

Original Research Article

Sevoflurane anesthesia induces apoptosis and cell cycle arrest in NPC-039 nasopharyngeal cells

Yan Zhang^{1*} and Hong Lin²

¹Department of Anesthesiology, Qilu Hospital of Shandong University, Jinan, Shandong 250012, ²Department of Anesthesiology Shandong Qianfoshan Hospital, Jinan, Shandong 250013, China

*For correspondence: **Email:** yanzhang134@hotmail.com; **Tel/Fax:** 0086-531-82169114

Received: 4 January 2017

Revised accepted: 21 March 2017

Abstract

Purpose: To determine the effects of sevoflurane on NPC-039 nasopharyngeal carcinoma cells.

Methods: WST-8 assays and flow cytometry with annexin V/PI staining were used to analyze the effects of sevoflurane on growth and induction of apoptotic changes in NPC-039 cells. Cell viability was performed on a microplate reader.

Results: Treatment of NPC-039 cells with 4 – 10 μ M sevoflurane significantly inhibited cell growth ($p < 0.005$) with a median inhibitory concentration of 6 μ M. NPC-039 cells incubated with sevoflurane showed prominent nuclear fragmentation and chromatin condensation. NPC-039 cells that exhibited apoptotic symptoms increased from 23.34 to 56.77 % when duration of treatment was increased from 24 to 48 h. Sevoflurane-treated cells also expressed increased levels of Bax and cleaved caspase-3. Reactive oxygen species formation was also higher in sevoflurane-treated NPC-039 cells than in control. Pre-treatment with Z-VAD-FMK followed by incubation with sevoflurane partly reduced the population of apoptotic NPC-039 cells compared with cells treated with sevoflurane alone. The proportion of cells in S- and G0/G1 phases decreased and increased, respectively, when sevoflurane concentration was increased from 2 to 10 μ M. Incubating NPC-039 cells with sevoflurane also markedly reduced levels of cyclin-dependent kinases including p15 INK4B, cyclin D1, p16 INK4A, and CDK-6. In contrast, pretreatment with ZVAD-FMK followed by incubation with sevoflurane increased p15 INK4B levels.

Conclusion: Sevoflurane inhibits nasopharyngeal carcinoma cell growth, activating apoptosis and inducing cell cycle arrest, thus suggesting its therapeutic potential for the treatment of nasopharyngeal carcinoma.

Keywords: Sevoflurane, Nasopharyngeal carcinoma, Chromatin, Condensation

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Nasopharyngeal carcinoma, which affects the head and neck tissues, has a high mortality rate; it is most common in Southeast Asia and south China [1]. According to a World Health Organization (WHO) report, more than 80 % of the globally detected cases of nasopharyngeal carcinoma occur in China. Nasopharyngeal carcinoma is caused by different epidemiological,

pathological, and virus-related mechanisms, and therefore differs from other common cancers of the head and neck [2].

At its early stage, the disease is currently treated using radiotherapy, but the relapse rate is very high, and the 5-year survival rate is very low [3]. In most cases, nasopharyngeal carcinoma undergoes metastasis to the neck lymph nodes, which worsens the prognosis [4]. The risk of

treatment failure is nasopharyngeal carcinoma metastasizes to distant organs such as the lungs and liver [5]. Therefore, physicians are focusing on the screening and identification of novel therapeutic molecules that can inhibit nasopharyngeal carcinoma cell growth.

Sevoflurane is a commonly used anesthetic [6,7]. Sevoflurane treatment was found to induce apoptosis in alveolar lung carcinoma cells *in vitro* [8]. In addition, pretreating animals with sevoflurane prevented injury to various organs during ischemia–reperfusion [9, 10]. Furthermore, volatile anesthetic compounds have been shown to exert anti-inflammatory effects both *in vitro* and *in vivo* [11,12].

Mechanistic studies have revealed that proinflammatory factors can suppress signal transduction pathways [13]. The induction of apoptosis via the intrinsic pathway is accompanied by reduced mitochondrial membrane potential, increased mitochondrial membrane permeability, and secretion of proapoptotic proteins from the mitochondria to the cytosol [14].

