RESEARCH COMMUNICATION

Sevoflurane Inhibits Proliferation, Induces Apoptosis, and Blocks Cell Cycle Progression of Lung Carcinoma Cells

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Abstract

Purpose: Sevoflurane, an inhalational anesthetic, is used extensively during lung cancer surgery. However, the effect of sevoflurane on growth of lung carcinoma cells remains unclear. The purpose of this study is to investigate effects on proliferation, apoptosis, and cell cycling in the A549 human lung adenocarcinoma cell line. Methods: A549 cells were treated with 1.7%, 3.4%, and 5.1 % sevoflurane for 2, 4, and 6 hours. Cell proliferation was evaluated by the MTT assay and colony formation assay. Apoptosis and cell cycle was analyzed by flow cytometry. Expression of X-linked inhibitor of apoptosis protein (XIAP), survivin, Bcl-2, Bax, caspase-3, cyclin A, cyclin B1, and cdc2 was measured by Western blotting. Results: Sgnificant inhibition of cell proliferation and induction of apoptosis were found in A549 cells after sevoflurane treatment. Simultaneously, expression of XIAP and survivin was surpressed, while that of caspase-3 increased significantly, but Bcl-2 and Bax were not altered. Sevoflurane caused cell cycle arrest at the G2/M phase. At the same time, data revealed that cyclin A, cyclin B1, and cdc2 expression was down-regulated after sevoflurane treatment. Conclusion: This study demonstrated that sevoflurane inhibited proliferation, and induced apoptosis in human lung adenocarcinoma A549 cells, associated with down-regulated expression of XIAP and suvivin, and activating caspase-3.

Keywords: Sevoflurane - lung cancer cells – proliferation – apoptosis - cell cycle

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Introduction

Lung cancer is one of the most common malignancy diseases. Non-small cell lung carcinoma account for 80% of all cases of lung cancer and can be treated with surgical resection (Jemal et al., 2010). However, metastasis or recurrence of tumor still remains the leading cause of death from lung cancer patients after surgery (Merritt et al., 2009; Yildizeli et al., 2009). Interventions occurring during the perioperative period have an effect on long-term outcome of patients with cancer surgery (Sessler et al., 2009). Anesthesiologists play an important role during cancer surgery period because anesthetics and anesthesia technique can exert influence on the growth of tumor cells (Gottschalk et al., 2010; Santamaria et al., 2010; Snyder et al., 2010). Anesthetics that have the anti-cancer effect should be used for cancer surgery in order to decrease the risk of recurrence and metastasis of patients after cancer surgery.

Sevoflurane, an inhalation anesthetic agent, is used widely for patients undergoing lung cancer surgery. Bronchi and pulmonary alveoli are exposed to sevoflurane directly during inhalational anesthesia of sevoflurane period. In vitro, sevofluane can inhibit the growth of colon cancer and larynx cancer cells (Kvolik et al., 2005; Kvolik et al., 2009). However, the effect of sevofluane on the growth of lung cancer cells is still not clear. Thus, the aim of the present study is to investigate the effects of sevofluane on proliferation, apoptosis, and cell cycle of human lung adenocarcinoma cell line A549 in a vitro model, and to elucidate the related molecules mechanism underlying the effects of sevoflurane on A549 cells.

Materials and Methods

Major reagents

RPMI-1640 and fetal bovine serum (FBS) was purchased from HyClone Corporation (NewYork, USA). 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Promega Corporation (USA). Annexin V-FITC and Propidium iodide (PI) double staining kit were purchased from KeyGEN Biotech Corporation (Nanjing, China). Sevoflurane was purchased from Maruishi Pharmaceutical Co., Ltd. (SVEOFRANE®, Japan). Mouse anti-human monoclonal antibody cyclin A and cyclin B1 (1/2000) were purchased from Cell Signaling Technology (CST, USA). Rabbit anti-human monoclonal antibody cdc2, Bax (1/1000), Bcl-2 (1/1000), cdc2 (1/1000), X-linked inhibitor of apoptosis protein

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(XIAP) (1/1000), and suvivin (1/1000) were purchased from Cell Signaling Technology. Mouse anti-human monoclonal antibody caspase-3 was purchased from Abcam Inc. (Cambridge, UK). Horseradish peroxidase (HRP) -conjugated secondary antibodies goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Southern Biotechnology Associates, Inc. (SBA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Abcam Inc. (Cambridge, UK). All other reagents were procured locally.

