Propofol suppresses proliferation, invasion and angiogenesis by down-regulating ERK-VEGF/MMP-9 signaling in Eca-109 esophageal squamous cell carcinoma cells

Y.-B. XU, Q.-H. DU, M.-Y. ZHANG, P. YUN, C.-Y. HE

Department of Anesthesiology, Provincial-Owned Hospital Affiliated to Shandong University, Jinan, Shandong Province, China

Abstract. – BACKGROUND AND AIMS: Propofol (2,6-diisopropylphenol), one of the most commonly used intravenous anaesthetic agents during cancer resection surgery, has been reported to have the ability of influencing the invasion of human cancer cells. However, the mechanisms are not very clear. In this study, we investigated the effects of propofol on the proliferation, invasion and angiogenesis of human Eca-109 cells, and explored the mechanism.

METHODS: The human Eca-109 cells was treated with propofol at the concentrations of 10-100 µmol/L for 72 hours or at the concentration of 100 µmol for 8-72 hours. Cell viability was determined by the MTT assay; the effect of propofol on apoptosis by 5′-triphosphate-biotin nick end labeling (TUNEL) staining. The effect of propofol on angiogenesis was determined by the chicken chorioallantoic membrane (CAM) angiogenesis assay. The effect of propofol on cell invasion using a modified Matrigel Boyden chamber assay. ERK1/2, MMP-9 and VEGF leves was detected by western blotting assay.

RESULTS: In human Eca-109 cells, propofol significantly promoted cell apoptosis and inhibited proliferation in a dose and time-dependent manner. Furthermore, propofol inhibited dose and time-dependent invasion and angiogenesis. Propofol significantly dose and time-dependently down-regulated gene expression and protein production of ERK/pERK, VEGF and MMP-9. The functional effects and MMP-9/VEGF inhibition were shown to be dependent on the ERK/VEGF and ERK/MMP-9 signaling pathways. It was noteworthy that the ERK activator (phorbol 12-myristate 13-acetate [PMA]) treatment increased the MMP-9/VEGF levels after propofol treatment, and led to significant increase of proliferation, invasion and angiogenesis.

CONCLUSIONS: These findings indicate that propofol inhibited proliferation, invasion and angiogenesis of human Eca-109 cells *in vitro* through modulation of ERK-VEGF /MMP-9 signaling. Propofol not only can be an anesthesia agent which reduces pain but plays an important role of inhibiting the migration and angiogenesis of ESCC cells in the therapy of ESCC patients.

Key Words:
　Esophageal squamous cell carcinoma, Angiogenesis, Metastasis, Propofol.

Abbreviations

MMPs = matrix metalloproteinases; VEGF = Vascular Endothelial Growth Factor; ERK = extracellular signal-regulated kinases; Eca 109 cells = esophageal squamous carcinoma cells; Rho A = gene ras homolog family member A; BSA = bovine serum albumin; FBS = fetal bovine serum; DMSO = dimethyl sulfoxide; TUNEL = Terminal deoxynucleotidyl transferase dUTP nick end labeling; CAM = chorioallantoic membrane; PMA = phorbal 12-myristate 13-acetate; ESCC = esophageal squamous cell carcinoma.

Introduction

Despite improvements in detection, surgical resection, and (neo-) adjuvant therapy, the overall survival for esophageal squamous cell carcinoma (ESCC), one of the most aggressive carcinomas of the gastrointestinal tract, remains lower than that of other solid tumors due to distant and lymph node metastasis[1]. Like other types of solid tumors, the development of ESCC is also the accumulation of the abnormal expression of metastasis oncogenes such as MMPs and VEGF[2-5].

Ras/Raf/mitogen-activated protein-extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK signaling is critical for many fundamental processes, including cell proliferation[6], survival[7], motility and migration[8]. In esophageal squamous cell carcinoma[9], transfection with

