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# Propofol suppresses invasion, angiogenesis and survival of EC-1 cells *in vitro* by regulation of S100A4 expression

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**Abstract. – OBJECTIVE: Propofol possess anticancer properties in several cancers. In the present study, we investigate the effect of propofol on the human esophageal squamous cell carcinomas (ESCC) EC-1 cells *in vitro* and its molecular mechanisms of action.**

**MATERIALS AND METHODS: EC-1 cells were explored to 10-100 μmol/L propofol for 72 h or 100 μmol/L/mL propofol for 24-72 h. EC-1 cells were explored to 100 μmol/L propofol for 24 h, then was transiently transfected into PcDNA3.1-S100A4 cDNA or PcDNA3.1 plasmid for 48 hrs. MTT, TUNEL, ELISA, migration, tube formation and immunoblotting were analized.**

**RESULTS: Propofol inhibits invasion, angiogenesis, proliferation and induces apoptosis in a dose and time-dependence manner, followed by deseased S100A4 expression by Western blot assay. Pre-transfection of PcDNA3.1-S100A4 cDNA inhibits propofol-induced apoptosis and promotes invasion and angiogenesis in EC-1 cells *in vitro*.**

**CONCLUSIONS: Propofol inhibited invasion, angiogenesis and induces apoptosis of human EC-1 cells *in vitro* through regulation of S100A4 expression. It not only can be an anesthesia agent, but also plays a important role of inhibiting the migration and angiogenesis of ESCC cells in the therapy of ESCC patients.**

*Key Words:*
Esophageal squamous cell carcinomas, Propofol, metastasis; NF-KB; S100A4.

## Introduction

Propofol is a short-acting intravenous anaesthetic agent, which has been widely used in operating rooms as well as in the Intensive Care Unit (ICU). Increasing experimental evidence suggests that propofol has the neuroprotective effect against ischemic neuronal injury in animal models of cerebral ischemia[1,2]. The mechanisms by which may be associated with apoptotic inhibition by a consequence of the regulation of Bcl-2, caspase-3 and Bax[3,4].

It has recently found that propofol possess anticancer properties in several cancers. In ovarian cancer OVCAR-3 cells and gallbladder cancer GBC-SD cells, propofol inhibited proliferation of OVCAR-3 and GBC-SD cells in a dose- and time-dependent manner. After exposure to propofol for 24-48 hours, these cells showed increased apoptosis and increased invasion[5-7]. Propofol also has the same anticancer properties in hepatocellular carcinoma[8], esophageal squamous cell carcinoma[9], osteosarcoma[10], and lung adenocarcinoma[11]. However, the propofol's molecular mechanism of action is unclear.

We have previously found propofol inhibited proliferation, invasion and angiogenesis of human esophageal squamous cell carcinomas (ESCC) Eca-109 cells *in vitro* through modulation of ERK-VEGF/MMP-9 signaling[9]. Wu et al[11] has reported that propofol inhibits MMP-2 and -9 mRNA and protein expressions, resulting in suppression of lung cancer cell invasion and migration *in vitro*. In breast cancer cells, propofol could inhibit the invasion and migration of breast cancer cells via regulation of NF-κB/MMPs signal[12].

S100A4 is a member of the S100 family. It has 101 amino acids and with a molecular weight of about 11.6 kDa. S100A4 possesses a wide range of biological functions, such as regulation of angiogenesis[13-15], cell survival[15-16], motility and invasion[16-18]. S100A4 functions through regulation of NF-κB, MMP-9 and VEGF[13,14,18]. Previous studies have found that NF-κB, MMP-9 and VEGF, which were related with invasion and migration of many experimental animal models,



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were positively regulated by propofol[9-12]. In the present study, we provide evidence to support the hypothesis that propofol inhibits invasiveness and survival of human ESCC cells through the transcriptional regulation of S100A4.

