the death receptor and mitochondrial pathway, in our study, even in the absence of propofol, spontaneous apoptosis was not inhibited by caspase inhibitors or BA, consistent with the findings of other studies [18]. The reason why caspase inhibitors and BA did not affect apoptosis is still unclear, further studies are needed to clarify the mechanism underlying spontaneous apoptosis. Second, the present study was an *in vitro* basic study to evaluate the effect of propofol on neutrophil apoptosis. In the clinical setting there are many factors, surgical trauma, infection or systemic inflammation response syndrome, to influencing neutrophil apoptosis [4-7,19]. Even clinical administrations of propofol reduce the postoperative counts of neutrophils in several kinds of procedures [9,10]. To confirm whether the dysregulation of neutrophil apoptosis at clinically relevant doses of propofol is involved, *ex vivo* studies are needed in which neutrophil status can be assessed in peripheral blood samples taken from surgical or ICU patients before and after propofol administration.

In conclusion, a clinically relevant concentration of propofol had no effect on human neutrophil apoptosis from healthy volunteers *in vitro*, although a high dose of propofol accelerated rate of apoptosis. The mechanism of propofol-induced apoptosis is involved with the mitochondrial pathway. We believe that propofol can be safely administered to patients without accelerated inflammation or immunodeficiency due to neutrophil apoptotic dysfunction.

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