Corresponding Author: Meng-Yuan Zhang, Ph.D; e-mail: slyyzmy@126.com

shRNA-ERK2 targeted ERK2 into Eca109 cells can inhibit growth of Eca109, inducing cell apoptosis and influencing cell-cycle, which suggested that ERK2 plays an important role in cell growth of Eca109. In human lung cancer models, (MEK)/ERK inhibition resulted in potent antiangiogenic effects with decreased VEGF expression and signaling[10], which suggested ERK plays an important role in regulation of angiogenesis. Many studies showed ERK signals promotes growth, invasion, angiogenesis and metastasis also by MMP-9 pathway[11-12]. Therefore, we suggested that ERK signal could be an effective target for cancer treatment. Propofol (2,6-disopropylphenol), one of the most commonly used intravenous anaesthetic agents during cancer resection surgery, has been reported to have the ability of influencing the invasion of human cancer cells[13-14]. In lung cancer cell, propofol inhibits invasion and migration *in vitro* by suppression of MMP-2 and -9 mRNA and protein expressions[14]. In addition, continuous infusion of propofol also inhibited pulmonary metastasis of murine osteosarcoma (LM 8) cells in mice by active Rho A[15], which is also a target for inducing MMP-9 malignant phenotype[16].

A recent studies found ERK pathway plays an important role in the regulation of propofol-mediated antioxidant effects in SH-SY5Y cells[17]. Propofol furthermore suppressed the ERK signaling pathway in cardiac H9c2 cells subjected to hypoxia/reoxygenation[18]. These results suggest that ERK pathway may be the target of propofol and this agent might be an ideal anesthetic for cancer surgery.

In this report we show that propofol suppresses proliferation, invasion and angiogenesis in Eca-109 esophageal squamous cell carcinoma cells *in vitro*. The proposed role of ERK1/2 signaling in regulation of MMP-9 and VEGF plays an important role in the course.

Materials and Methods

Cell Lines and Cell Culture

Human Eca-109 cells was obtained from the American Type Culture Collection and maintained in 5% CO_2 atmosphere at 37°C, and supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 5% glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells underwent serum starvation for 24 h before treatment with propofol or/and ERK activator (phorbol 12-myristate 13-acetate [PMA]) for the indicated time.

Reagents

Pure propofol was obtained from Sigma Chemical (St Louis, MO, USA) to exclude the influence of lipid emulsion. ERK activator (phorbol 12-myristate 13-acetate [PMA]) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against phosphorylated or total ERK1/2, MMP-9 and VEGF were purchased from Cell Signaling Technology (Beverly, MA, USA). Goat HRP-conjugated anti-rabbit and antimouse antibodies were purchased from Invitrogen (Carlsbad, CA, USA). PD98059 was purchased from Cayman Chemical Company, Ann Arbor, MI, USA.

Cell Viability Assay

Cell viability was determined by the MTT assay (Roche Diagnostics, Indianapolis, IN, USA) as the manufacture's instruction. Briefly, cells plated in 96-well plates (2000 cells/well) were treated with propofol at the indicated doses. At 72 hours after treatment, cell viability was determined.

TUNEL Assay

Eca-109 cells subjected to propofol or PMA or untreated cells were fixed with 4% paraformaldehyde. Terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick end labeling (TUNEL) staining was performed using an Apoptag Peroxidase in Situ Apoptosis Detection kit (Chemicon International, Temecula, CA, USA) as the manufacture's instruction.

Chicken Chorioallantoic Membrane (CAM) Angiogenesis Assay

Eca-109 cells were subjected to propofol or PMA or untreated cells were for the indicated time. The conditioned medium was filtered off for future research.White Leghorn chicken eggs were fertilized, and incubated at 37°C with 70% humidity. An artificial air sac was created over a region containing small blood vessels in the CAM as described previously[19]. To investigate the effect of propofol on tumor-induced angiogenesis, the conditioned medium above were placed onto the CAM (10-day-old). After placement of the discs, embryos were transferred back into the incubator. After 3 days, embryos were examined under a stereomicroscope for evaluation of angiogenesis. Angiogenesis was quantitated by counting vessel intersections.

In Vitro Cell Invasion Assay

The cellular invasiveness was quantified using a modified Matrigel Boyden chamber assay. The

BD Bio-Coat Matrigel invasion chamber (BD Biosciences, Bedford, MA, USA) was used according to the instructions of the manufacturer. Eca-109 cells were treated with propofol or PD98059 at the indicated doses and for the indicated time. Then 2.5×10^4 cancer cells treated above in serum-free media containing 0.15% bovine serum albumin (BSA) were seeded onto Matrigel-coated filters. Ten percent FBS was added to the lower chambers as a chemoattractant. After 24-hour incubation at 37°C, the filters were stained using a Diff-Quik kit (BD Biosciences, San Josè, CA, USA), and the number of cells that had passed through the filter was counted under magnification. The counting was performed in 5 randomly selected fields for each sample, and the mean values from 3 independent experiments were used.