## Materials and Methods

### Cell Culture

The human esophageal squamous cell carcinomas (ESCC) EC-1 cells, which has high S100A4 expression[19], was purchased from the American Type Culture Collection (ATCC, Shanghai, China). It was cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), nonessential amino acids, sodium pyruvate, penicillin/streptomycin antibiotics, and L-glutamine. Cells were maintained in a humidified incubator containing 10% $CO_2$ at 37°C. Before propofol treatment, the cells underwent serum starvation for 24 h.

### Propofol Treatment

EC-1 cells were explored to 10-100 μmol/L propofol for 72 h or 100 μmol/L/mL propofol for 24-72 h. The cells were collected and processed for analysis of MTT, TUNEL, ELISA, migration, tube formation and immunoblotting.

### S100A4 cDNA Transfection

PcDNA3.1-S100A4 cDNA and PcDNA3.1 plasmid was kindly gifted by Dr. Zhang, General Surgery, Affiliated Hospital of Medical College, QingDao University, QingDao[19]. EC-1 cells were explored to 100 μmol/L propofol for 24 h, then was transiently transfected into PcDNA3.1-S100A4 cDNA or PcDNA3.1 plasmid for 48 hrs using Lipofectamine 2000 (Invitrogen, Guangzhou, China) in accordance with the manufacturer's protocol. The cells were collected and processed for analysis of MTT, TUNEL, FACS, migration, tube formation and immunoblotting.

### Western Blot

Cellular proteins from treated EC-1 cells were prepared according to the manufacturer's protocol. For routine quantitation of proteins, following the manufacturer's protocol (Pierce, Rockford, IL, USA). 40 ug of protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on either 12% Tris-acetate gradient gels for S100A4 detection. After gel electrophoresis and transfer to nitrocellulose, the membranes were stained. Membranes were incubated at 4°C overnight in a blocking solution containing 5% bovine skim milk and 0.1% Tween 20 (Fischer Scientific, Pittsburgh, PA, USA) in TBS (10 mM Tris-HCl with 150 mM NaCl, pH 7.6), then probed with specific primary and secondary antibodies conjugated to horseradish peroxidase (HRP). Immunoreactive bands were visualized by chemiluminescence solution and exposure to X-ray film.

### TUNEL Assay for Apoptosis

Apoptosis of the EC-1 cells treated with propofol alone or combined with PcDNA3.1-S100A4 cDNA and PcDNA3.1 plasmid transfection was evaluated by the terminal transferase dUTP nick end labeling (TUNEL) assay according to the manufacturer's instructions. TUNEL-positive cells was assessed in three randomly selected fields each section. All assays were performed in quadruplicate.

### ELISA for Apoptosis

Apoptosis of the EC-1 cells treated with propofol alone or combined with PcDNA3.1-S100A4 cDNA and PcDNA3.1 plasmid transfection was evaluated by the Cell Apoptosis ELISA Detection Kit according to the manufacturer's protocol. The spectrophotometric absorbance of the samples was determined using ULTRA Multifunctional Microplate Reader (Tecan, San Jose, CA, USA) at 405 nm.

### MTT for Cell Growth Inhibition Assay

EC-1 cells treated with propofol alone or combined with PcDNA3.1-S100A4 cDNA and PcDNA3.1 plasmid transfection were seeded at a density of $3 \times 10^3$ cells per well in 96-well microtiter culture plates. After overnight incubation, 20 μL of MTT solution (5 mg/mL in phosphate buffered saline – PBS) were added to each well and incubated further for 2 h. Upon termination, the supernatant was aspirated and the MTT formazan formed by metabolically viable cells was dissolved in 100 μL of isopropanol. The plates were mixed for 30 min on a gyratory shaker, and absorbance was measured at 595 nm using a plate reader.

### Chicken Chorioallantoic Membrane (CAM) Angiogenesis Assay

EC-1 cells treated with propofol alone or combined with PcDNA3.1-S100A4 cDNA and PcDNA3.1 plasmid transfection for the indicated

time. The conditioned medium was filtered off for future research. White Leghorn eggs were incubated with 70% humidity at 37°C. 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. Blood vessels density was quantified by Image J software and represented as a bar diagram.