The *in vitro* production of reactive oxygen species (ROS) during oxidative stress also induces apoptosis via the mitochondrial pathway [15]. The present study aimed to evaluate the effects of sevoflurane on growth inhibition in nasopharyngeal carcinoma cells.

EXPERIMENTAL

Chemicals and reagents

Sevoflurane was purchased from Maruishi Pharmaceutical Co., Ltd, (Osaka, Japan). Annexin V was supplied by Becton Dickinson (Franklin Lakes, NJ, USA), and the caspase inhibitor Z-VAD was provided by Enzo Life Sciences (Plymouth Meeting, PA, USA). The disodium salt of 1,2-dihydroxy-3,5-benzenedisulfonic acid in its monohydrated form (Tiron) was supplied by Dojindo Laboratories (Kumamoto, Japan).

NPC-039 cell lines and culture

The NPC-039 cell line was purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and was cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were cultured at 37 °C in an incubator in a humidified atmosphere of 5 % CO₂ and 95 % air.

Analysis of cell growth

NPC-039 cells were cultured in 96-well plates at a density of 2×10^5 cells per well. The cells were incubated with 2, 4, 6, 8, and 10 μ M sevoflurane or DMSO for 48 h. Next, 10 μ L of the monosodium salt of WST-8 (Wako Chemical, Osaka, Japan) was added to each well, and the plates were incubated for 6 h in a humidified atmosphere of 5 % CO₂ and 95 % air at 37 °C. The absorbance at 450 nm was then recorded using an iMark microplate reader (Bio-Rad, Hercules, CA, USA) to determine cell viability.

Apoptosis analysis

Sevoflurane-induced apoptosis in NPC-039 cells was analyzed using flow cytometry with annexin V and propidium iodide. NPC-039 cells were plated in 50-mm culture dishes at a density of 2×10^6 cells per dish, cultured for 24 h, and then treated with 6 μ M sevoflurane with or without the caspase inhibitor Z-VAD (OMe)-FMK or DMSO alone. After incubation, the cells were collected, washed twice with ice-cold PBS, and treated with 1 \times binding buffer. Then, 10 μ L of annexin V and PI solution was added to each tube, and the cells were incubated in the dark for 20 min. They were then treated with binding buffer and analyzed using a FACSCalibur flow cytometer and CellQuest software (both from Becton Dickinson).

Measuring reactive oxygen species levels

ROS levels in sevoflurane-treated NPC-039 cells were analyzed using flow cytometry. Briefly, NPC-039 cells were either untreated or pretreated with the ROS scavenger Tiron, and then incubated in six-well plates for 48 h with sevoflurane. Then, the cells were treated with 10 μ M 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate acetyl ester for 45 min, and examined using a FACSCalibur flow cytometer and CellQuest software.

Western blotting analysis

NPC-039 cells were plated in 6-mm dishes at a density of 3×10^5 cells/dish in DMEM supplemented with 10 % FBS. After culture for 48 h, the cells were treated with sevoflurane for 48 h and harvested. Lysates were prepared using a NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's protocol. Proteins were separated by electrophoresis on 10 % sodium dodecyl sulfate–polyacrylamide gels, transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA), and incubated with primary

antibodies; β -actin was used as an endogenous control. The primary antibodies against β -actin, cyclin-dependent kinases-4 and -6, D1 p15-INK4B, and p16-INK4A were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against p-MEK and MEK were supplied by Sigma-Aldrich (St. Louis, MO, USA), and anti-caspase-3 and -Bax antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After overnight incubation, the membranes were washed with PBS and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (7074, Cell Signaling Technology). Protein-antibody complexes were visualized using an ECL plus kit (Amersham Biosciences, Hongkong) and a bio-image analyzer (Fuji Film, Tokyo, Japan).

Statistical analysis

The data are expressed as mean \pm standard deviation (SD), and data were analyzed using Student's *t*-tests. *P*-values < 0.005 were taken to indicate statistical significance.

RESULTS

Sevoflurane inhibits growth of NPC-039 cells

WST-8 assays revealed that incubation with sevoflurane for 48 h inhibited the growth of NPC-039 cells. Sevoflurane was added to cultures of NPC-039 cells at concentrations of 2, 4, 6, 8 and 10 μ M; the growth inhibition was significant ($p < 0.005$) after treatment with $\geq 4 \mu$ M sevoflurane for 48 h (Figure 1). The median inhibitory concentration of sevoflurane on NPC-039 cells was 6 μ M.

