

Propofol induces DNA damage in mouse leukemic monocyte macrophage RAW264.7 cells

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Abstract. Propofol is one of the most widely clinically used intravenous anesthetic, and it induces apoptosis in human and murine leukemia cell lines. Yet, whether propofol causes DNA damage and affects the mRNA expression of repair-associated genes in cancer cells remains undetermined. In the present study, we investigated the effects of propofol on DNA damage and associated mRNA gene expression in RAW264.7 cells. Comet assay and DNA gel electrophoresis were used to evaluate DNA damage in RAW264.7 cells and propofol-inhibited cell growth *in vitro*. The results revealed a longer DNA tail and DNA fragmentation. Real-time PCR assay was used to examine mRNA gene expression of DNA damage and DNA repair-associated genes. Following exposure to propofol for 48 h, a decrease in the mRNA expression of DNA-PK, BRCA1, MGMT and p53 was noted in the RAW264.7 cells. Results from the western blotting indicated that p53, MGMT, 14-3-3-σ, BRCA1 and MDC1 proteins were decreased while p-p53 and p-H2A.X(S140) were increased in the RAW264.7 cells following exposure to propofol. In conclusion, exposure

to propofol caused DNA damage and inhibited mRNA expression and protein levels of repair-associated genes in RAW264.7 cells.

Introduction

Hematological malignancies are a form of cancer that includes leukemia and lymphoma (1). Leukemia is one of the cancers that causes extensive mortality in the human population. It is known that leukemia starts in hematopoietic elements primarily in the bone marrow, the soft tissue inside most bones, then progresses to the blood, lymph nodes or other organs leading to serious health problems. In Taiwan, the 2009 Report of the Department of Health, R.O.C. (Taiwan) indicated that ~4 out of 100,000 individuals die each year of leukemia, and it is the 11th most common malignancy (2). At present, the treatment of patients with leukemia includes bone marrow transplant, radiotherapy and chemotherapy (3,4). However, these treatments are still unsatisfactory, and the exact mechanisms involved in leukemia are unclear.

Propofol (2,6-diisopropylphenol), is commonly used as an intravenous sedative-hypnotic (anesthetic). It has analgesic effects in inflammation-induced pain and facilitated pain (5) and has been clinically used for patients in rapid emergence after cessation of infusion (6). Propofol contains cardiac protection during ischemia-reperfusion (7-9), and it has been reported that propofol upregulates AQP1 expression (10) and inhibits nitric oxide synthase (11) in endotoxemia-mediated lung injury. However, animal studies suggest that propofol protects against endotoxemia-induced lung and kidney injuries (12,13).

A previous study found that propofol induces apoptosis, which is dependent on the mechanism that activates both the cell surface death receptor pathway and the mitochondrial pathway. It was also found that propofol may trigger neurodegeneration in neurons during development and its derivative may affect neuronal injury, including apoptotic cell death (14).

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It was also demonstrated that propofol inhibited the decarboxylase activity in three-dimensional primary cell cultures of fetal rat telencephalon (15). Recently, we found that propofol induced apoptosis in murine leukemia RAW264.7 cells *in vitro* through altered levels of apoptosis-associated proteins resulting in induction of apoptotic gene expression and inhibition of cell growth (13).

It was reported that the neuro-degeneration in age-related diseases, cerebral ischemia and brain trauma is associated with DNA damage (16), and if agents can induce DNA damage in cells, this may lead to cell mutations and to the development of malignancy. Although numerous studies indicate that propofol induces cell death, no information has addressed the effects of propofol-induced DNA damage in murine leukemia cells. Therefore, in the present study, we investigated the effects of propofol on DNA damage and expression (mRNA) of DNA repair-associated genes in murine leukemia RAW264.7 cells. The results revealed that propofol induced DNA damage and inhibited the expression of DNA damage and repair-associated genes in RAW264.7 cells *in vitro*.

Materials and methods

Chemicals and reagents. Propofol was obtained from B. Braun Melsungen AG (Schwarzenberger Weg, Melsungen, Germany). A stock solution of propofol was prepared in phosphate-buffered saline (PBS), and an equal volume of PBS (0.1%) was added to the controls. RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine and penicillin-streptomycin were obtained from Gibco®/Invitrogen Life Technologies (Grand Island, NY, USA).