### Cell Invasion Assay

EC-1 cells treated with propofol alone or combined with PcDNA3.1-S100A4 cDNA and PcDNA3.1 plasmid transfection for the indicated time were trypsinized and resuspended in DMEM and 0.1% bovine serum albumin (BSA) at $6 \times 10^5$ cells/ml. A 250 $\mu$l aliquot of the suspension was applied into the inner chamber of a cell invasion plate assembly in a QCM 24-well cell invasion assay kit (Chemicon International, Billerica, MA, USA). The cells were incubated at 37°C for 24 h. Invaded cells were fluorimetrically detected according to the manufacturer's instruction.

### Statistical Analysis

All statistical analyses were performed using the SPSS11.0 software (SPSS Inc., Chicago, IL, USA). The results were used as means ± SD of three replicate assays. Differences between different groups were assessed using ANOVA or also post hoc test. $p < 0.05$ was considered as statistical significance.

## Results

### Propofol Inhibits S100A4 Expression in EC-1 Cell in a Dose- and Time-Dependent Manner

S100A4 was overexpressed under normal growth conditions in EC-1 cells by Western blot assay. EC-1 cells treated with 10-100 μmol/L propofol for 72 h signifintly reduced the levels of S100A4 in EC-1 cells in a concentration-dependent fashion (Figure 1A). Furthermore, EC-1 cells treated with 100 μmol/L propofol for 24-72 h also significantly reduced the levels of S100A4 in EC-1 cells in a time-dependent fashion (Figure 1B).

### Effect of Propofol on Cell Proliferation

EC-1 cells were exposure to 10-100 μmol/L propofol for 72 h, and the cell proliferation was detected by MTT analysis. Treatment with 10, 50, and 100 μmol/L/mL of propofol for 72 h resulted in a dose-dependent inhibition of cell proliferation (Figure 2A). Treated with 100 μmol/L concentrations of propofol for 24-72 h also resulted in a time-dependent inhibition of cell proliferation (Figure 2B).

### Propofol Induces EC-1 cell Apoptosis in a dose and Time-Dependent Manner

EC-1 cells were treated with 0-100 μmol/L propofol over 72 h, or 100 μmol/L propofol for 0-72 h. TUNEL and ELISA technique was used to detect cell apoptosis. Treatment with propofol (10-100 μmol/L/Ml) for 72 h resulted in significant apoptosis relative to control by TUNEL assay (Figure 3A). Similarly results were also found when treated with propofol (100 μmol/L/mL) for 24-72 h (data not shown). The treatment of EC-1 cells with propofol resulted in a dose- and time-dependent promotion of cell apoptosis, suggesting that propofol was an effective promoyion of EC-1 cell death as a single agent. ELISA assay (Figure 3B) has the same results as the TUNEL results.

### Propofol Inhibits Invasion and Angiogenesis in EC-1 Cell

We first analyzed the effect of propofol treatment on the invasive capability of EC-1 cells. As

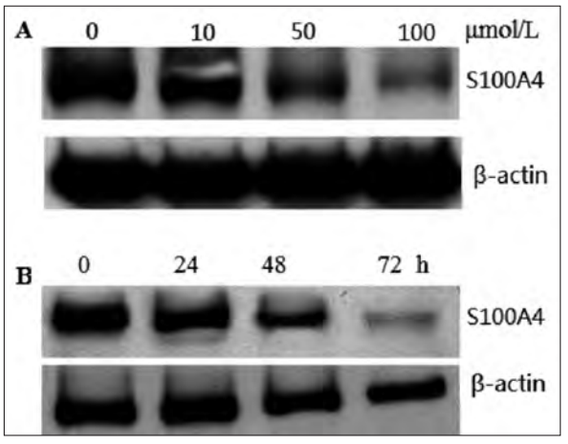


**Figure 1.** Effect of Propofol on S100A4 expression in EC-1 cells. ***A,*** EC-1 cells treated with 10-100 μmol/L propofol for 72 h, Western blot assay was used to detect S100A4 expression in the EC-1 cells. ***B,*** EC-1 cells treated with 100 μmol/L propofol for 24-72 h, Western blot assay was used to detect S100A4 expression in the EC-1 cells.

