

DNA damage effects of inhalation anesthetics in human bronchoalveolar cells

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Abstract

Background: The main objective was to evaluate and compare the local genotoxicity of sevoflurane and desflurane in bronchoalveolar cells, while the secondary outcome was to detect systemic oxidative DNA damage. To our knowledge, our study is the first one to evaluate the local effects of inhalation anesthetics in human bronchoalveolar cells in patients.

Methods: American Society of Anesthesiologists group I-II patients scheduled for lumbar discectomy surgery were enrolled in this randomized prospective study. Patients were randomized to sevoflurane or desflurane for anesthesia maintenance. Bronchoalveolar lavage samples and peripheral blood samples were taken at 2-time points: the first point (baseline, T1); and the second point (postexposure, T2). Final number of 48 samples were the sevoflurane (n=22) and desflurane (n=26) groups. Comet assay was applied to examine genotoxic properties. Oxidative DNA damage in plasma was measured with 8-hydroxy-2'-deoxyguanosine (8-OHdG).

Results: T2 values were higher than baseline values in both the desflurane group (tail-length: 66 ± 24 , %DNA in tail: 72 ± 60 , tail moment: 47.52 ± 14.4 ; $P = .001$, $P = .005$, $P = .001$, respectively) and the sevoflurane group (tail-length: 58 ± 33 , %DNA in tail: 88 ± 80 , tail moment: 51.04 ± 26.4 ; $P = .001$, $P = .012$, $P = .001$, respectively). T2 plasma 8-OHdG levels were also higher than baseline levels in the desflurane group (3.91 ± 0.19 ng/ml vs 1.32 ± 0.20 ng/ml, $P = .001$) and sevoflurane group (3.98 ± 0.18 ng/ml vs 1.31 ± 0.11 ng/ml, $P = .001$). There were no differences between the 2 groups in comet parameters and 8-OHdG levels.

Conclusion: Our results indicate that both inhalation agents cause DNA damage in the bronchoalveolar cells. Also, we detected increases in plasma 8-OHdG concentrations. Local genotoxicity and systemic oxidized DNA damage were similar in both groups.

Abbreviations: 8-OHdG = 8-hydroxy-2'-deoxyguanosine, ASA = the American Society of Anesthesiology, BAL = bronchoalveolar lavage, DNA = deoxyribonucleic acid.

Keywords: 8-hydroxy-2-deoxyguanosine, bronchoalveolar cells, comet assay, desflurane, DNA damage, inhalation anesthetics, sevoflurane

1. Introduction

Genotoxicity refers to a damaging effect on the genetic material including single-strand breaks, double-strand breaks, alkali labile sites, and DNA adducts (a covalent binding between a substance and DNA) induced by physical, biological, or chemical agents.

Unless the damage to the genetic material can be repaired, DNA sequence alterations, single or multiple nucleotide changes that can lead to chromosomal aberration may occur. Recombination, mutation, tissue damage, aging, and cancer may develop as a result of these changes.^[1]

The single-cell gel electrophoresis or comet assay is a sensitive, powerful, and safe method for detecting DNA strand breaks, which are a significant indicator of genotoxic and cytotoxic effects on cells caused by chemical and physical factors.^[2] The comet assay is commonly utilized to assess DNA damage in individual cells both in vitro and in vivo. It has proved its usefulness and versatility in human biomonitoring, ecogenotoxicology, genotoxicity testing, and basic research into the mechanisms of DNA damage and repair.^[3] The working group of the 6th International Workshop on Genotoxicity Testing (IWGT) focused solely on the in vivo comet assay and its use in regulatory genotoxicity testing.^[4] The in vivo comet assay is able to detect DNA damage in any tissue, even those with nonproliferating cells, and its high sensitivity makes it especially beneficial for the detection of genotoxicity. In vitro comet assay (cell culture) allows the screening of large numbers of genotoxic compounds.^[5] DNA damage is detected at the single-cell level using micro-gel technique including electrophoresis in alkaline (pH >13) conditions. The assay depends on