Cell culture and treatment. The RAW264.7 murine leukemia cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The RAW264.7 cells were maintained in RPMI-1640 medium with 2 mM L-glutamine, supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin and 100 µg/ml streptomycin, and incubated at 5% CO₂ and 37°C and 90% relative humidity. Propofol was diluted in sterile PBS before addition to the culture. The cells which were treated with PBS served as the control and were assigned to have 100% survival.

Cell viability analysis. Approximately 2x10⁵ cells/well of RAW264.7 cells in 12-well plates were maintained for 24 h in an incubator. Cells in each well were incubated with propofol at the final concentrations of 0, 25, 50, 100 and 200 µg/ml, vehicle (1 µl PBS) or 0.1% hydrogen peroxide (H₂O₂, positive control) for 48 h. Cells were also treated with 100 µg/ml propofol for 24, 48 and 72 h. Cells from each treatment group were stained with 4 µg/ml propidium iodide (PI) (Sigma-Aldrich Corp., St. Louis, MO, USA) and analyzed by FACSCalibur flow cytometry (Becton-Dickinson, San Jose, CA, USA). Cell viability was calculated as previously described (13,17).

Measurement of DNA damage by comet assay. RAW264.7 cells (2x10⁵/well) in 12-well plates were incubated with 0, 25, 50, 100 and 200 µg/ml of propofol, vehicle (1 µl PBS) or 0.1% H₂O₂ for 48 h, or cells were exposed to 100 µg/ml propofol for

Table I. Primer sequences used in the real-time PCR.

Primer name	Primer sequence
<i>DNA-PK-F</i>	TTCAAGACTTCAACCGCTTT
<i>DNA-PK-R</i>	TGCGGCTGGGTCAAGTGT
<i>BRCA1-F</i>	TTGAAGTCAAAGGAGACGTTG
<i>BRCA1-R</i>	TCTTTGGGCATGTTGGTGAA
<i>MGMT-F</i>	TCCCTTGCCTGCTCTCCAT
<i>MGMT-R</i>	AACCATTCTCCGAATTCACAA
<i>p53-F</i>	GGGTTAGTTTACAATCAGCCACATT
<i>p53-R</i>	GGGCCTTGAAGTTAGAGAAAATTCA
<i>GAPDH-F</i>	GGTGGACCTCATGGCCTACA
<i>GAPDH-R</i>	CAGCAACTGAGGGCCTCTCT

F, forward; R, reverse. DNA-PK, DNA-dependent serine/threonine protein kinase; BRCA1, breast cancer gene 1; MGMT, O⁶-methylguanine-DNA methyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

24, 48 and 72 h in RPMI-1640 medium and grown at 37°C in 5% CO₂ and 95% air. Cells from each treatment group were harvested by centrifugation for examination of DNA damage using the comet assay as previously described (18,19). The TriTek CometScore™ software image analysis system (TriTek Corp., Sumerduck, VA, USA) was used to calculate and quantify the comet tail length as previously described (18,19).

Evaluation of DNA fragmentation by DNA gel electrophoresis. RAW264.7 cells at a density of 2x10⁶ cells/well in 6-well plates were incubated with propofol (25, 50, 100 and 200 µg/ml) or vehicle (1 µl PBS) for 48 h at 37°C in 5% CO₂ and 95% air. Cells from each treatment group were individually isolated, and then DNA was extracted using a DNA isolation kit (Genemark Technology Co., Ltd., Tainan, Taiwan) (20,21). DNA electrophoresis was carried out on 1.5% agarose gel in Tris/boric acid buffer at 15 V for 2 h, and DNA was stained with ethidium bromide (EtBr; Sigma-Aldrich Corp.) and then examined and photographed under UV light box as previously described (18-20).

Analysis of gene expression by real-time PCR assay. RAW264.7 cells at a density of 2x10⁶/well in 6-well plates were incubated with 100 µg/ml propofol or without for 48 h. The total RNA from each sample was extracted using the Qiagen RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) as previously described (18,20). Briefly, the High Capacity cDNA reverse transcription kit was used for RNA samples which were reverse-transcribed for 30 min at 42°C according to the standard protocol of the supplier (Applied Biosystems, Carlsbad, CA, USA). The quantitative PCR from each sample was performed under the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C, 1 min at 60°C using 1 µl of the cDNA reverse-transcribed as described above, 2X SYBR-Green PCR Master Mix (Applied Biosystems) and 200 nM of forward and reverse primers as shown in Table I.