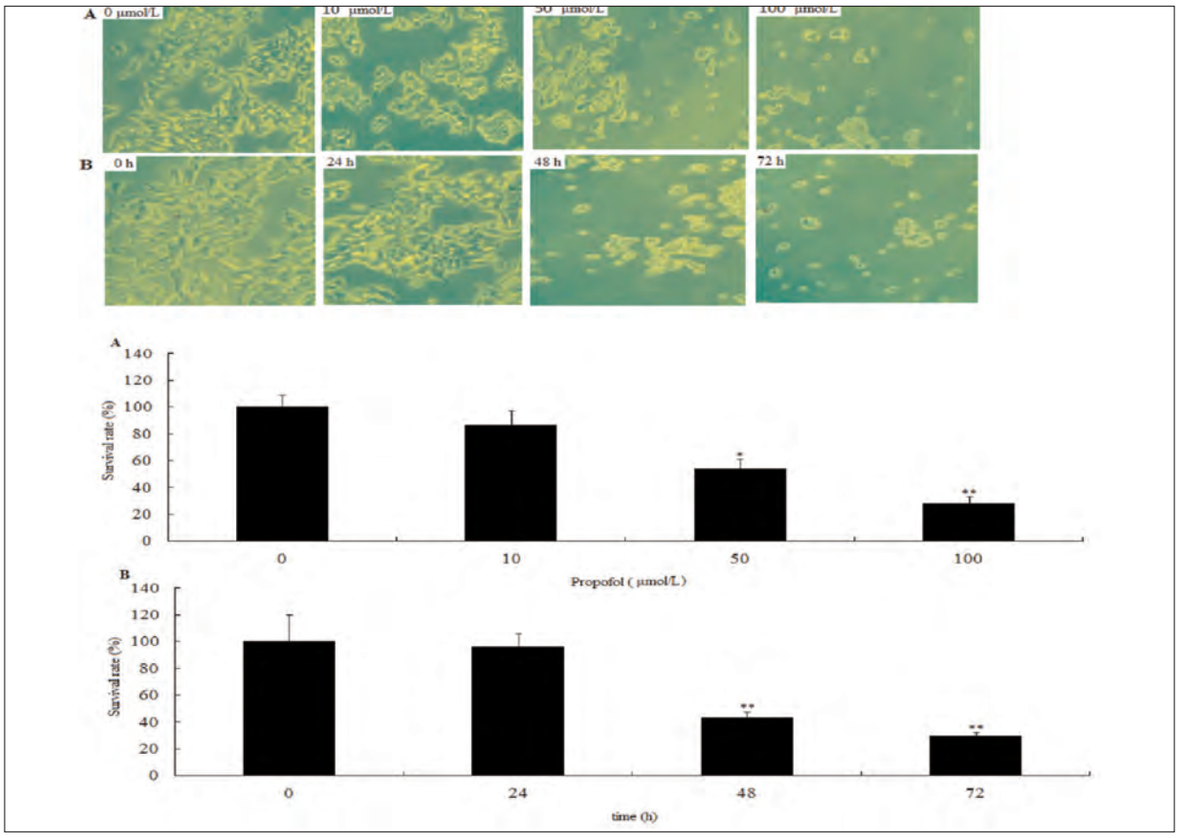


**Figure 2.** Effect of Propofol on survival in EC-1 cells. ***A,*** EC-1 cells treated with 10-100 μmol/L propofol for 72 h. ***B,*** EC-1 cells treated with 100 μmol/L propofol for 24-72 h. MTT assay was used to detect survival in EC-1 cells. *vs*. control **p* < 0.05, ***p* < 0.01 statistical significance.

shown in Figure 4A. treatment with 100 μmol/L propofol significantly decreased ($p < 0.05$) the number of invasive cells. Next we quantified the effect of propofol treatment on the angiogenic ability in CAM (chick chorioallantoic membrane). The images shows the presence of few scattered blood vessels around the sponge when treated with 100 μmol/L propofol in EC-1 cells compared to EC-1 cells alone on CAM. (Figure 4B). These data further support our hypothesis that propofol inhibits the invasive characteristics during human ESCC development.