Cell line and cell culture

The human pulmonary adenocarcinoma cell line A549 was obtained from Shanghai Cell Biology Medical Research Institute, Chinese Academy of Sciences, and maintained in RPMI-1640 supplemented with 10% FBS, 100 units/ml penicillin and 100μg/ml streptomycin. The cells were incubated in 5% CO_2 humidified at 37°C for growth.

Sevoflurane treatment

A549 cells in the exponential growth phase were seeded in plates, and were cultured in CO2 incubator (Thermo scientific, USA) for 48 hours. According to the experimental protocol as previously described (Loop et al., 2004; Roesslein et al., 2008), cell culture plates were placed in an air-tight glass chamber with inflow and outflow connectors. The chamber atmosphere was kept continuously saturated with water at 37°C. The entrance port of the chamber was connected to anesthetic machine (Cicero – EM 8060, Dräger, Germany). Sevoflurane was delivered into the chamber by a sevoflurane vaporizer (SEVORANE®, Abott, USA) attached to the anesthesia machine. The concentrations of sevoflurane in the chamber were detected at the chamber exit port by a gas monitor (PM 8060, Dräger, Gernany) that inlayed with the anesthetic machine. A549 cells were divided into 4 groups: control group, 1.7% sevoflurane group (S1), 3.4 % sevoflurane group (S2), and 5.1 % sevoflurane group (S3). The control group was exposed to 95% air/5% CO2 at 6 L/min for 2, 4, and 6 h. The sevoflurane group was exposed to 1.7%, 3.4%, or 5.1 % of sevoflurane mixed with 95% air/5% CO2 at 6 L/min for 2, 4, and 6 hours respectively. A stable sevoflurane concentration was achieved within 5 min.

Proliferation inhibition evaluated by MTT assay

A549 cells were seeded in 96-well plates (1×10^4 cells per well). After different concentration of sevoflurane treatment for 2, 4, and 6 hours, cells were placed in CO2 incubator for additional 48 h culture. Thereafter, MTT was added to each well and mixed. The wells were then incubated for an additional 4 hours. The medium was totally removed and 150 μl DMSO was added to each well to fully dissolve the blue crystals. Absorbance was measured at 570 nm (OD570) and the percentage of proliferation inhibition of A549 cells was calculated at each time point and for each concentration of sevoflurane according to the following formulae: % proliferation inhibition= (1- OD570 of test well) / (OD570 of control well) ×100%.

Proliferation inhibition in colony formation assays

A549 cells were seeded in 96-well plates (1×10^4 cells per well), and exposed to different concentration of sevoflurane treatment for 2, 4, and 6 hours. Thereafter, cells were prepared as single cell suspension, and seeded 100 cells of each group into 6-well plates. After 7 days of culture, cells were washed twice with phosphate buffered saline (PBS), fixed with methanol for 5 min, and stained with crystal violet for 30 min. Colony counting was performed with AID EliSpot Reader system (Autoimmun Diagnostika GmbH, Germany). The rate of colony formation was calculated according to the following formule: rate of colony formation = colony counts /seeded cells ×100%.

Apoptosis analysis by annexin V/PI flow cytometry

A549 cells were cultured in 12-well plates (2×10^5 cells per well). After different concentration of sevoflurane treatment for 2, 4, and 6 hours, cells were placed in CO_2 incubator for additional 48 h culture. Thereafter, apoptotic percentage of cells was detected by flow cytometry analysis using an Annexin V-FITC Apoptosis Kit. Cells were washed twice with cold PBS, and resuspended in 400μl with 1×binding buffer at a concentration of 1×10^6 cells/ml. This binding buffer was supplemented with 5 μl of annexin V-FIFC and incubated at room temperature in the dark for 15 minutes. Ten microliter of PI was then added and incubated at 4°C in the dark for 5 minutes. Cells were analyzed by a flow cytometer (FACS Caliber, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) within 1 hour.

