EFFECTS OF USING SEVOFLURANE WITH NITROUS OXIDE AND REMIFENTANIL ON DNA DAMAGE

FİLİZ ALKAYA SOLMAZ¹, ÖZLEM SELVI CAN², ELA KADIOGLU², SEMRA SARDAS³, OYA OZATAMER⁴
¹Ankara University Medical Faculty, Department of Anesthesiology and Reanimation, Ankara - ²Department of Toxicology, Faculty of Pharmacy, Gazi University, Ankara - ³Department of Toxicology, Faculty of Pharmacy, Marmara University, Istanbul - ⁴Ankara University Medical Faculty, Department of Anesthesiology and Reanimation, Ankara, Turkey

ABSTRACT

Aims: In this study, the effect of DNA damage in the course of sevoflurane anesthesia along with remifentanil and nitrous oxide, was investigated via Comet Assay technique.

Materials and methods: The study included 52 patients. After approving informed consent form all participants, patients were randomly divided into three groups to provide different anesthesia maintenance. Anesthesia induction was carried out with propofol and vecuronium bromide in all groups. After intubation, Group SN (Sevoflurane-Nitrousoxide) received 50%O₂+50% N₂O and sevoflurane 2-4%. Group SR (Sevoflurane-Remifentanil) received remifentanil by infusion at rate of 0.2mcg/kg/min, sevoflurane 2-4%+100% O₂, Group C (Control) sevoflurane 2-4% and 100% O₂ for maintenance. Blood samples were obtained from all patients before induction, 120th minute of operation, and on postoperative day one and five.

Results: Genotoxic damage was observed in all groups. When the groups were compared, no statistically significant difference was observed between the groups. However, while the DNA damage regressed to preoperative values on the 5th postoperative day in Group SR, it did not regress to preoperative values in Groups SN and C.

Conclusion: It has been demonstrated that nitrous oxide combined with sevoflurane for anesthesia administration is not an ideal agent, and that an alternative opioid with short-acting agent remifentanil, can be used. It has also been theorized, due to remifentanils contribution in Group SR’s anesthesia administration, that remifentanil can lead to using lower doses of sevoflurane and positively impact the recovery of genotoxic damage.

Key words: Sevoflurane, Remifentanil, Nitrous Oxide, DNA damage, Genotoxicity, Comet assay.

Received February 18, 2014; Accepted May 19, 2014

Introduction

In vivo and in vitro studies on chromosomal changes due to exposure to anesthetic gases have proven that inhalation anesthetics can have mutagenic and carcinogenic effects by effecting human genetic material¹⁴. The potential risks posed on operating room personnel who are exposed to inhalation agents at work place have raised interest in this topic. Russian anesthesiologist Vaisman was the first to report through experimental and epidemiologic studies in 1967, that it creates health risks to work in environments polluted with anesthetic gas waste, and that operating room personnel have certain health problems associated with anesthetic gases⁵⁶. Controlled studies investigating teratogenic and carcinogenic effects of anesthetics on personnel have followed and ASA (American Society of Anesthesiologists) data revealed that cancer risks increase in female operating room personnel⁸. Sevoflurane is a preferred anesthetic agent, especially in pediatric patients and in outpatient surgeries, because it is comfortable to use in induction, eliminated rapidly, and has muscle-relaxing effects.

However, the toxic byproduct compound A that is formed through degradation of soda lime and baralime during anesthesia, limits its use.

Compound A becomes significant especially in uses of low-flow anesthesia circuits and in long lasting interventions⁹₁⁰.
Increased use of Sevoflurane in the recent years, and the positive genotoxicity results of other halogenated anesthetics in similar structure have increased research on sevoflurane’s genotoxic effects. Though sevoflurane’s possible toxic effects on various organs have been determined, there is no clear evidence on the mechanisms of its interaction with genetic material. Still, the genotoxic effect is assumed to be caused by Compound A that is in vinyl ether structure and is one of the compounds formed via sevoflurane’s interaction with carbon dioxide absorbents (9).