### *Propofol-Mediated S100A4-Dependent Growth, Invasion, Angiogenesis and Apoptosis in EC-1 cells*

Propofol (100 μmol/L) inhibits angiogenesis and invasion, followed by S100A4 inhibition (Figure 1). When S100A4 cDNA was transfected into the EC-1/propofol cells, S100A4 expression was significantly increased (Figure 4C), and the effect of propofol on angiogenesis and invasion was increased (Figure 4).

EC-1 cells were treated with 0-100 μmol/L propofol over 72 h, or 100 μmol/L propofol for 0-72 h, resulted in a dose and time-dependent inhibition of cell proliferation (Figure 2), and increase of cell apoptosis (Figure 2), followed by decreased S100A4 expression (Figure 1). When S100A4 cDNA was transfected into the EC-1/propofol cells, propofol-induced apoptosis in the EC-1 cells was significantly decreased (Figure 5C-D), and survival rate was significantly increased (Figure 5A-B).

## Discussion

We investigated the effects of propofol on the behavior of human ESCC cells and the role of S100A4 in these effects. We showed that propofol inhibits invasion, angiogenesis, proliferation

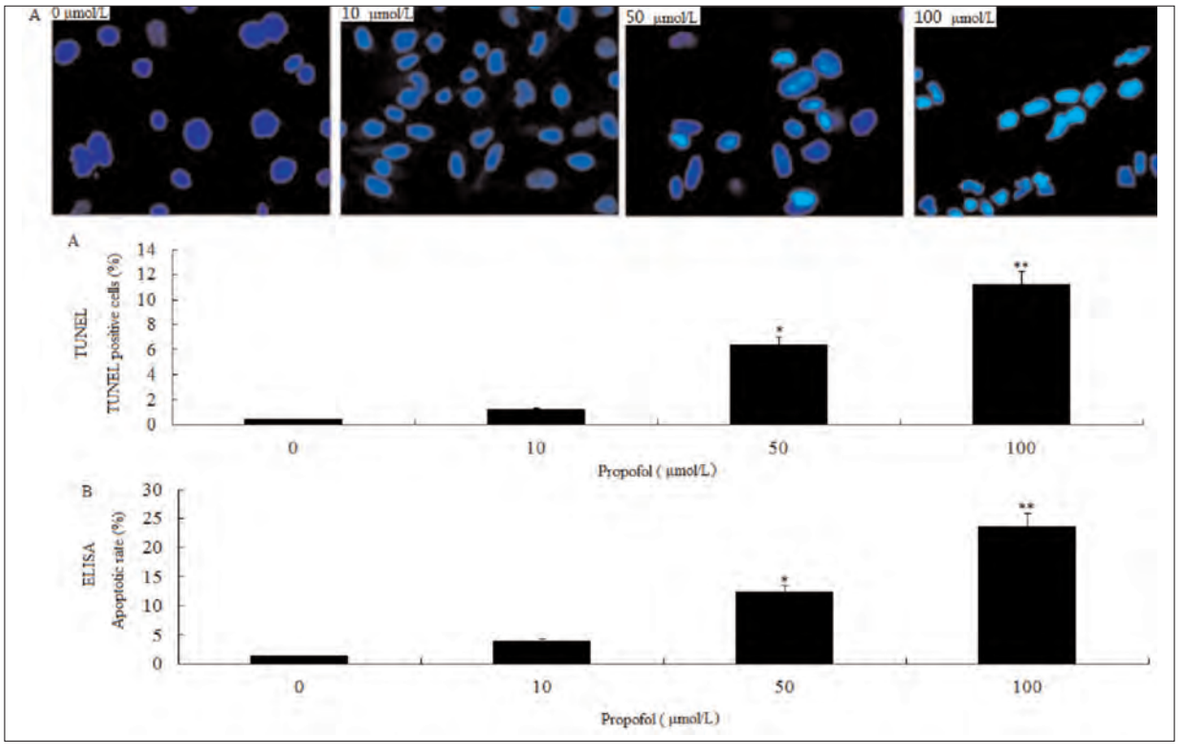


***Figure 3.*** TUNEL and ELISA assay was used to evaluate apoptosis in EC-1 cells. ***A,*** Propofol-induced apoptotic cell death by TUNEL after 72 h of treatment with 10-100 μmol/L propofol. ***B,*** Propofol-induced apoptotic cell death by ELISA after 72 h of treatment with 10-100 μmol/L propofol. *vs*. control, **p* < 0.05, ***p* < 0.01.

and induces apoptosis of EC-1 cells through inhibition of S100A4.

Propofol is extensively used as a sedative and general anesthetic for surgery. Additionally, recent studies demonstrated that propofol exerts anti-cancer effects by inducing cancer cell apoptosis[20-21]. Propofol may directly and indirectly suppress the viability and proliferation of cancer cells by promoting apoptosis in human lung cancer