Cell cycle assayed by flow cytometry

A549 cells were seeded in 6-well plates (1×10^6 cells per well). After different concentration of sevoflurane treatment for 2, 4, and 6 hours, cells were placed in CO_2 incubator for additional 48 h culture. Thereafter, distribution of cell cycle was examined using flow cytometry analysis. A549 cells were washed twice with

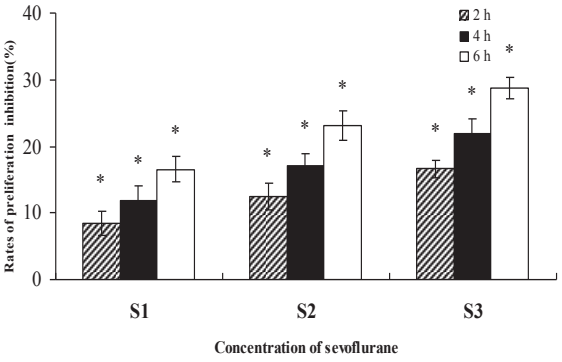

Figure 1. Effect of Sevoflurane on Proliferation of A549 Cells. A549 cells were treated with 0 (control), 1.7% (S1), 3.4% (S2) and 5.1% (S3) sevoflurane for 2, 4 and 6 hours, and proliferation inhibition was evaluated by MTT assay. The bars represent the rates of proliferation inhibition at different time points and at different concentrations of sevoflurane. The rate of proliferation inhibition in control group is zero. Data are shown as means ± standard deviation of 6 independent experiments. *Indicates a statistically significant difference (P<0.05) between the given group and its corresponding control group

PBS, and then centrifuged at 1200 g for 5 min, fixed in 70% ethanol at 4°C. Thereafter, the cells were washed again with PBS, treated with RNase (50 μg/ml) at 37°C for 30 min, and stained with PI (100 μg/ml) in the dark at 4°C for 30 min. The cell cycle of cells was then analyzed by flow cytometry.

Western blotting analysis

A549 cells were cultured in 6-well plate (1×10^6 cells per well). After different concentration of sevoflurane treatment for 4 hours, cells were placed in CO2 incubator for additional 48 h culture. Thereafter, cells were washed with PBS and lysed with lysis buffer. The lysate were incubated on ice for 30 minutes and centrifuged at 14,000 g for 15 minutes. The supernatants were collected and measured for protein concentration using bicinchoninine acid assay. Protein samples were separated by to a 12.5% SDS-polyacrylamide gel, and then electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, USA). The membrane was blocked overnight at 4°C in TBS-Tween 20 (TBST) buffer containing 5% non-fat milk. The membrane was then washed with TBST (3 × 5 minutes), and was incubated for 2 h at 37°C with 1/1000 dilution of survivin antibodies, 1/500 dilution of caspase-3 antibodies, and 1/10000 dilution of GAPDH antibodies respectively. After being washed with TBST (3 × 5 minutes), the membrane were incubated with HRP-conjugated goat anti-rabbit IgG (1/5000 dilution) or rabbit anti-mouse IgG secondary antibody (1/4000 dilution) at 37°C for 1 h. Protein signals were detected using enhanced chemiluminescence reagent (Millipore, USA) and quantified by densitometry using Bio-Rad Quantity One software (Bio-Rad, Hercules, USA).

Data analysis

Statistical analysis was performed by SPSS for Windows® (v.11.0; SPSS Inc, Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD). Differences in group were assessed by using repeated measure analysis of variance. Differences among groups were analyzed by using one-way analysis of variance, followed by Duncan's test for post hoc comparisons. P<0.05 was considered statistically significant.

Results

Sevoflurane inhibits the proliferation of A549 cells

To observe the proliferation inhibition effects of sevoflurane, A549 cells were treated with different concentrations of sevoflurane for 2, 4 and 6 hours, and the rate of proliferation inhibition was detected by MTT assay, and the colony formation rate was evaluated by colony formation assay. As shown in Figure 1 and 2, results showed that sevoflurane could significantly inhibit the proliferation of A549 cells. The effect of inhibition was enhanced with increased sevoflurane concentration and action time.

Sevoflurane induces apoptosis of A549 cells

To investigate the apoptosis percentage, the cells were treated with different concentrations of sevoflurane for 2, 4 and 6 hours, and the percentage of apoptosis was detected by flow cytometry. As shown in Figure 3, data revealed that the numbers of early apoptotic cells in sevoflurane group were significantly increased as compared to control group. The effect of sevoflurane induced A549 cells apoptosis was augmented with increased sevoflurane concentration and action time .