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relaxation of supercoiled loops by strand breaks; only those loops that are relaxed are able to move into a tail under electrophoresis, which often resembles a comet, observed by fluorescent microscope.^[6,7]

Sevoflurane (fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl) ethyl ether) and desflurane (1,2,2,2-tetrafluoroethyl-difluoromethylether) are halogenated ethers and they are quite different from conventional volatile agents due to their low solubility. The blood/gas partition coefficient is a function of solubility of the agent in blood, and both of these agents have a lower blood/gas partition coefficient than other halogenated ethers.^[8] Clinical studies have verified that concentrations of sevoflurane and desflurane can be easily regulated and their recovery from anesthesia is rapid.^[9,10] Although sevoflurane was first synthesized in the 1970s, it was not used in clinical practice until the end of 1993 due to questions regarding its metabolism to 2% to 5% inorganic fluoride and the extent of sevoflurane degradation in the presence of CO₂ absorbents.^[11] These carbon dioxide absorbents can degrade to compounds A, B, C, D, and E (an isomer of Compound D). Compound A can interact with nuclear DNA because of its highly reactive nature and alkylating activity.^[12] Desflurane is fluorinated methyl ether differing from isoflurane only in the substitution of fluorine for chlorine on the α -ethyl carbon. Fluorination increases the molecular stability and thus reduces the toxicity.^[13]

There are studies in the literature examining the systemic genotoxic effects of similar anesthetic agents in peripheral blood cells.^[14–18] Although bronchoalveolar cells are the first to get in contact with the inhalation anesthetics, data showing the effects of inhalation anesthetics at cellular level is insufficient. We found only a few studies conducted by the same researchers evaluating local genotoxic effects of different anesthetic agents (halothane and penthrane) in bronchoalveolar and lung cells during *in vitro* exposure.^[19,20] They showed that both anesthetics provoked DNA fragmentation in bronchial epithelial cells and halothane exerts genotoxic and cytotoxic effect on the alveolar cells *in vitro*. In other study, researchers evaluated the genotoxicological effects of isoflurane in the lymphocytes and organs of rats. Their study has demonstrated that isoflurane exposure results in significant DNA damage in rat lymphocytes, bone marrow, spleen, brain, livers, and lung.^[21] Bronchoalveolar lavage (BAL) samples include cellular and acellular components of the distal bronchioles and gas exchange units. Bronchoalveolar cells play important roles in host defense, inflammation, and regulation of immune responses.^[22] Determining the local genotoxic effects of inhalation anesthetics on bronchoalveolar cells would be helpful to better understand the risk of DNA damage. To our knowledge, our study is the first one to evaluate the local effects of inhalation anesthetics in human bronchoalveolar cells in patients.

The safety of anesthesia can be determined by its impact on oxidative stress and inflammation and various biomarkers have been developed for this purpose. For example, oxidative stress-induced DNA double-strand breaks can be detected by upregulation of 8-hydroxy-2-deoxyguanosine (8-OHdG).^[23] 8-OHdG is generated after the repair of reactive oxygen species-mediated DNA damage and is, therefore, one of the most widely recognized biomarkers of oxidative damage of DNA.^[24] Secondly, we evaluated plasma (8-OHdG) level is an indicator of the balance between oxidative DNA damage and repair mechanisms in the systemic circulation.^[25]

Thus, the aim of this study was to investigate DNA damage in bronchoalveolar cells using comet assay in order to determine the local effects of the 2 most commonly used inhalation anesthetics. The main objective was to evaluate and compare sevoflurane and desflurane in terms of their genotoxicity in bronchoalveolar cells, and the secondary objective was to detect systemic oxidative DNA damage.

2. Methods

This study was a randomized self-controlled prospective clinical study. Following ethics committee approval (protocol no: 2012/92) and in accordance with the Declaration of Helsinki, the study included 54 American Society of Anesthesiology (ASA) Class I or II patients between 18 and 65 years of age who were scheduled for lumbar discectomy surgery. The duration of anesthesia was at least 60 minutes.