and neuroblastoma cells[22-23]. We have previously found that treating pancreatic cancer cells (MIA-PaCa-2) with propofol in concentrations of 10, 25, 50, and 100 mmol/mL resulted in dose- and time-dependent promotions of apoptosis. It was a single effective promoter of the death of pancreatic cancer cells[24]. Wang et al[7] has reported that treatment with propofol induced apoptosis and increased paclitaxel killing of all paclitaxel-sensitive and -resistant ovarian cancer cells followed by significant decrease in the Slug levels. Treatment with propofol inhibits invasion and migration. We also found that propofol inhibited proliferation, invasion and angiogenesis of human Eca-109 cells *in vitro* through modulation of ERK-VEGF/MMP-9 signaling[9].

Our data showed that propofol-induced apoptosis and survival inhibition in EC-1 cells with high S100A4 expression occurs in a dose and time-dependent manner and significantly rises at the concentration of 100 μmol/L propofol treatment for 72 hours. We also found in the present study that treatment with 100 μmol/L propofol significantly decreased the number of invasive cells, and decreased the angiogenic ability in CAM (chick chorioallantoic membrane).

Studies in rodents[25] have provided evidence supporting the direct involvement of S100A4 in tumor progression and metastasis. Based on observations in transgenic mice, S100A4 has been identified as a potent stimulator of angiogenesis[26]. Otherwise, both extracellular and intracellular S100A4 also participates in the regulation of cell death[27].

In the present study, we found that treatment with propofol inhibits S100A4 expression in a dose- and time-dependent manner in the EC-1 cells. Although EC-1 cells treated with propofol resulted in a dose and time-dependent inhibition of cell proliferation, and increase of cell apoptosis**.** When S100A4 cDNA was transfected into