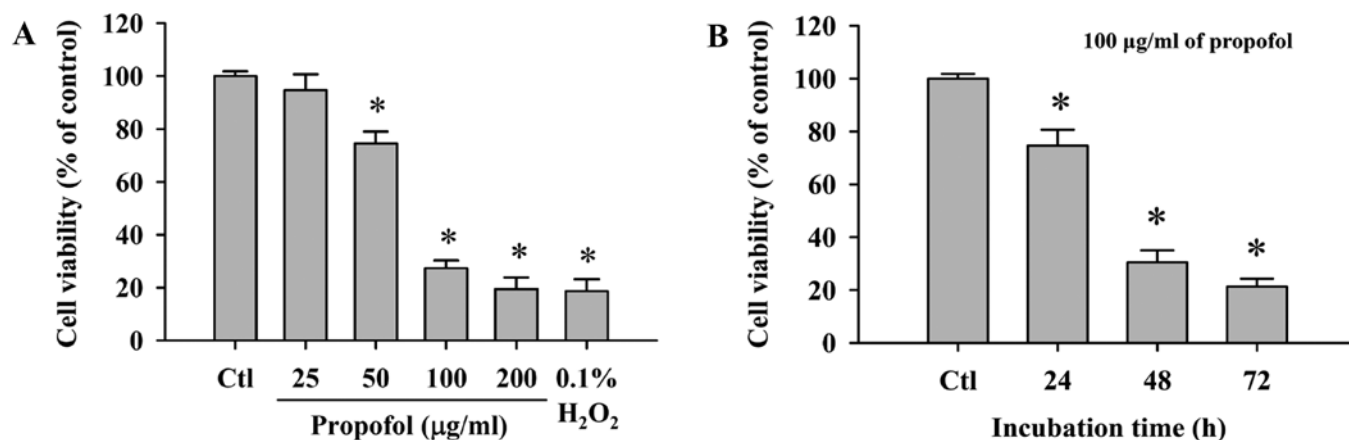


Figure 1. Propofol decreases the percentage of viable murine leukemia RAW264.7 cells. (A) Cells at a density of 2×10^5 /well were placed in 12-well plates and then incubated with propofol at final concentrations of 25, 50, 100 and 200 $\mu\text{g/ml}$, vehicle (1 μl PBS) or 0.1% H_2O_2 (positive control) for 48 h or (B) cells were treated with 100 $\mu\text{g/ml}$ propofol for 24, 48 and 72 h. Cells from each treatment group were analyzed by flow cytometry as described in Materials and methods. * $P < 0.05$ was considered to indicate a significant difference between the experimental and control groups.

Finally, the DNA sequence was evaluated using the Primer Express software and each assay was run on an Applied Biosystems 7300 real-time PCR system in triplicates to ensure reproducibility. Expression fold-changes were derived using the comparative C_T method (22).

Analysis of protein expression by Western blot assay. RAW264.7 cells (2×10^6 /well) were placed in a 6-well plate and then propofol was added to the cells at final concentrations of 2.5, 5 and 10 μM , while DMSO (solvent) alone was added to the wells as a vehicle control. Cells were incubated with propofol in medium with 10% FBS at 37°C for 0, 6, 12 and 24 h. Cells were harvested and resuspended in lysis buffer [ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 0.1% Triton X-100]. The collected cells were sonicated and centrifuged at 13,000 \times g for 20 min at 4°C to remove cell debris, and the supernatant was collected for determination of total protein concentration using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA) as the standard. SDS gel electrophoresis and western blotting were performed as previously described (22,23) for determining the effects of propofol on the protein levels of p53, p-p53, MGMT, p-H2A.X(S140), 14-3-3- σ , BRCA1 and MDC1.

Statistical analysis. All data are presented as the means \pm SD, and the Student's t-test was used to analyze differences between the propofol-treated and untreated (control) groups. All statistical analyses were performed, and $P < 0.05$ was considered to indicate a statistically significant result.