Patients with ASA Class 3 or 4 disease, malignancy, or chronic pulmonary disorders and those who required blood transfusion were excluded. Smokers, alcoholics, obese subjects, and those who had recently received radiation, medications, and/or antioxidant supplements were excluded from the study. Patients with known occupational exposures (operating room personnel, chemical plant workers) were also excluded. All patients signed a consent form before surgery. Patients were randomly allocated into the 2 groups according to the protocol number given from the hospital information system was odd number or even number: sevoflurane group (27) and desflurane group (27). The samples were coded with patient protocol number at 2-time points; first sample "T1" and second sample "T2." Six patients (5 sevoflurane and 1 desflurane) were excluded from the study because their BAL samples that did not have sufficient living cells for comet analysis, resulting in a final number of 48 patients between the sevoflurane (n=22) and desflurane (n=26) groups.

2.1. General anesthesia

In preoperative preparation rooms, intravenous midazolam (1.5 mg) was given for sedation. Electrocardiogram, arterial blood pressure (systolic, diastolic, and mean), heart rate, and peripheral oxygen saturation were monitored and recorded at 10-minute intervals during surgery for all patients. General anesthesia was induced with 1.5 mcg/kg fentanyl, 2 mg/kg propofol, and 0.6 mg/kg rocuronium. After endotracheal intubation, patients received either 2% sevoflurane or 6% desflurane plus remifentanyl infusion at 0.01 to 0.1 mcg/kg/min for maintenance of anesthesia. The lungs were mechanically ventilated using the volume-controlled mode with a tidal volume of 6 ml/kg and respiratory rate of 10 to 15 breaths/min to maintain an end-tidal carbon dioxide tension between 30 and 35 mm Hg. Fresh gas flow rate with FiO₂ 50% was adjusted to 3 liters per minute. N₂O was not used in order to avoid any possible additional DNA damage in the patients. Intraoperative normothermia (>35.0°C) was maintained using forced air warming devices (Covidien Warm-Touch, model WT-5800, Covidien llc, 15 Hampshire Street, Mansfield, MA) with a specific blanket on the lower limbs, set to deliver forced-air at 42 to 46C following prone position. Postoperative pain relief was provided by a intravenous patient-controlled analgesia device delivering morphine 20 to 40 μ g/kg at 10-minutes lockout intervals, after the last sampling. No routine medication was used for postoperative nausea and vomiting prophylaxis.