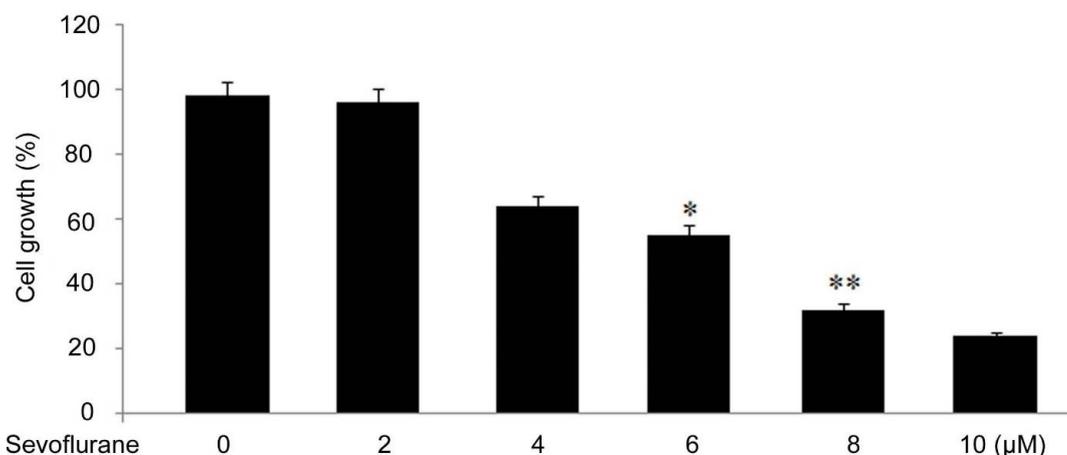


Figure 1: Sevoflurane inhibits the growth of NPC-039 cells. Cells were incubated with 2, 4, 6, 8 or 10 μ M of sevoflurane and then analyzed using WST-8 assays. Control cells were incubated with DMSO alone; ** $p < 0.005$ vs. control cells

Effect of sevoflurane treatment on NPC-039 cell apoptosis

After incubation with sevoflurane, cells were analyzed for any alterations in their morphology. Cells treated with sevoflurane for 24 h exhibited prominent nuclear fragmentation and chromatin condensation (Figure 2). In addition, increasing the treatment duration from 24 to 48 h led to a marked increase in the percentage of apoptotic NPC-039 cells.

Sevoflurane induced NPC-039 cell apoptosis

The percentage of NPC-039 cells showing signs of apoptosis increased from 23.34 to 56.77 % when the treatment time as increased from 24 to 48 h (Figure 3). The annexin V/PI-stained NPC-039 cell population also increased as the treatment time increased from 24 to 48 h (Figure 3).

Sevoflurane activates caspase-3 and elevates ROS levels in NPC-039 cells

NPC-039 cells treated with 6 μ M sevoflurane for 48 h expressed increased levels of Bax and cleaved caspase-3 compared with untreated cells (Fig. 4A). Reactive oxygen species formation was also higher in sevoflurane-treated NPC-039 cells after 48 h of treatment (Figure 4B). Pre-treatment with Z-VAD-FMK (caspase inhibitor) for 30 min followed by 48 h incubation with 6 μ M sevoflurane reduced the population of apoptotic NPC-039 cells compared with cells treated with sevoflurane alone (Figure 4C). Moreover, pretreatment with Tiron (a ROS scavenger) followed by incubation with sevoflurane also reduced the size of the apoptotic NPC-039 cell population (Figure 4C).