Results

Effect of propofol on the viability of RAW264.7 cells. Cells were treated with 0, 25, 50, 100 and 200 $\mu\text{g/ml}$ of propofol for 48 h or treated with 100 $\mu\text{g/ml}$ propofol for 0, 24, 48 and 72 h. All cells from each treatment group were collected and then flow cytometric assay was used to evaluate the percentage of viable RAW264.7 cells. Propofol decreased the percentage of

viable cells when compared to the control in a dose-dependent (Fig. 1A) and time-dependent manner (Fig. 1B).

Propofol induces DNA damage in RAW264.7 cells. We investigated whether propofol induces DNA damage in RAW264.7 cells *in vitro*. The comet assay was selected for determining DNA damage. The results indicated that propofol induced DNA damage in RAW264.7 cells. Higher concentrations of propofol led to a longer DNA migration smear (comet tail), and these effects occurred in a dose- (Fig. 2A and B) and time-dependent manner (Fig. 2C and D). As shown in Fig. 2A, 0.1% H_2O_2 induced the occurrence of a comet tail, and H_2O_2 is well documented as a highly reactive oxygen species and it has been used as a positive control for numerous studies involving agent-induced DNA damage.

Propofol induces DNA fragmentation in RAW264.7 cells. As shown in Fig. 2, propofol induced DNA damage in RAW264.7 cells, and therefore, DNA gel electrophoresis was used to investigate whether propofol causes DNA fragmentation in RAW264.7 cells. Cells were treated with various concentrations of propofol for 48 h, and then DNA was isolated from each treatment group. DNA fragmentation was assessed by DNA gel electrophoresis. As shown in Fig. 3, propofol induced DNA fragmentation in RAW264.7 cells.

Effects of propofol on expression of DNA damage and repair-associated genes in RAW264.7 cells. Based on the above results from the comet assay and DNA gel electrophoresis, we found that propofol induced DNA damage and fragmentation in RAW264.7 cells. Thus, we further investigated the effects of propofol on the expression of DNA damage and repair-associated genes in RAW264.7 cells. mRNA expression of all examined genes associated with DNA damage and repair including DNA-PK, BRCA1, MGMT and p53 was decreased following 48 h of exposure to propofol (Fig. 4).

Effects of propofol on the expression of DNA damage and repair-associated proteins in RAW264.7 cells. To further