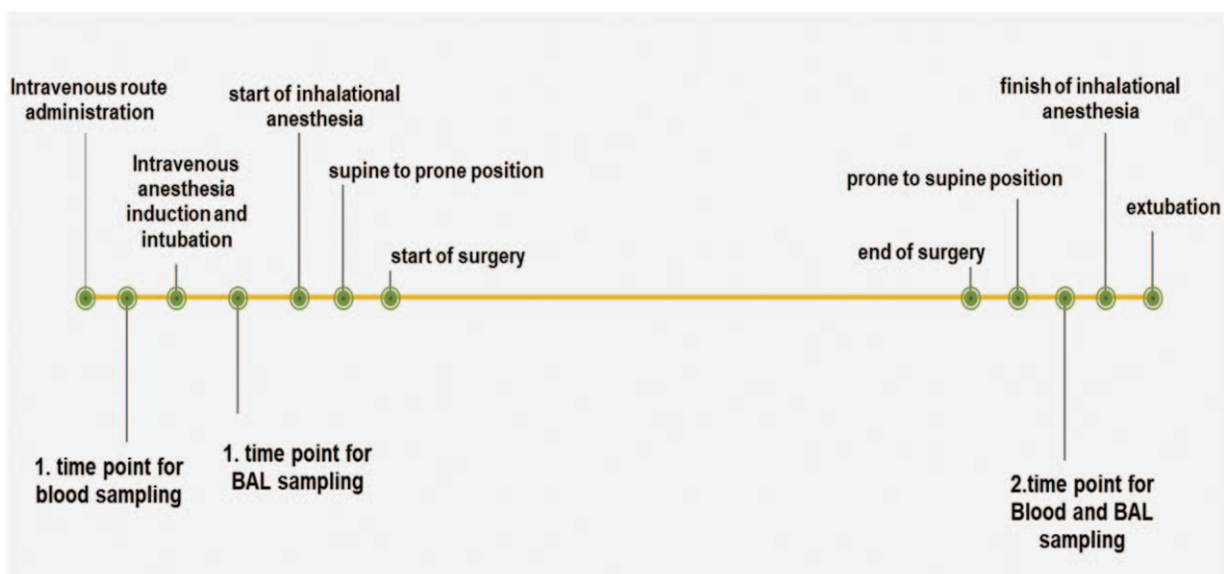


Figure 1. Sampling time point graphic.

2.2. Sampling

The first peripheral blood was taken before the anesthesia (baseline; T1 blood). After intubation, the first BAL sample was taken (baseline; T1 BAL). The second BAL and peripheral blood samples were taken at the end of surgery (postexposure; T2 blood and T2 BAL) (Fig. 1). BAL samples were obtained using 30 ml of saline in sterile conditions (Muco-Safe w. Filter; Unomedical, Convatec Limited, CH5 2NU, UK) and were immediately taken to the laboratory. Peripheral blood samples were obtained from upper extremity veins following local disinfection with betadine. Peripheral blood samples (2 ml) were transported to the laboratory in tubes (BD-Plymouth, PL6 7BP.UK) containing lithium/heparin (68 IU).

2.3. Comet analysis

BAL material was immediately centrifuged at 4°C for 10 minutes. The supernatant was removed and phosphate-buffered saline was added on the pellet to bring the volume to 1 ml. Viability test was performed in order to confirm the presence of adequate living cells. For this purpose, 10 µl of sample was mixed with the same amount of trypan blue stain and counted on Thoma slide. Samples showing approximately 15.000 living cells per 10 µl

were included in our study (bright and nonstained cells are viable). The cell viabilities were >80% in 2 groups. At this stage, 6 samples that did not have sufficient cells for analysis were excluded from the study. The protocol used followed the general procedures described by Singh et al^[26] and Tice et al^[27] with some modifications. Firstly, 500 µl of 1% agarose was spread onto the slide and cooled. Then, 75 µl of 1% low-melting-point agarose and 20 µl of sample were mixed and spread onto the slides. Slides were incubated at 4°C for a couple of minutes, and then in lysis solution for 4 hours. After 20 minutes in alkaline electrophoresis solution, samples were run in electrophoresis (300 A, 25 V) for 15 minutes in a cool environment. All steps of the technical experiments were performed under dim yellow light to prevent further induction of DNA damage. After electrophoresis, slides were washed with phosphate-buffered saline and stained for 10 minutes with 20 µg/ml ethidium bromide. Comets were visualized with a Zeiss fluorescence microscope (Meta-system Isis) using to 200× magnification. Images from 100 nucleoids (50 from each replicate slide) per time point per patient were scored using TriTek Comet Slide v1.5 Freeware software (Fig. 2). DNA damage was expressed as Arbitrary Units (AU) based on pixel intensity. Of the 17 parameters provided by the