Sevoflurane down-regulated XIAP and survivin expressions

To investigate the related molecules mechanism underlying the effect of sevoflurane on proliferation, apoptosis, and cell cycle of A549 cells, we observed the effect of sevoflurane on XIAP, suvivin, Bcl-2, and Bax expression in protein level. The cells were exposed to different concentration of sevoflurane for 4 hours. As shown in Figure 4A and 4B, the protein level of XIAP, suvivin, Bcl-2, and Bax were then detected by Western blotting. The analysis of Western blotting revealed that sevoflurane down-regulated the protein level of XIAP and surviving, but did not alter the expression of Bcl-2 and Bax.

Downregulation of XIAP and survivin affected the expression of caspase-3

As shown in Figure 4C, Western blotting analysis revealedthe expression of cleaved caspase-3 was increased significantly after down-regulation of survivin

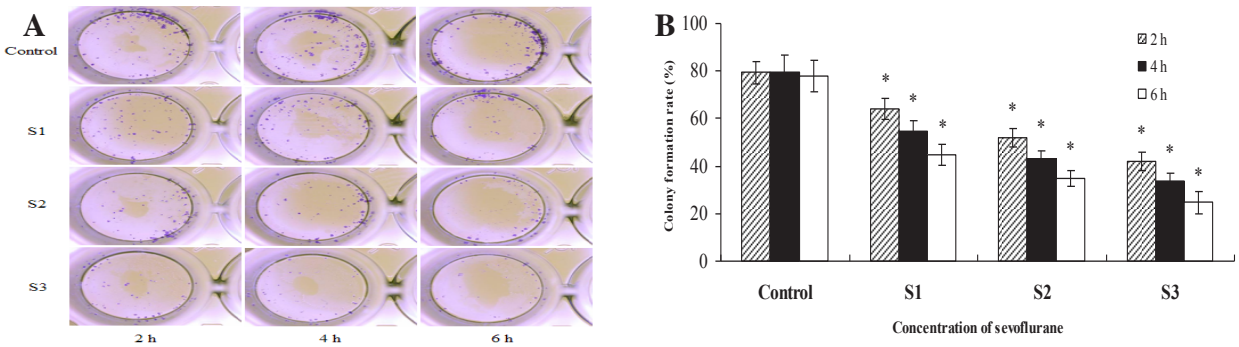

Figure 2. Effect of Sevoflurane on Colony Formation rate of A549 Cells. A549 cells were treated with 0 (control), 1.7% (S1), 3.4% (S2) and 5.1% (S3) sevoflurane for 2, 4 and 6 hours, and colony formation rate assay was evaluated by colony formation assay. The bars represent colony formation assay at different time points and at different concentrations of sevoflurane. Data are shown as means ± standard deviation of 6 independent experiments. *Indicates a statistically significant difference (P<0.05) between the given group and its corresponding control group

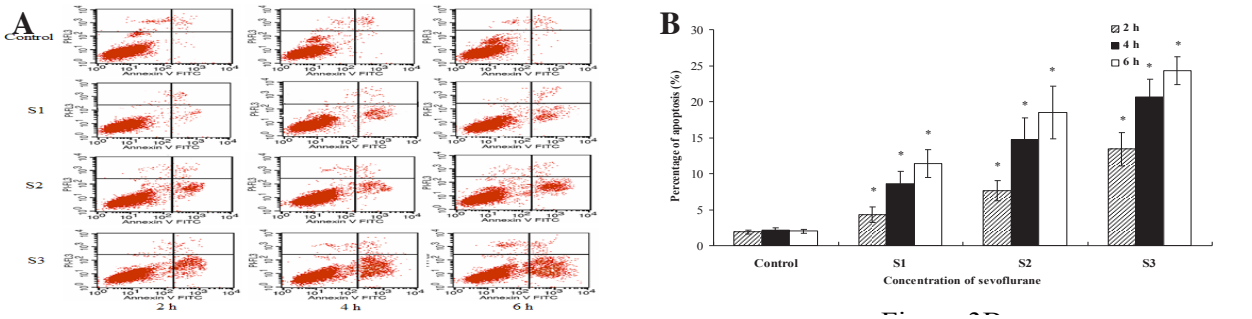

Figure 3. Effect of Sevoflurane on Apoptosis of A549 Cells. (A) A549 cells were treated with 0 (control), 1.7% (S1), 3.4% (S2) and 5.1% (S3) sevoflurane for 2, 4, and 6 hours. Apoptotic percentage was analyzed by Annexin V/PI staining. (B) the bars represent the percentage of apoptotic A549 cells at different time points and at different concentrations of sevoflurane. Data are shown as means ± standard deviation of 6 independent experiments. *Indicates a statistically significant difference (P<0.05) between the given group and its corresponding control group