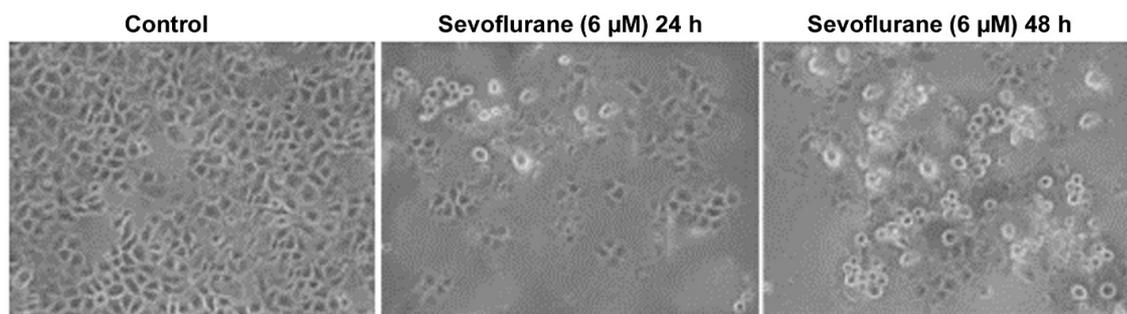


Figure 2: Morphological alterations in NPC-039 cells following treatment with 6 μM sevoflurane

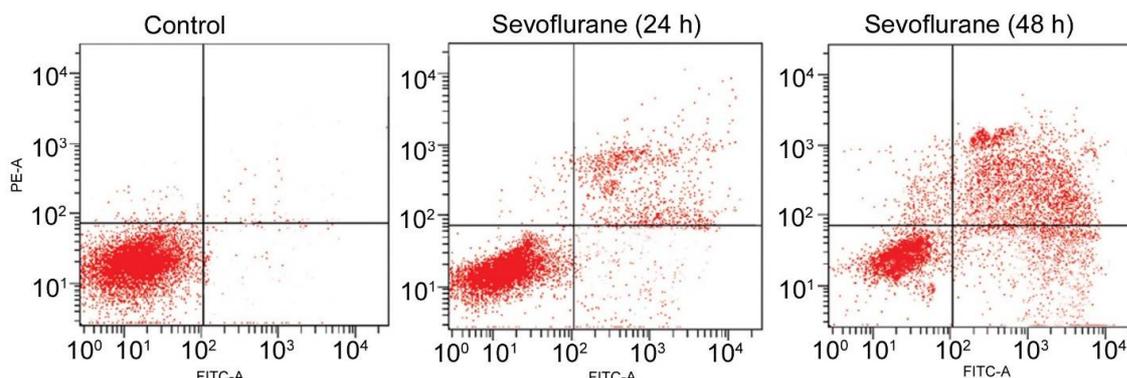


Figure 3: Sevoflurane induces apoptosis in NPC-039 cells. The cells were incubated with 6 μM sevoflurane for 24 and 48 h and then analyzed by flow cytometry after staining with annexin V/PI. The induction of apoptosis was compared in sevoflurane-treated and control NPC-039 cells