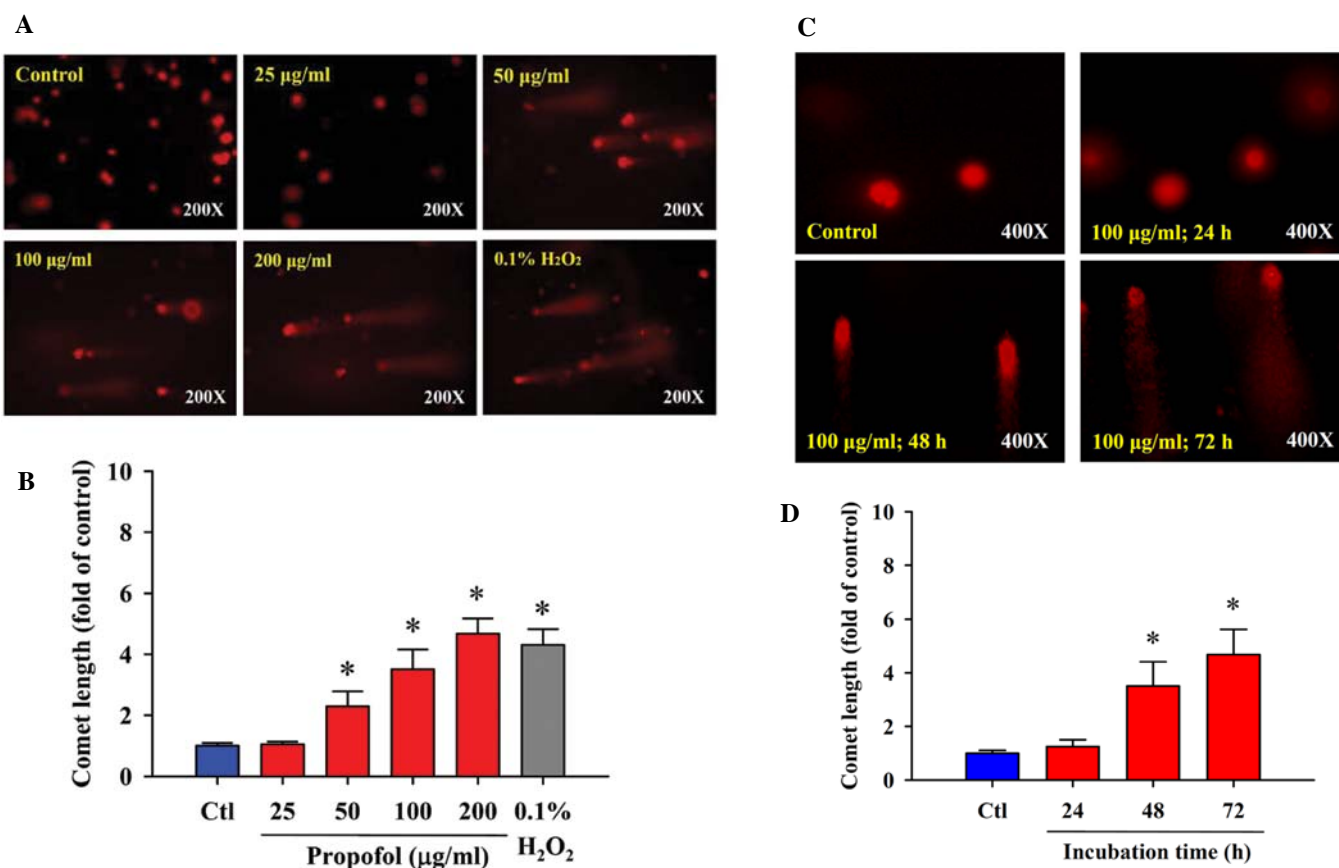


Figure 2. Propofol-induced DNA damage in RAW264.7 cells was determined by comet assay. Cells at a density of 2×10^5 /well in 12-well plates were incubated with propofol at final concentrations of 25, 50, 100 and 200 $\mu\text{g/ml}$, vehicle (1 μl PBS) or 0.1% H_2O_2 (positive control) for 48 h; or cells were treated with 100 $\mu\text{g/ml}$ propofol for 24, 48 and 72 h. DNA damage was determined by comet assay as described in Materials and methods. Representative images of the comet assay for (A) dose- and (C) time-dependent effects; (B and D) comet lengths (folds of control). Comet tail shows DNA damage. * $P < 0.05$ was considered to indicate a significant difference compared to the control sample.

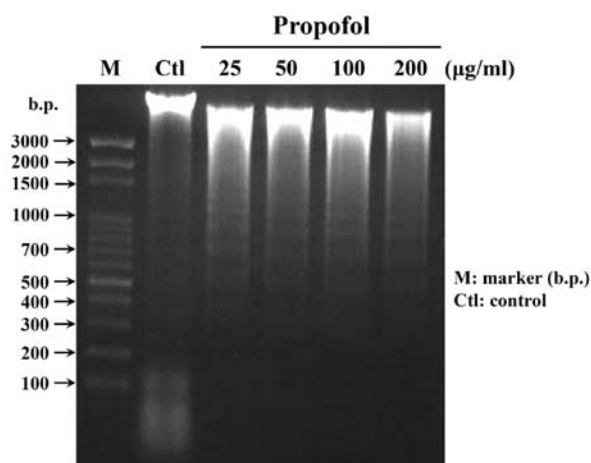


Figure 3. Propofol-induced DNA fragmentation in RAW264.7 cells as determined by DNA gel electrophoresis. Cells at a density of 2×10^6 /well in 6-well plates were incubated with propofol at final concentrations of 25, 50, 100 and 200 $\mu\text{g/ml}$ for 48 h. Cells were collected, and DNA was isolated from the cells of each treatment group for gel electrophoresis as described in Materials and methods.