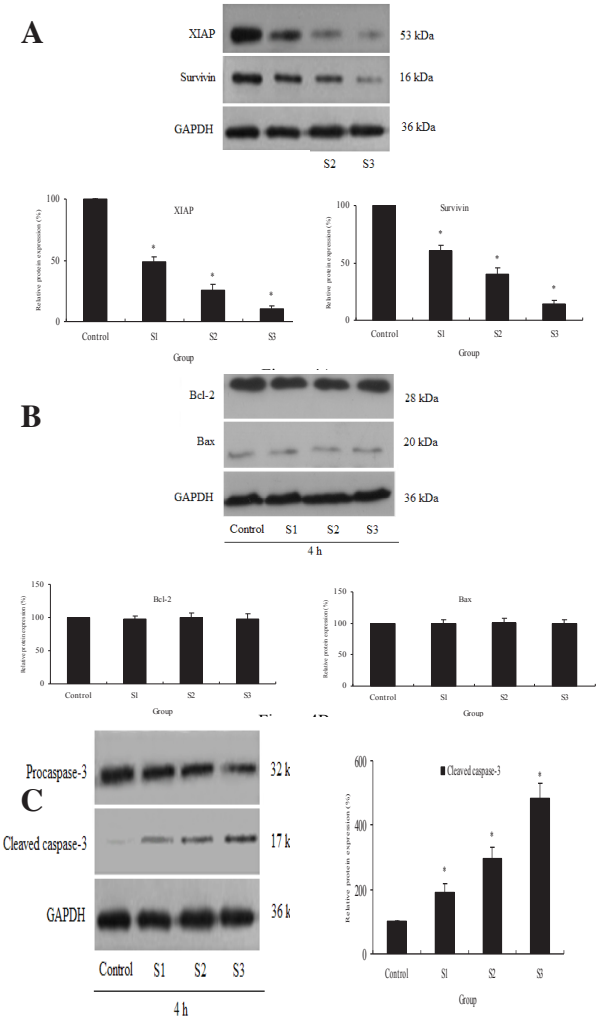

Figure 4. Effect of Sevoflurane on the Expressions of Apoptosis-related Protein in A549 Cells. A549 cells were treated with 0 (control), 1.7% (S1), 3.4% (S2) and 5.1% (S3) sevoflurane for 4 hours. The protein level of XIAP, survivin, Bcl-2, Bax, and caspase-3 was analyzed by Western blotting. The bars represent the protein level of XIAP, survivin, Bcl-2, Bax, and caspase-3 at different concentrations of sevoflurane. Data are shown as means ± standard deviation of 6 independent experiments. *Indicates a significant difference (P<0.05) between the given group and its corresponding control group

by sevoflurane.

Sevoflurane induces G 2/M arrest of A549 cells

To determine cell cycle distribution in A549 cells,

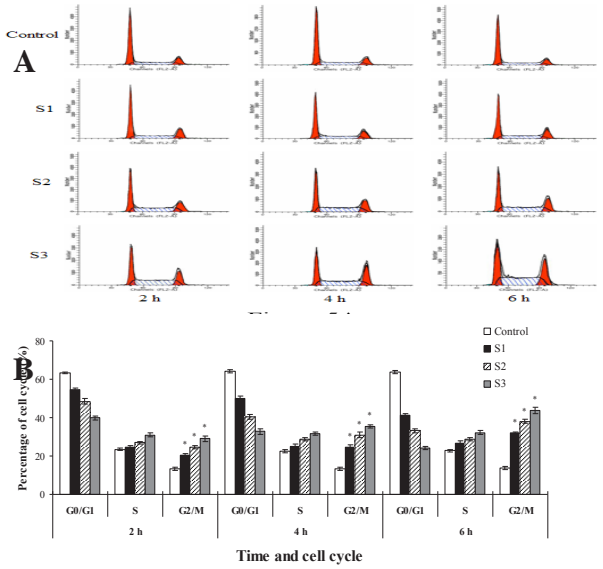

Figure 5. Effect of Sevoflurane on Cell Cycle of A549 Cells. (A) A549 cells were treated with 0 (control), 1.7% (S1), 3.4% (S2) or 5.1% (S3) sevoflurane for 2, 4 and 6 hours andcell cycling was analyzed by flow cytometry. (B) the bars represent the phases of cell cycle A549 cells at different time points and at different concentrations of sevoflurane. Data are shown as means ± standard deviation of 6 independent experiments. *Indicates a statistically significant difference (P<0.05) between the given group and its corresponding control group

the cells were exposed to different concentrations of sevoflurane for 2, 4, and 6 hours, and the cell cycle distribution was analyzed by flow cytometry. As shown in Figure 5, results showed that sevoflurane could increase the percentage of cells in G2/M phase, and decrease the percentage of cells in G0/G1 phase. The result indicated that cell cycle was arrested at G2/M phase by sevoflurane. The effect of sevoflurane blocked the cell cycle at G2/M phase was strengthened with increased sevoflurane concentration and action time .