Comet assay technique is more and more accepted in genotoxic screenings. Comet technique is used in many fields of toxicology from aging to genetic toxicology and molecular epidemiology. While there are numerous genotoxicity studies on inhalation anesthetics use on non-cancerous cases, there were not any studies found in the literature on genotoxicity caused by opioid added to sevoflurane.

The goal of this study is to determine the ratio of DNA damage and its postoperative course of the genotoxic effects of sevoflurane’s combined use with nitrous oxide and remifentanil on patients undergoing tympanoplasty and myringoplasty due to benign pathologies such as chronic otitis media.

Material and methods

Patients

The study was performed on 52 patients (ASA grades 1 and 2), aged 20-50 years, who were undergoing operation for elective myringoplasty and tympanoplasty. All patients were assigned randomly into one of the three groups. The study was approved by the local ethics committee and a signed informed consent form was obtained from participants after the purpose and the methods of the study were explained at the preoperative visit.

A survey (Form I), particularly investigating exposure to potential risk factors that may cause DNA damage, was developed and administered. Smokers, and those with malignancy and another systemic or genetic conditions likely to increase DNA damage were excluded from the study. All patients were otherwise healthy and had not received regular medication before operation.

Anesthetics management

Following an 8-hour fasting period, a 5 cc heparinized blood sample was collected from all patients included in the study group and did not receive premedication. Electrocardiogram, non-invasive blood pressure (systolic, diastolic and mean), heart rates, end tidal sevoflurane (ET$_{SVF}$), end tidal carbon dioxide (ET$_{CO2}$) and peripheral transcutaneous oxygen saturation (SpO$_2$) were monitored. Propofol 1.5-2.5 mg/kg and vecuronium bromide (Norcuron-Organon, 0.1 mg/kg) were administered intravenously for anaesthesia induction. After intubation ventilation was mechanically conducted with intermittent positive pressure ventilation (IPPV) ensuring the ET$_{CO2}$ values would be 34±4 mmHg. Anaesthesia maintenance in Group SN (Sevoflurane-Nitrous Oxide group) was provided with 50% O$_2$ + 50% N$_2$O and sevoflurane (2-4% concentration).

Group SR (Sevoflurane-Remifentanil group) received remifentanil by infusion at rate of 0.2 mcg/kg/min, sevoflurane (2-4% concentration), and 100% O$_2$; N$_2$O was not administered.

Group C (Control group) sevoflurane (2-4% concentration), in 100% O$_2$ and N$_2$O or opioid agents were not administered. The neuromuscular blockage was reversed with neostigmin 0.07 mg/kg and atropine 0.5 mg intravenously if neuromuscular blockage persisted after surgery.

Blood sampling

Venous blood was collected using a heparinized syringe in patients before anesthesia (sample BA).

Additional venous blood samples were taken from all patients at minute 120 (sample 1) after the start of anesthesia. Postoperative samples were taken on the following first (sample 2) and fifth postoperative day (sample 3).

All samples were coded so that the scorer was unaware of the exposure status of the subject and the time of withdrawal. Blood samples were processed on the same day since there was a risk that prolonged storage of the sample (more than 4 days) releases proteolytic enzymes and causes loss of cell viability (10,11).

Chemicals

All chemicals were purchased from Sigma Chemical unless otherwise stated. Superfrost 1.0-1.2 mm thick microscope slides from Merck were used. Normal and low melting point agarose were obtained from Gibco. Dulbecco’s phosphate-buffered saline (PBS), without Mg and Ca, was from ICN Flow.
**Cell preparation**

30 μl blood from each donor was added to phosphate buffer saline (PBS) in an Eppendorf and was underlayed with Histopaque 1077 for lymphocyte isolation. Cells were immediately centrifuged at 200xg for 3 minutes at 4°C. Lymphocytes were retrieved from just above the boundary between PBS and Histopaque and were added to PBS and centrifuged at 200xg for 3 minutes at 4°C. Cells were checked for viability by trypan blue exclusion test. Each analysis was done in duplicate, and carried out immediately after sample collection without freezing or storing.