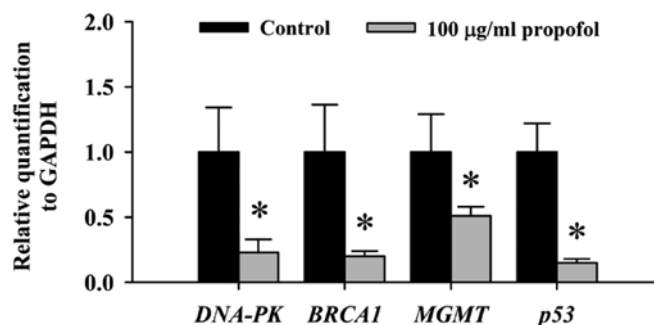


Figure 4. Propofol alters mRNA expression of DNA damage and repair-associated genes in RAW264.7 cells as determined by real-time PCR. Total RNA was extracted from the RAW264.7 cells following treatment with or without 100 $\mu\text{g/ml}$ propofol for 24 h. RNA samples were reverse-transcribed to cDNA and real-time PCR was conducted as described in Materials and methods. The ratios of DNA-PK, BRCA1, MGMT and p53 mRNA/GAPDH are presented in the bars of the histogram. Data represents mean \pm SD of three experiments. * $P < 0.05$ was considered to indicate a significant difference when compared to the control.

confirm whether propofol inhibits DNA repair gene expression and whether it also affects the associated protein expression, RAW264.7 cells were treated with propofol and then harvested

for western blotting. As shown in Fig. 5, levels of p53, MGMT, 14-3-3- σ , BRCA1 and MDC1 proteins were decreased while p-p53 and p-H2A.X(S140) were increased in the RAW264.7 cells following exposure to propofol.

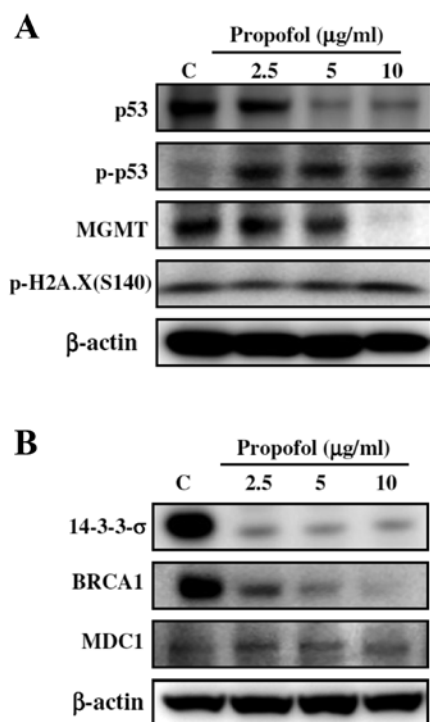


Figure 5. Propofol affects levels of DNA damage and repair-associated proteins in RAW264.7 cells. RAW264.7 cells were treated with propofol (0, 2.5, 5 and 10 μg/ml) and were then harvested for western blotting as described in Materials and methods. The protein levels of p53, p-p53, MGMT and p-H2A.X(S140) (A), 14-3-3-σ, BRCA1 and MDC1 (B) were examined from cells after exposure to propofol. The blots shown are representative from three independent experiments.

Discussion

Cytotoxic drugs and radiation are well-known DNA-damaging agents commonly used for cancer therapy and associated with the development of therapy-related myeloid neoplasms (23). Based on reports from other investigators, propofol was found to induce cytotoxic effects on human leukemia HL-60 cells via induction of apoptosis (24). Our previous study found that propofol induced apoptosis in murine leukemia RAW264.7 cells (13). However, there is no available information that has addressed propofol-induced DNA damage in murine leukemia cells. In the present study, we confirmed that propofol has cytotoxic effects on RAW264.7 cells, and we also demonstrated that propofol induced DNA damage (Fig. 2A and C) and DNA fragmentation (Fig. 3) and these effects were dose-dependent, and associated with loss of cell viability (Fig. 1). Furthermore, western blotting assay was used to examine the expression of DNA repair-associated protein. The results indicated that levels of p53, MGMT, 14-3-3-σ, BRCA1 and MDC1 proteins were decreased while p-p53 and p-H2A.X(S140) were increased in RAW264.7 cells following exposure to propofol. In the present study, the dose of propofol for inducing DNA damage was 25 μg/ml which is lower than that of other reports showing the blood concentration of propofol to be <25 μg/ml during and after anesthesia (24). Possibly the *in vitro* studies used culture plates, and the propofol directly affected the cells.