Statistical Analysis

All values in the present study were reported as Mean ± SD from three independent experiments. Two-sided student's unpaired *t* test was used for statistical analyses. *p* values less than 0.05 were considered statistically significant.

Results

Propofol Inhibits Eca-109 Cell Proliferation in a Dose and Time-Dependent Manner

To determine whether propofol may regulate the proliferation of Eca-109 cells, we examined the effect of propofol on cell viability in various kinds of human Eca-109 cell line by a MTT analysis. The results showed that treatment with propofol at the concentrations of 10-100 μmol/L for 72 hours significantly inhibitted cell proliferation in a concentration-dependent manner, resulting in a 8-47% decrease in cell viability in Eca-109 cell (Figure 1A). We also examined the effect of propofol on Eca-109 cell proliferation at the concentration of 100 μmol at a serial of different treatment time (8-72 hours), and found that propofol significantly inhibited cell proliferation in a time-dependent manner in the Eca-109 cell lines (Figure 1B).

Propofol Promotes Eca-109 Cell Apoptosis in a Dose and Time-Dependent Manner

We examined the effect of propofol on cell apoptosis in various kinds of human Eca-109 cell line by TUNEL analysis. The results showed that treatment with propofol at the concentrations of

10-100 μmol/L for 72 hours significantly promoted cell apoptosis in a concentration-dependent manner, resulting in a 1.8-10.6% increase in cell apoptosis in Eca-109 cell (Figure 2A). We also examined the effect of propofol on Eca-109 cell apoptosis at the concentration of 100 μmol at a serial of different treatment time (8-72 hours), and found that propofol significantly promoted cell apoptosis in a time-dependent manner in the Eca-109 cell lines (Figure 2B). This suggests that propofol inhibits proliferation of Eca-109 cells in part by promoting apoptosis.