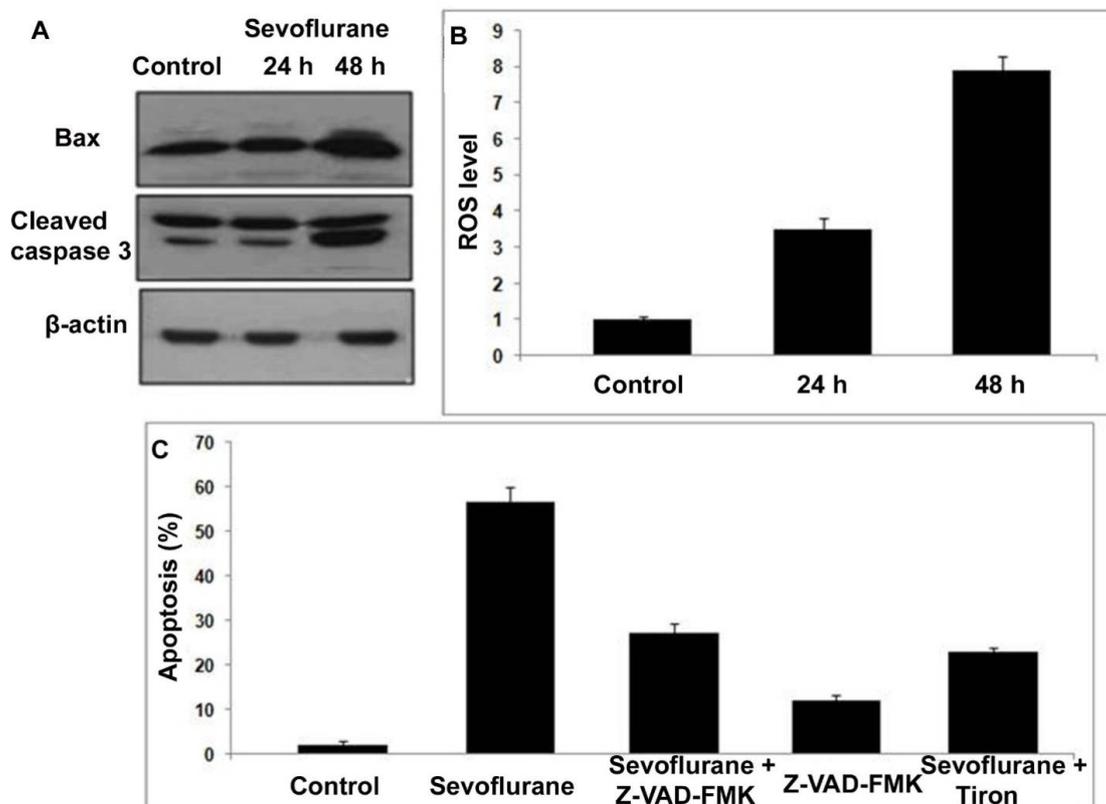


Figure 4: Sevoflurane treatment activates caspase-3 and Bax and induces ROS production; (A) Western blotting was performed after cells were treated with sevoflurane to determine the levels of caspase-3 and Bax; (B) ROS production was analyzed in NPC-039 cells that had been incubated with 6 μM sevoflurane with or without pretreatment with Z-VAD-FMK; (C) Apoptosis induction was analyzed in NPC-039 cells that had been incubated with 6 μM sevoflurane with or without pretreatment with Tiron. Comparisons were made with control cells

Sevoflurane induces NPC-039 cell cycle arrest

Incubating NPC-039 cells with sevoflurane for 48 h altered the size of the cell population in various phases of the cell cycle. The proportion of cells in the S-phase and G0/G1 phase was decreased and increased, respectively, by treatment with increasing concentrations of sevoflurane (2 to 10 μM) for 48 h (Figure 5). The population of cells in the sub-G1 peak was increased significantly after sevoflurane treatment.

Sevoflurane alters expression of CDK and its inhibitors

In NPC-039 cells, incubation with sevoflurane for 48 h markedly reduced the levels of various cyclin-dependent kinases including p15 INK4B, cyclin D1, and CDK-6 (Figure 6). In contrast, pretreating NPC-039 cells with ZVAD-FMK followed by incubation with sevoflurane for 48 h increased the levels of p15 INK4B (Figure 6).

DISCUSSION

In the present study, the inhibitory effects of sevoflurane on NPC-039 cell growth were assessed *in vitro*. The results revealed that sevoflurane significantly inhibited the growth of NPC-039 nasopharyngeal carcinoma cells.

Previous studies demonstrated that sevoflurane treatment induced apoptosis *in vitro* in alveolar lung carcinoma cells [8]. In the current study, the addition of sevoflurane to NPC-039 cells inhibited their growth. Further examination of the cells revealed that sevoflurane treatment led to prominent nuclear fragmentation and chromatin condensation. The mechanism by which sevoflurane inhibited NPC-039 cell growth was also investigated. Flow cytometric analysis of NPC-039 cells revealed an increase in the percentage of cells with apoptotic characteristics from 23.34 to 56.7 % when the treatment duration was increased from 24 to 48 h.

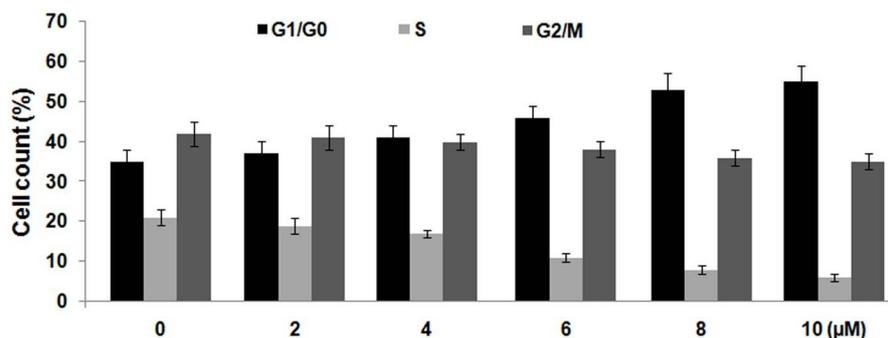


Figure 5: Sevoflurane alters the proportion of NPC-039 cells in different phases of the cell cycle. Sevoflurane (2 to 10 μM) was added to cultures of NPC-039 cells and incubated for 48 h. Flow cytometry was then used to analyze the proportion of cells in various phases of the cell cycle