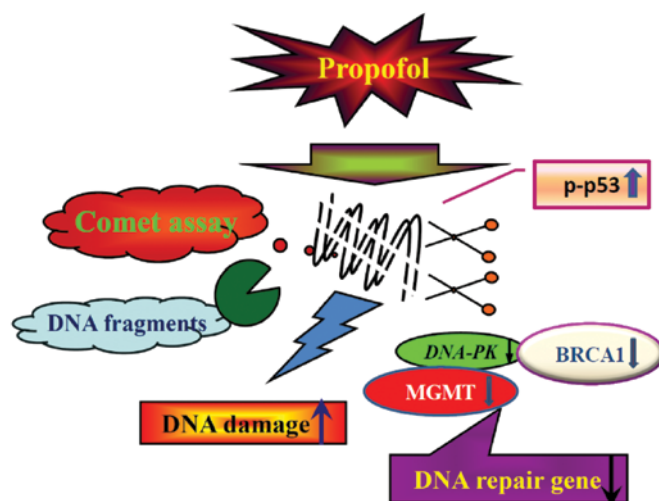


Figure 6. Possible signaling pathways involved in the inhibition of expression of DNA damage and repair-associated genes in murine leukemia RAW264.7 cells by propofol.

In the present study, the comet assay revealed that propofol induced nuclear DNA damage. It has been reported that various types of DNA damage are associated with cancer development, and therefore, mutations resulting from DNA damage are considered to be a hallmark of cancer (25). Thus, whether or not propofol is a carcinogen requires further investigation. However, numerous studies have used the comet assay to examine whether agents induce DNA damage based on its high sensitive technique for DNA damage assessment (26-28). Other investigators also showed that the comet assay can measure the strand-break formation during the process of excision repair of DNA (29,30).

In our previous study, we found that propofol-induced cell death was mediated through induction of apoptosis in RAW264.7 cells, as determined by DAPI staining (13). In the present study, we also confirmed that propofol caused DNA fragmentation as determined by DNA gel electrophoresis (Fig. 3). Our previous study showed that propofol induced apoptosis through the activation of caspase-3 in RAW264.7 cells, not via reactive oxygen species (ROS) (13). This is in agreement with other reports which found that propofol was capable of scavenging hydrogen peroxide (H₂O₂) (31). Thus, we suggest that propofol-induced DNA damage occurs independently of ROS production. Further studies are needed to establish the role of the interaction of propofol with DNA in cancer cells.

Agent-induced DNA damage can result in the loss of DNA repair capacity and accumulation of DNA damage. It was reported that agent-induced DNA damage can be reduced by DNA repair system through eliminating DNA lesions (32-34). In the present study, propofol-induced DNA damage in RAW264.7 cells is not clear. Therefore, to our knowledge this is the first report on propofol-induced DNA damage in murine leukemia cells. The mechanism involved in the DNA damage observed in the present study remains to be determined.

The results from real-time PCR revealed that propofol inhibited expression of DNA repair-associated genes

including DNA-PK, BRCA-1, MGMT and p53 (Fig. 4) dose-dependently in the RAW264.7 cells. Importantly, it has been reported that if agents cause DNA damage in checkpoints of the cell cycle, then there are signal transduction pathways involved in the cell cycle and cellular responses to DNA damage for maintaining genomic integrity (35-37). In human breast and ovarian cancer, it was reported that BRCA1 (tumor suppressor) plays critical roles in DNA repair, cell cycle checkpoint control and maintenance of genomic stability (38). DNA-PK plays an important role in DNA damage repair (39). Furthermore, MGMT reduces cytotoxicity of therapeutic or environmental alkylating agents (40,41). Another report demonstrated that anesthesia with propofol did not directly influence the expression of the DNA repair genes hOGG1 and XRCC1 in blood cells (42). Herein, we demonstrated that propofol inhibited expression of several of the DNA repair-associated genes including DNA-PK, BRCA-1 and MGMT in RAW264.7 cells. Furthermore, western blotting showed that propofol inhibited levels of DNA repair-associated proteins such as MGMT, 14-3-3- σ , BRCA1 and MDC1, and DNA damage-associated p53 proteins. Notably, propofol promoted p53 phosphorylation.

Our proposed flow chart for propofol-induced DNA damage in murine leukemia RAW264.7 cells is illustrated in Fig. 6, which shows that propofol induces DNA damage followed by the inhibition of expression (mRNA) of DNA repair-associated genes including DNA-PK, BRCA1, MGMT and p53 which then leads to DNA damage as shown by the comet assay and quantitative real-time PCR analysis.

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