Propofol Inhibits Eca-109 Cell Angiogenesis in the Chorioallantoic Membrane

Eca-109 cells were subjected to 10-100 μmol/L propofol for 72 hours, or at the concentration of 100 μmol/L for 8-72 hours. The conditioned medium was filtered off for the CAM assay. The results showed that the conditioned medium from Eca-109 cells treated with propofol at the concentrations of 10-100 μmol/L for 72 hours significantly inhibited angiogenesis in a concentration-dependent manner (Figure 3A). We also examined the effect of the conditioned medium from Eca-109 cells treated with 100
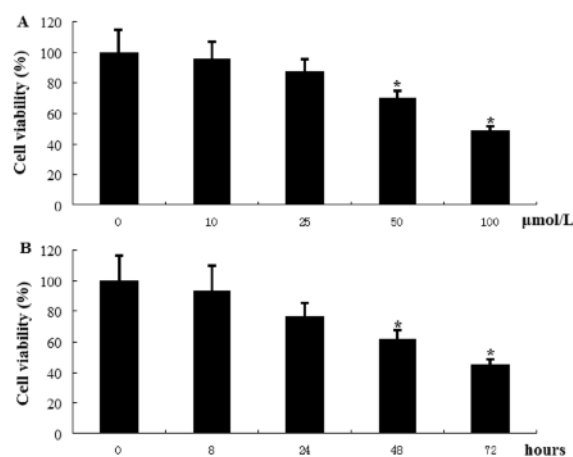
Figure 1. Propofol inhibits proliferation of Eca-109 cells. Human Eca-109 cells were treated with propofol at the doses of 10-100 μmol/L for 72 hours *(A)* or at the concentration of 100 μmol/L for 8-72 hours *(B)*. The cell viability was determined by MTT assay *(A)*. Cells treated with vehicle control DMSO were used as the referent group with cell viability set at 100%. The percent cell viability in each treatment group was calculated relative to cells treated with vehicle control. The data are presented as mean ± SD of three separate experiments. **p* < 0.05, significant differences between treatment groups and control groups.
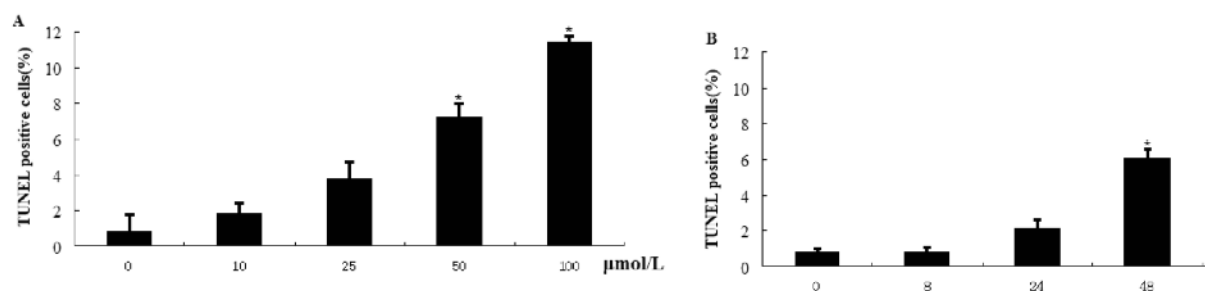
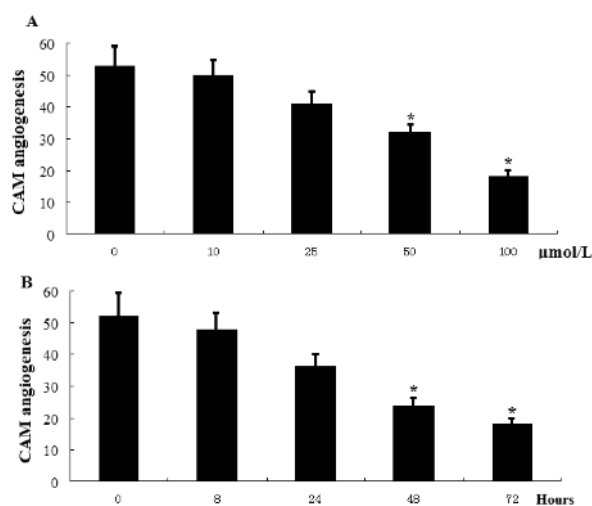**Figure 2.** Propofol promotes apoptosis of Eca-109 cells. Human Eca-109 cells were treated with propofol at the doses of 10-100 μmol/L for 72 hours *(A)* or at the concentration of 100 μmol/L for 8-72 hours *(B)*. The cell apoptosis was determined by TUNEL assay *(A)*. Cells treated with vehicle control DMSO were used as the referent group. The data are presented as mean ± SD of three separate experiments. *$p < 0.05$, significant differences between treatment groups and control groups. TUNEL immunostaining showed more TUNEL-positive Eca-109 cells. Scale bar, 50 μm.

μmol/L propofol for 8-72 hours, and found that the conditioned medium significantly inhibited angiogenesis in a time-dependent manner (Figure 3B).

Propofol Inhibits Eca-109 Cell Migration

To address the role of Propofol in Eca-109 invasion, Eca-109 cells were subjected to 10-100 μmol/L propofol for 72 hours, or at the concentration of 100 μmol/L for 8-72 hours. Contrast to control cells, the relative invasive cells in Eca-109

cells treated 10-100 μmol/L propofol was 0.94, 0.82, 0.64, 0.41 (control was defined to 1) (vs control, *$p < 0.01$, Figure 4A). The results showed Propofol treatment showed dose-dependently decreased in the invasive Eca-109 cells. We also examined the effect of propofol on Eca-109 cell invasion at the concentration of 100 μmol at a serial of different treatment time (8-72 hours), and found that propofol significantly inhibited cell invasion in a time-dependent manner in the Eca-109 cell lines (Figure 4B).
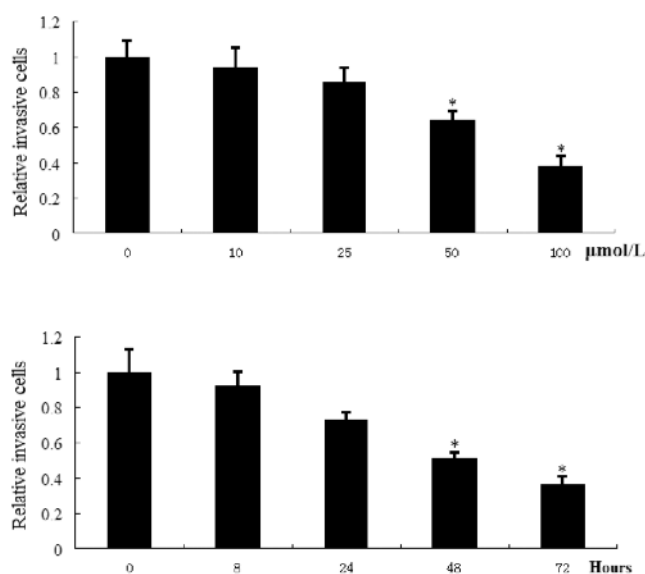
Propofol Inhibits MMP-9 and VEGF Expression