**Comet assay**

The basic alkaline technique of Singh et al. was followed with minor modifications (12). Briefly, the cells were mixed with 75 μl of 0.5% low-melt ing agarose in phosphate-buffered saline and immediately pipetted onto agarose-coated slides, spread out with a cover slip, and maintained at 4°C for 10 minutes to solidify. After removal of the cover slip, the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, 1% sodium sarcosinate, pH 10.0) with 1% Triton X-100 and 10% DMSO added just before use for a minimum of 1 hour at 4°C.

The slides were removed from the lysing solution, drained, and placed in a horizontal gel electrophoresis tank (Pharmacia GNA 100) with fresh electrophoresis buffer (1 mM Na2EDTA and 300 mM NaOH, pH 13) for 40 minutes to allow the unwinding of the DNA and the expression of alkali-labile damage. Electrophoresis was conducted at 1.6 Vcm⁻¹ for 20 minutes (300 mA). After electrophoresis, the slides were washed three times with neutralizing buffer (0.4 M Tris, pH 7.5) and stained for 10 minutes with 20 µg/ml ethidium bromide.

**Microscopic analysis and slide scoring**

A total of 100 cells were analyzed using two slides per subject for comet assay at 400 magnification under a fluorescent microscope (Zeiss, Oberkochen, West Germany, and RG) equipped with an excitation filter of 546 nm and a barrier filter of 590 nm. One hundred cells were classified by eye into three categories on the basis of the extent of migration as; no migration (NM), low migration (LM) and high migration (HM).

The number of comets in each sample was calculated (0x number of comets in category NM +1x number of comets in category LM+2x number of comets in category HM) and expressed as total comet score (TCS), which summarizes the damage frequencies as referred by Collins (13).

The overall score for each slide was therefore between 0 (undamaged) and 200 (maximally damaged) in arbitrary units. One slide reader performed analysis blindly, without knowledge of the groups in order to minimize variability.

**Statistical analysis**

Statistical analysis was performed using a statistical package programme (SPSS 11.5, Chicago, IL, USA). Chi-square test and one-way ANOVA for demographic and anamnestic evaluations; and t-test for within group and one-way ANOVA for between group comet essay comparisons between the groups. All data displayed is in mean ± standard deviation (SD) form. P< 0.05 was considered as statistically significant.

**Results**

There was no significant difference between groups in terms of patients’ demographic characteristics (Table 1) and hemodynamic variables in all patients during this study.

### Table 1: Demographic characteristics of patients and control group.

<table>
<thead>
<tr>
<th>Gender (female/male)</th>
<th>Group SN (n=18)</th>
<th>Group SR (n=18)</th>
<th>Group C (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12/6</td>
<td>12/6</td>
<td>15/1</td>
</tr>
<tr>
<td>Age (years)</td>
<td>33.7± 12.7</td>
<td>37.1± 10.3</td>
<td>33.7± 9.1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67.1± 12.5</td>
<td>68.4± 11.9</td>
<td>69± 15.9</td>
</tr>
<tr>
<td>Duration of anaesthesia (min)</td>
<td>163.5± 34.7</td>
<td>178± 39.7</td>
<td>161.8± 39.5</td>
</tr>
<tr>
<td>Duration of operation (min)</td>
<td>151.5±34.7</td>
<td>171.6± 39.2</td>
<td>155.3± 40.6</td>
</tr>
</tbody>
</table>

Data are displayed as mean ± S.D.; P< 0.05 (between groups, by one-way ANOVA and Chi-square test).
None of the participants consumed cigarettes or alcohol. Between the groups, the number of smokers at home was not different.

History of cancer and hereditary disease in patients and their primary and secondary relatives; no difference was observed between the groups. Other than the reasons for the surgery, the groups did not differ significantly with respect to the diseases they had within the past two years and the time since recovery. The number of radiologic assessments was 2.11 ± 1.64, 1.50 ± 0.61 and 1.81 ± 1.32 for groups SN, SR, and C respectively and there was no statistically significant difference between the groups (p > 0.05).

Also no significant difference was observed in terms of days for last radiologic assessment among groups.

The groups did not differ in terms of medicine use and types of medication used within the past two months. None of the cases reported regular vitamin use history for longer than 6 months. The dietary choices and cooking method preferences did not show a difference between the groups.