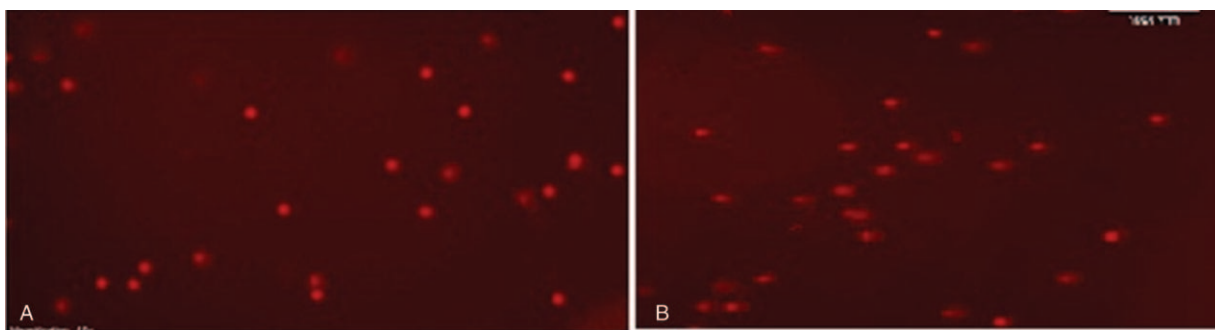


Figure 2. Evaluation of DNA damage by comet assay. (A) Microscopic image of baseline (T1) bronchoalveolar cells; (B) Microscopic image of post-exposure (T2) bronchoalveolar cells showing moderate DNA damage (200×).

Table 1

Comparison of the demographic and intraoperative characteristics. The data are shown in mean and standard deviation.

	Desflurane Group (n = 26)	Sevoflurane Group (n = 22)	P-value
Age, yr	54.88 ± 8.86	56.14 ± 10.09	.649*
Gender	Male	18 (69.20%)	10 (45.50%)
	Female	8 (30.80%)	12 (54.5%)
Operation* time (min)	129.62 ± 76.04	134.09 ± 75.87	.840*

* Unpaired *t* test.

† Chi Square test.

program, we selected tail length, % DNA in the tail (tail intensity), and tail moment to estimate the extent of DNA damage. Cells with the core completely fragmented were not counted during analysis.

2.4. 8-OHdG measurement

Blood samples collected at the 2-time points were centrifuged at 1000 g for 15 minutes. Plasma was removed and stored frozen at -70°C until analyzed. Plasma 8-OHdG assays were performed using competitive enzyme-linked immunosorbent assay (ELISA) from NWLSS 8-OHdG ELISA Kit Northwest (Vancouver, WA, Canada). The lower limit of detection was 0.125 ng/ml. The results are expressed in ng/ml.

2.5. Statistical methods

Statistical calculations were performed with NCSS (Number Cruncher Statistical System) 2007 software (Utah) program for Windows. Standard descriptive statistical calculations were expressed as mean ± standard deviation. For the variables indicate a normal distribution; unpaired *t* test was used in the comparison of groups and paired *t* test was employed in the assessment of baseline and postexposure values. For the variables does not indicate a normal distribution; Mann-Whitney *U* test was used in the comparison of groups and Wilcoxon test was employed in the assessment of baseline and postexposure values. Chi square test was performed during the evaluation of

Table 2

Comparison of the hemodynamic parameters (the data are shown in mean and standard deviation).

Time	Hemodynamics	Sevoflurane group	Desflurane group	P-value*
First sampling time	MAP	94.9 ± 8.7	94.3 ± 9.3	.848
	HR	79.3 ± 10	77.7 ± 9.36	.572
Second sampling time	MAP	88.4 ± 12.7	87.7 ± 10.7	.839
	HR	77.1 ± 8	73.3 ± 7.62	.101

MAP = mean arterial pressure, HR = heart rate.

* Unpaired *t* test.

qualitative data. Statistical significance level was established at $P < .05$.

In the power analysis of our study, the difference in Tail intensity between groups was found as 11% to 38% (with a probability of alpha error = 0.05) carried out by using G-power 3.1 program; the sample size calculation has revealed that the required subject number was found 22, assuming value for power is 0.8.^[28] We increased by 20% to accommodate missing data.