MMP-9 and VEGF overexpression is associated with angiogenesis and tumor cell proliferation and invasion[2-3,5]. We determined whether the effect of propofol in inhibiting Eca-109 cells proliferation, invasion and angiogenesis was realized through inhibiting VEGF and MMP-9 pathway. The effect of propofol on VEGF and MMP-9 protein expression was analyzed using western blot. As shown in Figure 5A and 5B, treatment with propofol significantly inhibited the expression of VEGF and MMP-9 protein in a dose- and time-dependent manner in Eca-109 cells.

Propofol Inhibits Activation of ERK1/2

It has shown that MAPK is required for the transactivation activity of VEGF and MMP-9. To determine whether propofol-mediated inhibiting proliferation, invasion and angiogenesis in Eca-109 cells is through the inhibition of MAPK signaling pathway, we evaluated the effect of propofol on the inhibition of ERK, one of the key signaling proteins in MAPK pathway, by western blot.

As shown in Figure 5C, treatment with propofol at 10-100 μmol/L for 72 hours significantly

Figure 3. Effect of propofol on angiogenesis in the CAM assays. Human Eca-109 cells were treated with propofol at the doses of 10-100 μmol/L for 72 hours *(A)* or at the concentration of 100 μmol/L for 8-72 hours *(B)*. Representative image of the conditioned medium above-treated CAM shows decreased angiogenesis compared to control. The data are presented as mean ± SD of three separate experiments. *$p < 0.05$, significant differences between treatment groups and control groups.

Figure 4. Propofol inhibits invasion of Eca-109 cells. Human Eca-109 cells were treated with propofol at the doses of 10-100 µmol/L for 72 hours *(A)* or at the concentration of 100 µmol/L for 8-72 hours *(B)*. The cell invasion was determined by using a modified Matrigel Boyden chamber assay. Cells treated with vehicle control DMSO were used as the referent group. The data are presented as mean ± SD of three separate experiments. *$p < 0.05$, significant differences between treatment groups and control groups.

decreased the levels of the phosphorylated ERK1/2 protein in a dose-dependent manner, whereas the levels of total ERK1/2 protein did not change. Furthermore, treatment with propofol at the concentration of 100 µmol/L for 8-72 hours significantly decreased the levels of the phosphorylated ERK1/2 protein in a time-dependent manner (Figure 5D), whereas the levels of total ERK1/2 protein did not change (Figure 5D).

Propofol-Mediated Inhibition of VEGF and MMP-9 is ERK-Dependent

To determine whether the propofol-mediated decrease of VEGF and MMP-9 is through regulating the ERK/MAPK signaling, we analyzed the effect of ERK activator (phorbol 12-myristate 13-acetate [PMA]) on VEGF and MMP-9 in propofol-treated Eca-109 cells. Pretreatment with 40 µmol/L ERK activator (phorbol 12-myristate 13-acetate [PMA]) for 12 hours dramatically increased VEGF and MMP-9 expression after propofol treatment (Figure 6). These results indicated the ERK signaling pathways played important roles in mediating propofol's effect on inhibiting VEGF and MMP-9 in Eca-109 cells.

Propofol-Mediated Inhibition of Proliferation, Invasion and Angiogenesis is ERK-Dependent MMP-9 and VEGF Downregulation

As shown above, treatment of Eca-109 cells with propofol inhibits proliferation, invasion and angiogenesis in a dose- and time-dependent manner. However, pretreatment with 40 µmol/L ERK

activator (phorbol 12-myristate 13-acetate [PMA]) to activate pERK increased MMP-9 and VEGF expression greatly increased the proliferation, invasion and angiogenesis in Eca-109 cells after propofol treatment (Figure 7).