Postoperative analgesic and antibiotic use and duration of use were not different between the groups. Throughout the surgery, ETCO₂ values were
Effects of using sevoflurane with nitrous oxide and remifentanil on DNA damage

Table 4: Individual grade of DNA damage evaluated by Comet (100 cells) in Group C and the mean ± S.D. comet response in Group C.

Table 5: The mean ± S.D. comet response in control and patients groups.

Discussion

Many in vivo and in vitro experimental and clinical cytogenetic studies have been conducted, demonstrating that anesthetic gas exposure causes chromosomal changes. Current data from a majority of these studies suggest that anesthetic gases have genotoxicity potential.

Alkaline Comet Assay is applicable for studying DNA damage and reparation, biomonitorization, and genotoxicity. This technique can be used on various somatic cells.

In our study, we preferred to examine lymphocytes obtained from peripheral blood as it is easier to collect and there is more knowledge/experience about/on them. While no genotoxic feature of inhalation agents were observed in studies using prior techniques, DNA damage has been detected more clearly and distinctly using this technique. Sensitivity of this technique allows for observing DNA damage and reparation, biomonitorization, and genotoxicity.

Parallel to advancements in genotoxicity screenings, while initial studies reported that halothane had a slight mutagenic effect and that enflurane, isoflurane and nitrous oxide did not have mutagenic effects. This is consistent with the findings of our study, where no genotoxic effects were observed with sevoflurane/nitrous oxide/remifentanil.
Sardas et al. used Alkaline Comet technique and studied DNA damage in non-smoking patients administered isoflurane anesthesia. As a result, using the comet method, they concluded that genotoxic effect starts in peripheral blood lymphocytes of patients exposed to isoflurane before DNA repair begins.

Additionally considering that damage reparation in not always successful in patients with DNA reparation genetic defects with conditions such as diabetes, malignancy, advanced age, genetic variations, chemical exposure, it has been reported that isoflurane anesthesia may lead to irreversible DNA damage and serious health problems and that it is important to investigate DNA damage in patients who receive repeated isoflurane anesthesia.

In another study on a group of benign and malignant patients administered sevoflurane anesthesia, it was reported that the nucleic structure of their lymphocytes were effected; the damage was at maximized especially at 120th minute of operation and while this damage regressed to preoperative values on postoperative day 5 in benign patients, malignant patient groups’ lymphocyte cell DNA damage was not fully repaired, so the conclusion was that the genotoxic effects of sevoflurane, like those of many other inhalation anesthetics, were reversible in benign patients with intact DNA repairation mechanisms. Since the reparation and regression time would be longer due to defects in repairation mechanisms, patients who could have accumulating damage effects due to genetic diseases or malignancy that could trigger the mutagenicity and carcinogenicity of agents with known genotoxicity were excluded from the study.

Hoerauf et al. studied on the SCE frequency of non-smoking operating room personnel exposed to isoflurane and nitrous oxide for a minimum of 3 months, determined that SCE frequency significantly increases among these individuals compared to the control group. Additionally, they also emphasized that the genetic damage of being exposed to anesthetic gas waste exposure may be equivalent to that of a smoker who smokes 11-20 cigarettes a day. In our study, the total migration ratio of non-smoking patients in Groups SN and K was maximized on postoperative day 1 and this ratio had not regressed back to preoperative values on day 5.

In the remifentanil group, total migration was maximized on postoperative day 1 as well but had regressed back to preoperative values by postoperative day 5. Husum B. et al., halothane, isoflurane combined with general anesthesia and spinal anesthesia were administered to smokers only; pre-anesthesia and next day blood samples from the three groups did not have a difference with respect to SCE values. As a result of these studies, they concluded that smoking alone emerged a factor increasing DNA damage. We also excluded smokers from our study as we had theorized that it would contribute to DNA damage.

Studies on sevoflurane use in humans are limited; there was study on sevoflurane using the Comet technique found in the literature review. Karabiyik et al. compared damage in patients who received sevoflurane and isoflurane using the comet technique and have not detected any difference between the two agents.

A study published in 1997, conducted on Chinese hamster ovary cells, mention the DNA damage caused by Compound A, a toxic metabolite of sevoflurane. This is the only cytogenetic study of compound A conducted on mammals. In both studies, the genotoxic effect of sevoflurane is explained by compound A’s ability to cause structural changes by binding to DNA molecules. Similar effects in our study can possibly be associated with toxic features of compound A.