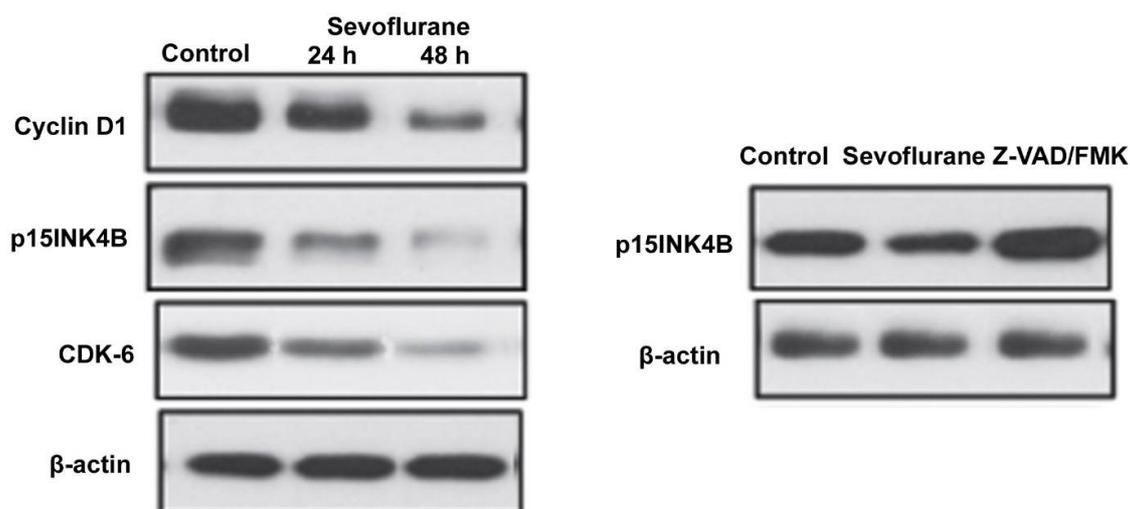


Figure 6: Effects of sevoflurane on the levels of cyclin-dependent kinase and its inhibitors. Western blotting was used to determine the expression of p15 INK4B, cyclin D1, and CDK-6 in NPC-039 cells after 48 h of treatment with sevoflurane. The levels of p15 INK4B were also analyzed after cells were pretreated with ZVAD-FMK

The size of the annexin V/PI-stained NPC-039 cell population also increased when the treatment time was increased from 24 to 48 h.

The factors that contribute to the anti-cancer properties of chemotherapeutic agents include increasing the levels of ROS and p27 [16, 17]. The current results showed that NPC-039 cells treated with sevoflurane expressed higher levels of ROS. Sevoflurane also increased the concentrations of Bax and cleaved caspase-3 compared with untreated cells. However, pretreatment with the caspase inhibitor Z-VAD-FMK followed by exposure to sevoflurane partly reduced the population of apoptotic NPC-039 cells compared with sevoflurane treatment alone. In addition, pretreatment with the ROS scavenger Tiron followed by incubation with sevoflurane also reduced the apoptotic NPC-039 cell population. Thus, the present study demonstrated that sevoflurane inhibited NPC-039 cell growth via two pathways. The first involves the induction of apoptosis via ROS generation and increased caspase-3 and Bax levels. The second pathway involves inducing cell cycle arrest via the increased expression of CDK inhibitors and decreased levels of CDKs.

CONCLUSION

The ability of sevoflurane to inhibit nasopharyngeal carcinoma cell growth by activating apoptosis and arresting the cell cycle indicates that it is a potent chemotherapeutic agent that could be an effective treatment for nasopharyngeal carcinoma.