Discussion

This study has investigated the mechanisms underlying the anti-metastatic and proliferation function of propofol in ESCC Eca-109 cells *in vitro*. We provide evidence that in ESCC cells, propofol downregulates expression of matrix metalloproteinase MMP-9 and VEGF via a mechanism involving ERK1/2. Overexpression of ERK activator [phorbol 12-myristate 13-acetate [PMA]) block downregulation of MMP-9 and VEGF by propofol treatment. Propofol suppresses tumor proliferation, angiogenesis and metastasis in the ESCC Eca-109 cells, the effect of which was by inhibiting pERK dependent downregulation of MMP-9 and VEGF.

Propofol is an intravenous anesthetic with neuroprotective, myocardial-protective and intestinal mucosal-protective effects against cerebral ischemia-reperfusion, myocardial ischemia/reperfusion injury, and the underlying mechanisms was by inhibition of cell apoptosis[20-22]. In this study, we found treatment with propofol inhibited cell proliferation in a dose and time-dependent manner in the Eca-109 cell lines. Furthermore, we found treatment with propofol promoted cell apoptosis in a dose and
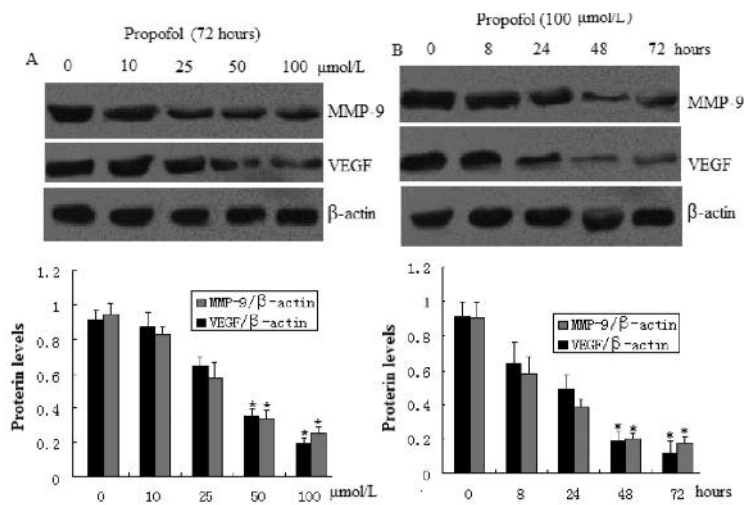
Figure 5. Propofol downregulates pERK1/2, MMP-9 and VEGF expression in Eca-109 cells. **(A),** Human Eca-109 cells were treated with propofol at the concentration of 10-100 μmol/L for 72 hours. **(B),** Treated with propofol at the concentration