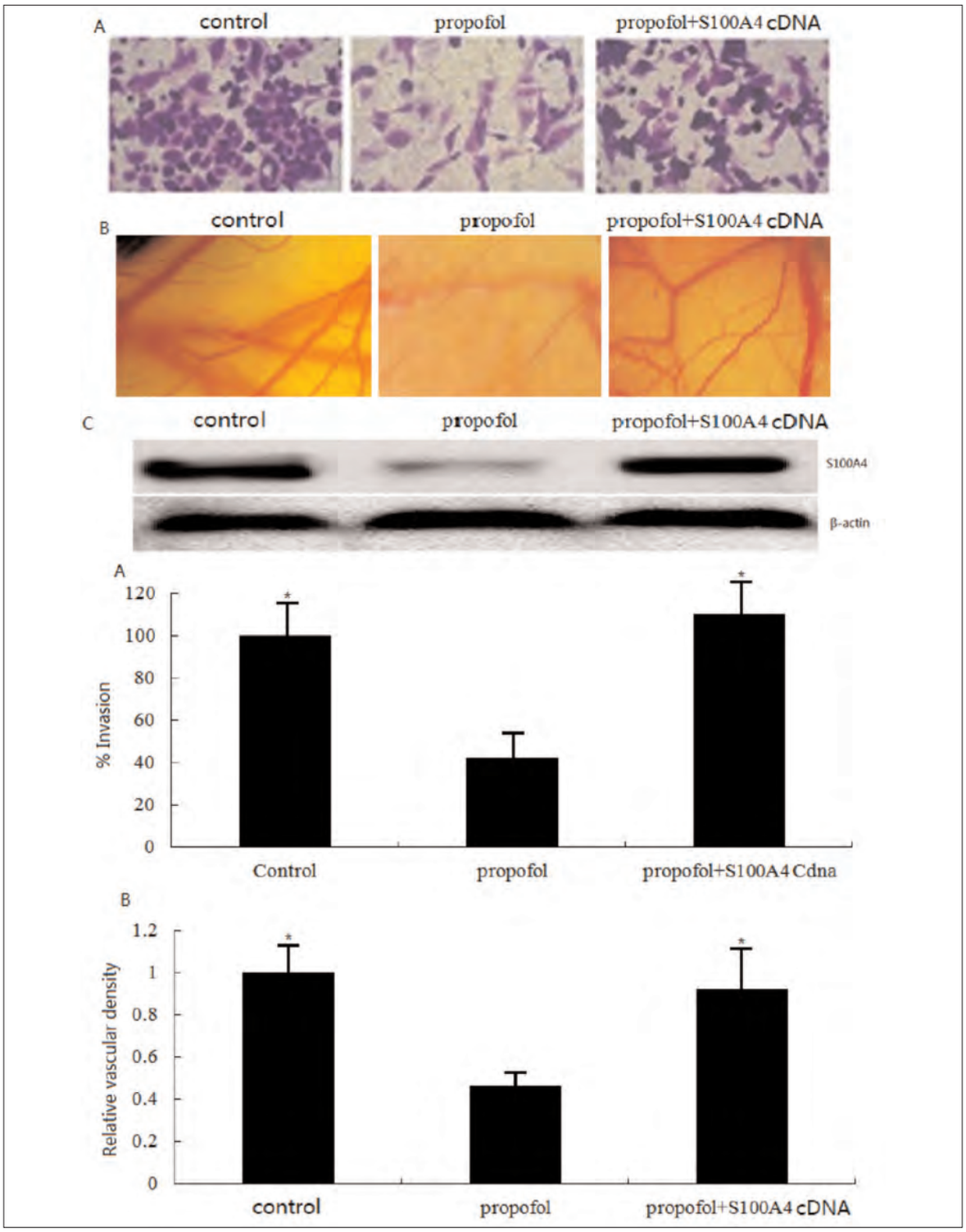


**Figure 4.** Propofol inhibited angiogenesis by CAM and invasion in matrigel assay. ***A,*** Propofol inhibits *in vitro* invasion by matrigel assay. ***B,*** Propofol inhibits in vivo angiogenesis through CAM assay. Blood vessels density was quantified. Values are means ± SD. ***C,*** Western blot assay for S100A4 in S100A4 cDNA transfected EC-1 cells. Vs. propofol, $*p < 0.05$. Although propofol inhibits angiogenesis and invasion, when S100A4 cDNA was transfected into the EC-1 cells, the effect of propofol on angiogenesis and invasion was restored.