Sevoflurane down-regulated the expressions of cyclin A, cyclin B1, and cdc2

To investigate the related molecules mechanism underlying the effect of sevoflurane on cell cycle of A549 cells, we observed the effect of sevoflurane on cyclin A, cyclin B1, and cdc2 expression in protein level. The cells were exposed to different concentration of sevoflurane for

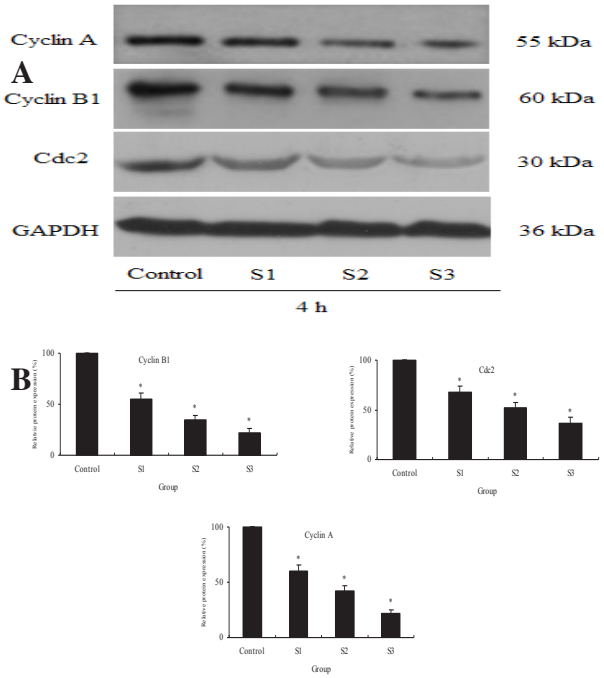

Figure 6. Effect of Sevoflurane on the Expression of Cell Cycle-related Proteins in A549 Cells. (A) A549 cells were treated with 0 (control), 1.7% (S1), 3.4% (S2) and 5.1% (S3) sevoflurane for 4 hours. The protein level of cyclin A, cyclin B1, and Cdc2 was analyzed by Western blotting. (B) the bars represent the protein level of cyclin A, cyclin B1, and Cdc2 at different concentrations of sevoflurane. Data are shown as means ± standard deviation of 6 independent experiments. *Indicates a statistically significant difference ($P<0.05$) between the given group and its corresponding control group

4 hours. As shown in Figure 6, the protein level of cyclin A, cyclin B1, and cdc2 were then detected by Western blotting. The analysis of Western blotting revealed that sevoflurane down-regulated the protein level of cyclin A, cyclin B1, and cdc2.

Discussion

Sevoflurane, an inhalation anesthetic, is used extensively during lung cancer surgery as anesthesia maintenance or anesthesia induction. Sevoflurane can induce T lymphocytes, Jurkat T-cells, and H4 human neuroglioma cells apoptosis (Loop et al., 2004; Roesslein et al., 2008; Dong et al., 2009). Moreover, the anti-cancer effect of sevofluane has been reported in two papers. In vitro studies showed that sevoflurane had an effect of inhibiting growth and promoting apoptosis of colonic and laryngeal cancer cells (Kvolik et al., 2005; Kvolik et al., 2009). However, the effect of sevoflurane on proliferation, apoptosis, and cell cycle of lung cancer cells remains unclear. We selected human lung adenocarcinoma A549 cells as the experimental object because bronchi and pulmonary alveoli are exposed to sevoflurane directly during inhalational anesthesia of sevoflurane period. To simulate the clinical anesthesia setting of lung cancer surgery, we adopted three commonly-used clinical concentration of sevoflurane to treat A549 cells for 2-6 hours in this study. The 1.7%, 3.4%, and 5.1% sevoflurane are equal to 1 minimal alveolar concentration (MAC), 2 MAC, and 3 MAC respectively. In our study, we demonstrated that sevoflurane inhibited cells proliferation, induced apoptosis, and blocked cell cycle progression of A549 cells.