DECLARATIONS

Acknowledgement

None declared

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

Open Access

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the

terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>) and the Budapest Open Access Initiative (<http://www.budapestopenaccessinitiative.org/read>), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

REFERENCES

1. Chang ET, Adami HO. The enigmatic epidemiology of nasopharyngeal carcinoma. *Cancer Epidemiol Biomarkers Prev* 2006; 15: 1765-1777.
2. Guigay J. Advances in nasopharyngeal carcinoma. *Curr Opin Oncol* 2008; 20: 264-269.
3. Wei WI, Sham JS. Nasopharyngeal carcinoma. *Lancet* 2005; 365: 2041-2054.
4. Chua DT, Sham JS, Wei WI, Ho WK, Au GK. The predictive value of the 1997 American Joint Committee on cancer stage classification in determining failure patterns in nasopharyngeal carcinoma. *Cancer* 2001; 92: 2845-2855.
5. Cheng SH, Jian JJ, Tsai SY, Chan KY, Yen LK, Chu NM, Tan TD, Tsou MH, Huang AT. Prognostic features and treatment outcome in locoregionally advanced nasopharyngeal carcinoma following concurrent chemotherapy and radiotherapy. *Int J Radiat Oncol Biol Phys* 1998; 41: 755-762.
6. Chandler JR, Myers D, Mehta D, Whyte E, Groberman MK, Montgomery CJ, Ansermino JM. Emergence delirium in children: a randomized trial to compare total intravenous anesthesia with propofol and remifentanyl to inhalational sevoflurane anesthesia. *Paediatr Anaesth* 2013; 23: 309-315.
7. Bi SS, Deng CH, Zhou TY, Guan Z, Li L, Li HQ, Zhang LP, Yang L, Lu W. Remifentanyl-sevoflurane interaction models of circulatory response to laryngoscopy and circulatory depression. *Br J Anaesth* 2013; 110: 729-740.
8. Guui-Hua W, Juan Z, Da-Qing L, Zhuo L, Jing Y, Nan-Fu L, Yan G. The common anesthetic, sevoflurane, induces apoptosis in A549 lung alveolar epithelial cells. *Mol Med Rep* 2014; 9: 197-203.
9. Oka M, Hirazawa K, Yamamoto K, Iizuka N, Hazama S, Suzuki T, Kobayashi N. Induction of Fas-mediated apoptosis on circulating lymphocytes by surgical stress. *Ann Surg* 1996; 223: 434-440.
10. Sasajima K, Inokuchi K, Onda M, Miyashita M, Okawa KI, Matsutani T, Takubo K: Detection of T cell apoptosis after major operations. *Eur J Surg* 1999; 165: 1020-1023.
11. Giraud O, Seince PF, Rolland C, Lecon-Malas V, Desmots JM, Aubier M, Dehoux M. Halothane reduces the early lipopolysaccharide-induced lung inflammation in mechanically ventilated rats. *Am J Respir Crit Care Med* 2000; 162: 2278-2286.

12. Helmy SA, Al-Attayah RJ. The effect of halothane and isoflurane on plasma cytokine levels. *Anaesthesia* 2000; 55: 904-910.
13. De Rossi LW, Brueckmann M, Rex S, Barderschneider M, Buhre W, Rossaint R. Xenon and isoflurane differentially modulate lipopolysaccharide-induced activation of the nuclear transcription factor κ B and production of tumor necrosis factor- α and interleukin-6 in monocytes. *Anesth Analg* 2004; 98: 1007-1012.
14. Ralph SJ, Rodríguez-Enríquez S, Neuzil J, Moreno-Sánchez R. Bioenergetic pathways in tumor mitochondria as targets for cancer therapy and the importance of the ROS-induced apoptotic trigger. *Mol Aspects Med* 2010; 31: 29-59.
15. Telsler J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007; 39: 44-84.
16. Akamatsu H, Komura J, Asada Y, Niwa Y. Effects of cepharanthin on neutrophil chemotaxis, phagocytosis, and reactive oxygen species generation. *J Dermatol* 1991; 18: 643-648.
17. Harada K, Supriatno, Yamamoto S, Kawaguchi S, Yoshida H, Sato M. Cepharanthine exerts antitumor activity on oral squamous cell carcinoma cell lines by induction of p27Kip1. *Anticancer Res* 2003; 23: 1441-1448.