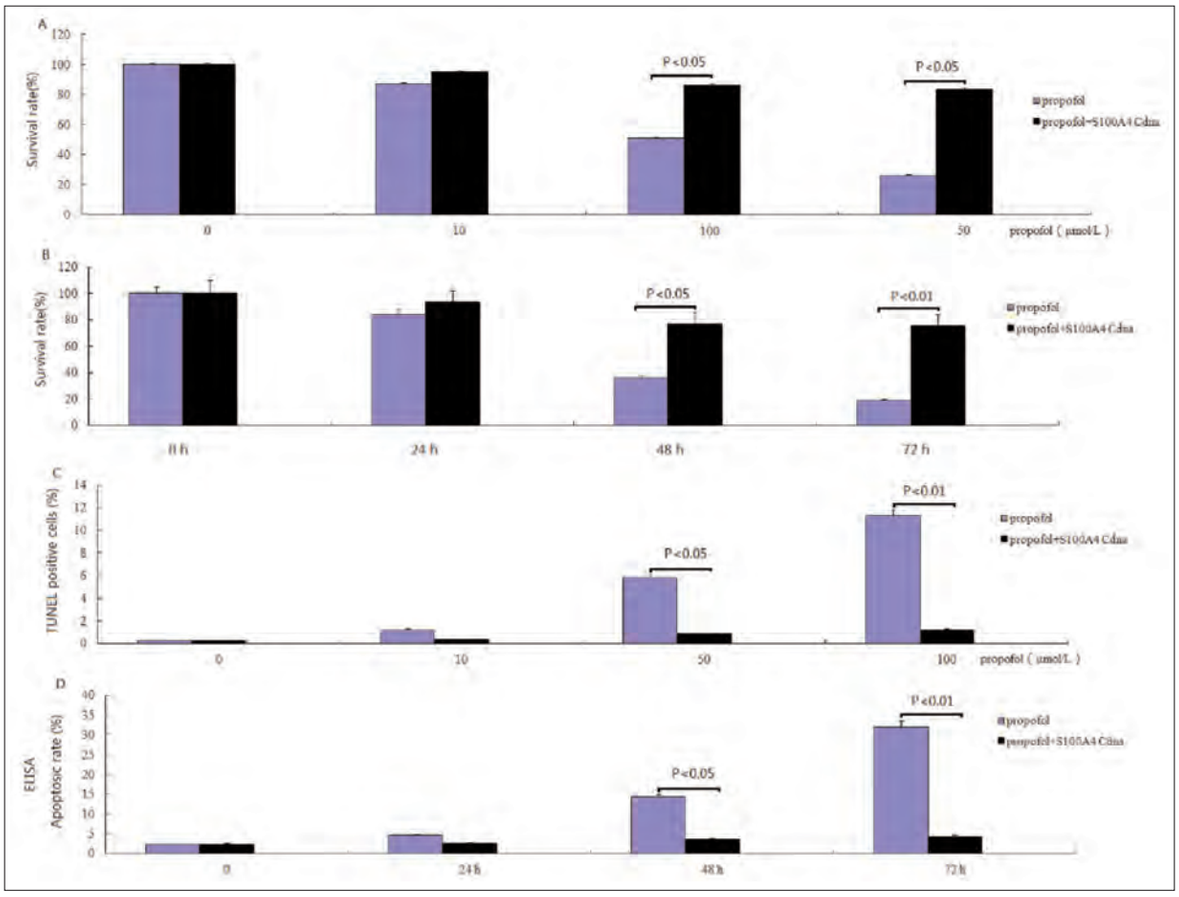


**Figure 5.** Effect of S100A4 on propofol-induced apoptosis and survival. S100A4 cDNA was transfected into the EC-1 cells, then treated with 0-100 μmol/L propofol over 72 h, or 100 μmol/L propofol for 0-72 h. ***A, B,*** MTT assay was used to detect survival in EC-1 cells. C-D: TUNEL and ELISA assay was used to evaluate apoptosis in EC-1 cells.

the EC-1/propofol cells, propofol-induced apoptosis in the EC-1 cells was significantly decreased, and survival rate was significantly increased. Chicken chorioallantoic membrane (CAM) angiogenesis assay and cell invasion assay also showed that S100A4 cDNA was transfected into the EC-1 cells, the effect of propofol on angiogenesis and invasion was restored.

## Conclusions

This study provides new insights into effect of propofol on behavior of ESCC cells and the related mechanism. Our present study suggests that propofol inhibits invasion, angiogenesis, proliferation and induces apoptosis of EC-1 cells through inhibition of S100A4. However, this should be verified in further studies, including animal trials and prospective clinical studies.


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## Conflict of Interest

The Authors declare that there are no conflicts of interest.

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