of 100 μmol/L for 8-72 hours. The expression of ERK1/2, MMP-9 and VEGF protein levels were detected by Western blotting. β-actin was used as a control for sample loading. *$p < 0.05$, versus 0 min timepoint.
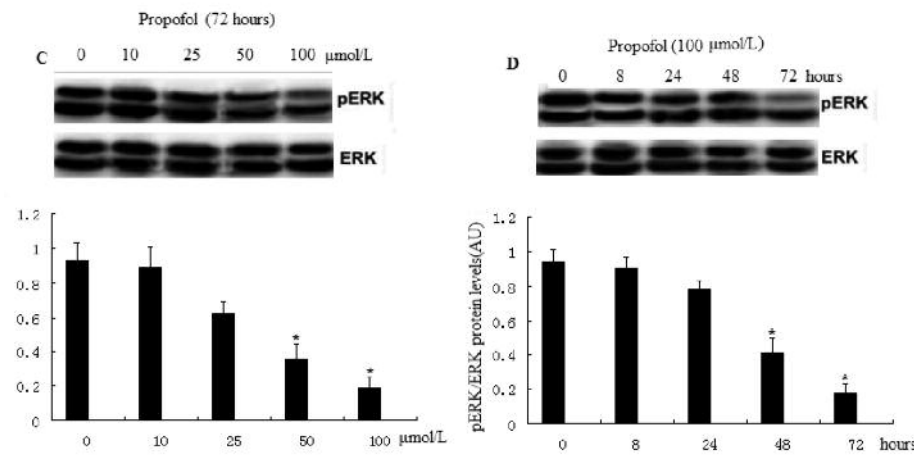
Figure 6. Propofol-mediated inhibition of VEGF and MMP-9 is ERK-dependent. Eca-109 cells were pretreated with 40 μmol/L ERK activator (phorbol 12-myristate 13-acetate [PMA]) for 12 hours, after which the cells were then treated with **(A)** 10-100 μmol/L for 72 hours, **(B)** 100 μmol/L for 8-72 hours. Relative VEGF and MMP-9 protein value was detected by western blot assay. *$p < 0.05$. versus 0 min timepoint.
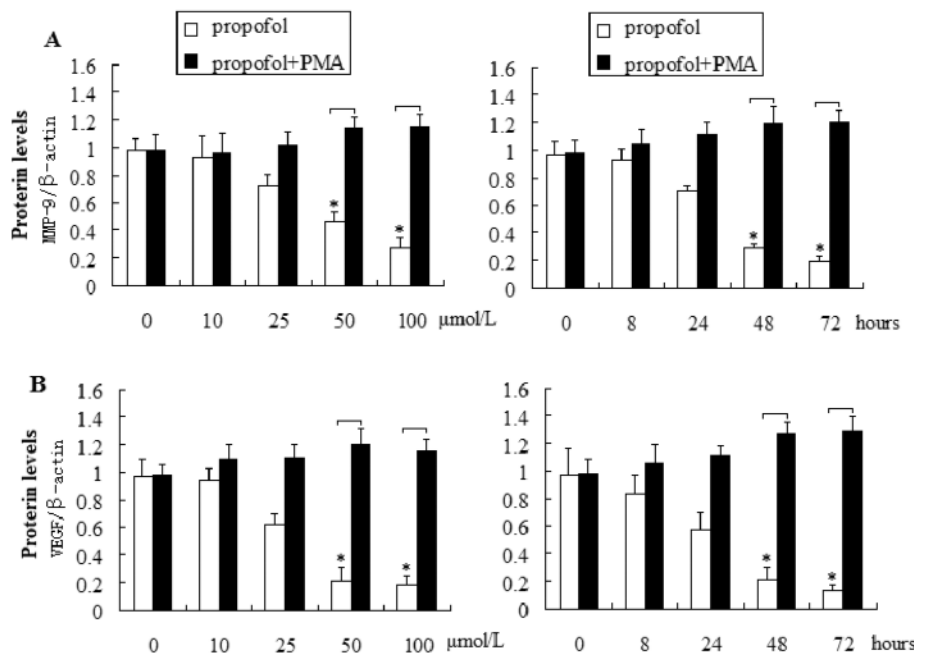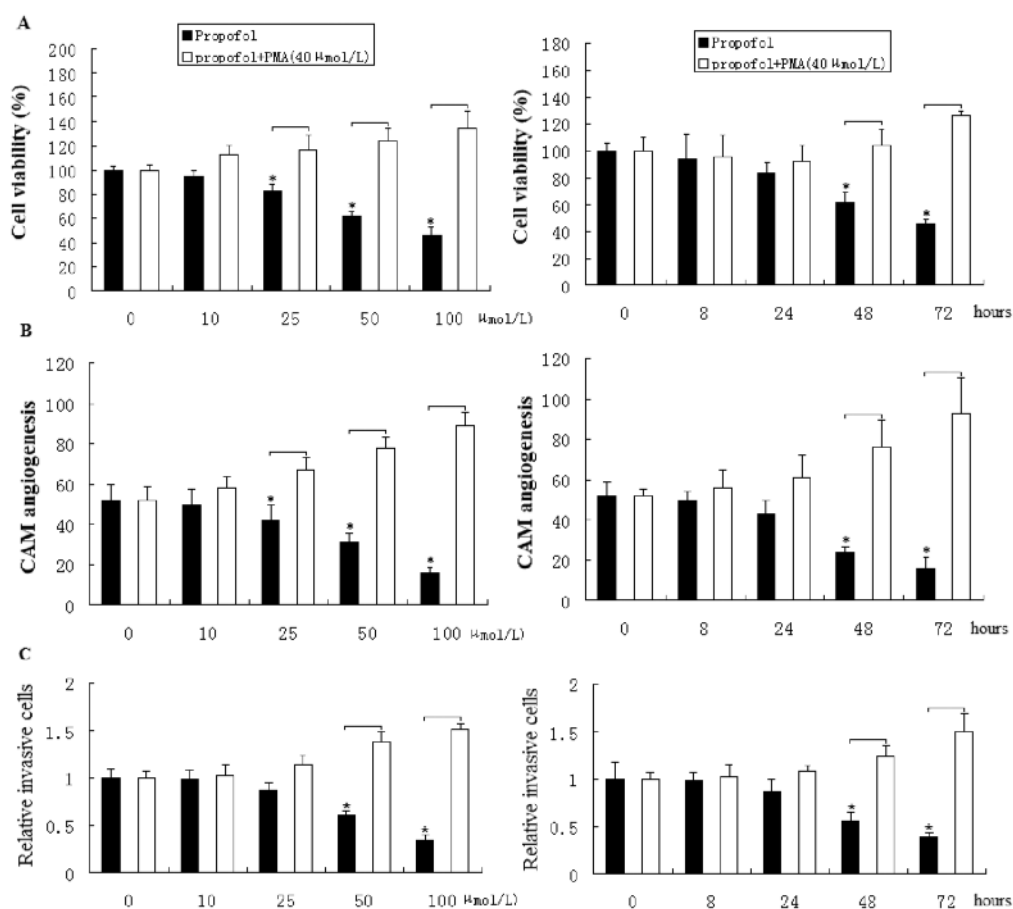
Figure 7. Propofol-mediated increase of proliferation, invasion and angiogenesis is ERK-dependent MMP-9 and VEGF downregulation. Human Eca-109 cells were pretreated with 40 μmol/L ERK activator [phorbol 12-myristate 13-acetate [PMA])] for 12 hours, after which the cells were then treated with 10-100 μmol/L for 72 hours, or 100 μmol/L for 8-72 hours. The cell viability was determined by MTT assay *(A)*. The cell invasion was determined by using a modified Matrigel Boyden chamber assay *(B)*. The angiogenesis was determined by the CAM assays *(C)*. The data are presented as the mean±SD of three separate experiments. * *p* < 0.05, versus 0 min timepoint.