While there are numerous studies on inhalation anesthetics’ genotoxicity on humans conducted on benign patients, there are no studies investigating how of adding opioids to inhalation agents impacts genotoxicity. Prior SCE and comets tests on inhalation anesthetics revealed that genotoxic effects are maximized at 120th minute. Start to decrease by postoperative day 1, and return to preoperative values on day 5. Therefore, these time periods were taken as reference points for the second, third and fourth blood samples.

Genotoxic effects were maximized on day 1 and started to decrease afterwards in all groups of our study as well.

While Allen et al. aimed to investigate the genotoxicity of opioids only, did not observe genotoxicity in an Ames test. It was reported that the genotoxic effect of remifentanil (in the form of mini mutant colony formation) in vitro L5178 tk+-mouse lymphoma test, was present only in mouse KCS9 metabolic activation and over the dose of 308mcg/ml. Such activity pattern observed in genetic toxicology tests has been reported to be not specific to remifentanil and is observed with other opioids as well.
In our study, while comet technique was used to examine sevoflurane’s genotoxic damage, we aimed at investigating the damage caused by added remifentanil and how much of this damage is reversible postoperatively. As a result, we demonstrated that the group receiving remifentanil has damage increase in 120th minute of the operation, that this damage is maximized on postoperative day 1, and that it regresses to preoperative values on postoperative day 5. In nitrous oxide and control groups, the damage was detected at 120th minute of the operation, was maximized on postoperative day 1, and had regressed on postoperative day 5 but had not returned to preoperative values yet.

It is observed that using nitrous oxide to lower the inhalation agent dose and to provide a more stable anesthesia is not harmless and it adds to the sevoflurane damage. Propofol was used as intravenous agent during induction in our study. Since this medication has antioxidant features and the dose was equal across the groups, it was reasoned that it would not impact the results. The type and duration of analgesics and antibiotics used postoperatively was not different between the groups and no genotoxic characteristic of these medications was referenced in literature\(^{(23,27)}\). We reasoned that radiologic assessments would not impact conclusions, as the groups did not differ in terms of number of assessments and the number of days since last assessment.

We were scrupulous to avoid preoperative hypoxia in patients during the study. By doing so, we aimed at eliminating the negative impact hypoxic cell damage or oxidative damage could have on the test. To minimize exposure to chemical agents in our study, we surveyed out participants to evaluate environmental factors, and did not find a difference between the groups. Since DNA damage is reportedly higher in smokers than non-smokers, smokers were excluded from the study\(^{(28,29)}\).

As a result of this study, while calling attention to sevoflurane’s possible genotoxic characteristics, we also demonstrated that: Comet test is applicable in determining and following genotoxic behavior in patients with genetic diseases, malignancy potential and additional risk factors; remifentanil and nitrous oxide added to sevoflurane anesthesia of minimum of 2 hours impacts the lymphocyte nucleus structure in benign patients, but this impact does not vary across groups; and is highest in all three groups on postoperative day 1 and regresses to preoperative values in Group SR on
postoperative day 5, while it did not regress to preoperative values in the other two groups.

Total migration values between the groups were not different between the groups across each blood sample (p > 0.05).

We determined that the genotoxic effects of sevoflurane are reversible in individuals with intact DNA reparation mechanism, as is the case for most of the other inhalation anesthetics. However, with addition of nitrous oxide, the damage did not change on day 5 that is accepted as a reference point in most studies. In Group SR, sevoflurane concentration used was lower due to hemodynamic instability and this lead to an increase in the recovery rate.

Based on our findings, we believe that there may be drawbacks of using sevoflurane in benign cases especially for prolonged and repeated anesthetic applications; in such cases, additional medications to lower the sevoflurane concentration and/or alternative methods should be preferred so the damage could be lowered. Similarly, we think that, the potential risks that sevoflurane anesthesia use, which has almost become a routine in inductive and administration in pediatric applications using open systems, poses on the anesthesiologist and other operating room personnel should be reassessed.

References