An imbalance between anti-apoptotic gene expression and pro-apoptotic gene expression happens during tumor progression period, which inhibits apoptosis and promotes proliferation of tumor cells (Lockshin et al., 2001). XIAP and survivin are two members of the inhibitor of apoptosis protein (IAP) family that is potentially involved in both facilitating tumor cell proliferation and inhibiting apoptosis (Li, 2005; Zheng et al., 2010). The results of MTT assay, colony formation assay, and flow cytometer analysis demonstrated respectively that sevoflurane significantly inhibited the proliferation and induced apoptosis of A549 cells. Simultaneously, data showed that expression of XIAP and survivin in A549 cells was down-regulated at protein level after sevoflurane treatment, which suggested that sevoflurane-mediated anti-proliferative and pro-apoptotic activity were related to down-regulating the expressions of XIAP and survivin. XIAP and survivin are two potent modulators of apoptosis, and have been shown to directly block the process and activation of the cell death terminal effectors caspase-3, which induce the apoptosis of cells (Altieri, 2003; Oost et al., 2004; Rödel et al., 2005; Dean et al., 2010). In this study, the results of Western blotting indicated that the cleaved caspase-3 expression was increased after inhibition of XIAP and suvivin expression, which caused apoptosis of A549 cells.

The Bcl-2 family is involved in the regulation of cell apoptosis, including a series of anti-apoptotic and pro-apoptotic members. Bcl-2 is an anti-apoptotic member of Bcl-2 family, which prevents apoptosis by inhibiting the release of mitochondrial apoptogenic factors into the cytoplasm. In contrast, Bax is a pro-apoptotic member of this family, which promotes apoptosis by activating caspases (Leber et al., 2010; Llambi and Green, 2011). Results revealed that the expressions of Bcl-2 and Bax did not alter after sevoflurane treatment, suggesting sevoflurane-mediated apoptosis was not associated with modulating the expressions of Bcl-2 and Bax.

Regulation abnormality of cell cycle is an important mechanism of cell canceration. The checkpoint in G2/M phase of cell cycle takes the responsibility of maintaining the precision of heredity. It prohibits that cells of damaged or incomplete DNA from entering into mitosis and allows for the repair of DNA that was damaged in late S or G2 phases prior to mitosis (Hartwell and Kastan et al., 1994). Cyclin A, cyclin B and cdc2 play critical role in regulating cell cycle progression. They belong to 'mitotic and G2 cyclins'. The activation of cdc2 is required for its interaction with cyclin A and cyclin B, which are related to the control of G2/M transition and mitosis (Nigg, 1995; De Falco and De Luca, 2010). In this study, cell-cycle analysis revealed that sevoflurane caused A549 cells accumulation in G2/M phase and decreased the number of cells in G0/G1 phase, suggesting sevoflurane blocks cell-cycle progression at the G2/M phase. At the same time, sevoflurane induced G2/M arrest that was accompanied by down-regulation expression of cyclin A, cyclin B1,

and cdc2. It implies that sevoflurane-mediated G2/M arrest was through down-regulating these molecules expressions.

Incidence of recurrence and metastasis after lung cancer surgery remains high because surgery may contribute to tumor cells growth and invasion ability (Yamaguchi et al., 2000; Coffey et al., 2003). Nowadays, it is increasingly recognized that anesthetic technique and anesthetics have potential to effect long-term outcome of patients undergone cancer surgery (Exadaktylos et al., 2006; Biki et al., 2008; de Oliveira et al., 2011). The findings in this study that sevoflurane inhibited growth, induced apoptosis, and blocked cell cycle progression of A549 cells may have practical implications for lung cancer surgery.

In conclusion, results from the present study demonstrated that sevoflurane, an inhalation anesthetic, inhibited proliferation, induced apoptosis and blocked cell cycle arrest at G2/M phase in human lung adenocarcinoma A549 cells. Moreover, this study indicated that the anti-proliferative and pro-apoptotic effects of sevoflurane were associated with down-regulating the expressions of XIAP and suvivin, and activating caspase-3; the effect of sevoflurane blocking cell cycle arrest at G2/M phase was associated with down-regulating the expressions of cyclin A, cyclin B1, and cdc2.

Acknowledgements

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