time-dependent manner in the Eca-109 cell lines. These results indicated that propofol inhibits proliferation of Eca-109 cells in part by promoting apoptosis.

It has reported that ERK signaling is activated in both prosurvival and proapoptotic conditions[23-24]. In the hepatic L02 cells, propofol protects hepatic L02 cells from hydrogen peroxide-induced apoptosis via activation of extracellular signal-regulated kinases (ERK) pathway[25]. We, therefore, explored whether propofol has an impact on ERK signals in the Eca-109 cells. The data showed propofol could inhibit phosphorylation of extracellular signal-regulated kinase 1 and 2 (pERK1/2) in a dose and time-dependent manner in the Eca-109 cell lines followed by increased proliferation and decreased apoptosis. However,

pretreatment with ERK activator [PMA]) to activate pERK greatly increased the proliferation in the Eca-109 cell lines after propofol treatment. We, therefore, suggested that the propofol-mediated regulation of proliferation in the Eca-109 cell lines is through regulating the pERK expression.

It has showed propofol could inhibit the migration and invasion of human lung cancer A549 cells and HeLa cells *in vitro*[14-15]. The data here showed propofol could inhibit angiogenesis and metastasis in the ESCC Eca-109 cells *in vitro* in a dose and time-dependent manner, and the propofol-mediated regulation of angiogenesis and metastasis in the Eca-109 cell lines is through regulating the pERK expression. However, there is no information on how propofol affects migration and invasion of cancer cells.

Multiple molecular mechanisms regulate invasion and migration of cancer cells. MMPs and VEGF are involved in ESCC angiogenesis, metastasis and stimulation of tumor growth. Increased expression of MMPs and VEGF is involved in ESCC invasion and metastasis (26-29). In the ESCC cell line EC9706, plasma FN was able to phosphorylate Raf and further activate Erk, but it did not alter MMP-2 protein expression or activity, indicating that MMP-2 may not be the downstream target gene of the Erk pathway in the ESCC cell line EC9706[30]. Here, we demonstrated that propofol inhibited the protein expression of MMP-9 and VEGF in the ESCC Eca-109 cells in a dose and time-dependent manner. However, pretreatment with ERK activator [PMA]) to activate pERK greatly increased the MMP-9 and VEGF expression in the Eca-109 cell lines after propofol treatment.

Conclusions

Propofol inhibited proliferation, angiogenesis and invasion by inhibition of the ERK pathway and further repression of particular MMP-9 and VEGF gene expression. Our findings suggest that propofol inhibited proliferation, angiogenesis and invasion in the Eca-109 cell lines. The possible signaling pathways of propofol may be *via* blocking ERK-dependent MMP-9 and VEGF expressions. The results suggest that propofol might be an ideal anesthetic for cancer surgery.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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