3. Results

Twenty-six patients were included in the desflurane group and 22 in the sevoflurane group. There were no statistically significant differences in mean age, sex distribution, and the mean surgery duration between the sevoflurane and desflurane groups ($P > .05$) (Table 1). There was also no significant difference in the hemodynamic parameters (mean arterial pressure and heart rate) between 2 groups ($P > .05$) (Table 2). With regard to comet parameters, T_1 (baseline) and T_2 (postexposure) values were significantly different in both groups. Comet parameters (tail length, % DNA in tail, tail moment) showed significantly higher values at T_2 compared to baseline levels ($P < .05$ for desflurane and $P < .05$ for sevoflurane). However, there were no statistically significant differences between the 2 groups in T_1 and T_2 values of comet parameters ($P > .05$) (Table 3) The ranges of tail length, % DNA in Tail and tail moment were given as AU in this study. Plasma 8-OHdG levels were also higher at T_2 than at baseline in both the desflurane group and sevoflurane group ($P < .05$) and

Table 3

Comparison of the comet parameters of bronchoalveolar cells in baseline (T1) and post-exposure (T2) periods of both drug groups (the data are shown in mean and standard deviation). The ranges of tail length, %DNA in tail and tail moment were given as arbitrary unit in this table.

Comet parameters	Time point	Desflurane group (n = 26)	Sevoflurane group (n = 22)	P-value*
Tail length	Baseline (T_1)	31 ± 13	34 ± 14	.524
	Post-exposure (T_2)	66 ± 24	58 ± 33	.388
	P-value†	.001	.001	
% DNA in tail	Baseline (T_1)	39 ± 25	50 ± 31	.173
	Post-exposure (T_2)	72 ± 60	88 ± 80	.424
	P-value†	.005	.012	
Tail moment	Baseline (T_1)	12.09 ± 3.2	17 ± 4.34	.088
	Post-exposure (T_2)	47.52 ± 14.4	51.04 ± 26.4	.311
	P-value†	.001	.001	

* Mann-Whitney *U* test was used in the comparison of 2 groups.

† Wilcoxon test was employed in the assessment of baseline values (T_1) and post-exposure values (T_2).

Table 4
Comparison of the baseline and post-exposure plasma 8-OHdG levels in desflurane and sevoflurane group (the data are shown in mean and standard deviation).

	Time point	Desflurane group (n=26)	Sevoflurane group (n=22)	P-value*
8-OHdG, ng/ml	Baseline (T ₁)	1.32 ± 0.20	1.31 ± 0.11	.381
	Postexposure (T ₂)	3.91 ± 0.19	3.98 ± 0.18	.394
	P-value†	.001	.001	

8-OHdG = 8-hydroxy-2-deoxyguanosine.

* Unpaired *t* test.

† Paired *t* test.

did not differ significantly between the 2 groups at either time point ($P > .05$) (Table 4).

4. Discussion

The systemic genotoxic effects of inhalation anesthetics have been reported in previous studies evaluating DNA damage in peripheral leukocytes as in vivo or in vitro (in cell culture).^[14–18,27–33] Also, there is a study evaluating local genotoxic effects of halothane and penthrane in bronchoalveolar cells during in vitro exposure.^[19–20] In this study, the local effects of the 2 most commonly used inhalation agents were investigated on bronchoalveolar cells in patients. We analyzed DNA damage using comet method to estimate local genotoxic effect of inhalation anesthesia with sevoflurane and desflurane on bronchoalveolar cells in patients.

During case selection for this study, we avoided severe diseases (diabetes, hypertension, cardiovascular diseases, cancer, etc) that may augment effects of DNA damage. Therefore, only patients scheduled for elective surgery for spine (lumbar discectomy surgery) were included in the study. Patients without systemic disease were the majority in this group. Anesthetic exposure time of the bronchoalveolar epithelium was the main parameter affecting the study outcomes. However, we could not fix the second sampling time because we could not get the expected operation time from the surgical team. The duration of these surgeries was at least 60 minutes. A fixed time could not be established for taking the second sample due to technical difficulties. To take the second sample, the BAL procedure was planned for the end of the operation, when the patients switched from prone position to supine position. However, when the durations of operations were compared, there was no statistically significant difference between groups in terms of duration of anesthesia.

Previous studies investigating whether inhalation anesthetics cause DNA damage in peripheral blood cells have yielded conflicting results. Orosz et al^[14] evaluated possible toxic effects of balanced anesthesia maintained with sevoflurane and showed that sevoflurane appears neither to damage DNA nor to alter redox status. Karabiyik et al^[17] evaluated the genotoxic properties of sevoflurane in human lymphocytes using in vivo comet assay. The authors reported detecting DNA damage due to sevoflurane, but concluded that cellular DNA repair occurred within 5 days. Alleva et al^[29] evaluated the genotoxicity of sevoflurane on DNA of lymphocytes and reported that the risk caused by sevoflurane was considerably low. Szyfter et al^[32] conducted a study of sevoflurane genotoxicity in vivo and in vitro conditions by using the comet assay through comparison with halothane and isoflurane. They concluded that sevoflurane was

not genotoxic in vivo or in vitro. Otherside Brozovic et al^[34] evaluated the DNA damage and repair in kidney cells of mice after repeated exposure to sevoflurane and isoflurane and showed that sevoflurane was slightly more genotoxic than isoflurane. Lüleci et al^[31] showed that sevoflurane administration may affect cell division and have a mutagenic effect on DNA. Karpinski et al^[33] demonstrated that the genotoxicity of desflurane was capable of increasing DNA migration in a dose-dependent manner under experimental conditions applied. Nogueira et al^[15] showed with the comet assay that surgical patients anesthetized with desflurane (6%) had increased damage of lymphocyte DNA. Akin et al^[18] revealed that exposure to desflurane increased sister chromatid exchange in human lymphocytes, thereby this agent may evoke genetic damage.

Our study showed that sevoflurane and desflurane induce strand breaks or alkali-labile sites in bronchoalveolar cells from patients undergoing lumbar discectomy surgery. DNA single-strand breaks demonstrated by comet assay can indicate damage before DNA repair complete. Therefore, detected DNA single-strand breaks may be reversible. In addition the types of surgeries could have influenced the results. Also, the types of surgeries along the drugs used during surgeries and the halogenated anesthetics could influenced the findings. Those issues couldn't be separated in this study. However, DNA repair is not always successful and an increased number of DNA single-strand breaks could lead to irreversible DNA damage.^[30] We did not observe differences regarding local genotoxicity and systemic oxidized DNA damage when comparing patients under sevoflurane or desflurane. In our study, the similar genotoxic effect of sevoflurane and desflurane can be explained by the similarity of chemical structures.

Previous studies have demonstrated that 8-OHdG formation is correlated with DNA modifications. This formation is independent of reactive oxygen species type (peroxides, superoxide, hydroxyl radical, etc) and exposure time.^[35] Jalszynski et al^[36] hypothesized that polyfluorinated anesthetics can alkylate the N-7 position of purines. They believe that anesthetic genotoxicity might also be due to their metabolic oxidation or reduction, giving rise to reactive metabolites and reactive oxygen species. To evaluate systemic DNA damage induced by oxygen radicals in the present study, we measured 8-OHdG levels. We opted to use the comet method for cell-level analysis because our primary objective was to investigate damage to bronchoalveolar cell DNA locally induced by inhalation anesthetics. However, we preferred plasma 8-OHdG analysis to assess the systemic situation because it has been established as a valid marker of the equilibrium between DNA damage and repair mechanisms.^[23,37] In our study, we observed increases in plasma 8-OHdG concentrations as a marker of oxidative DNA damage

with both desflurane and sevoflurane. Also, the study showed that sevoflurane and desflurane may induce DNA damage effects on bronchoalveolar cells.

One limitation of our study was that a fixed time could not be established for taking the second sample due to technical difficulties. The BAL procedure was planned for the end of the operation, when the patients switched from prone position to supine position for access to adequate lavage material. The other limitation of our study is that BAL is an invasive procedure and is performed twice during the course of our study (before and immediately after anesthesia), but the DNA damage associated with this procedure could not be assessed.

In conclusion, our study indicates that inhalation anesthetics may exert genotoxic effects on bronchoalveolar cells. Also, we observed increases in plasma 8-OHdG concentrations in both groups. There were no differences regarding local genotoxicity and systemic oxidized DNA damage when comparing patients under sevoflurane or desflurane.

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