UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA

Maria Vitória Destro

Potencial genotóxico e mutagênico da exposição ao sevoflurano em camundongos

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Mestra em Anestesiologia, na área de Ciências da Saúde.

Orientadora: Profa. Dra. Mariana Gobbo Braz
Coorientador: Prof. Associado Leandro Gobbo Braz

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Câmpus de Botucatu

ATA DA DEFESA PÚBLICA DA DISSERTAÇÃO DE MESTRADO DE MARIA VITÓRIA DESTRO, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM ANESTESIOLOGIA, DA FACULDADE DE MEDICINA.

Aos 29 dias do mês de julho do ano de 2024, às 14:00 horas, no(a) Via sistemas de videoconferência e outras ferramentas para comunicação a distância (Google Meet), realizou-se a defesa de DISSERTAÇÃO DE MESTRADO de MARIA VITÓRIA DESTRO, intitulada Potencial genotóxico e mutagênico da exposição ao sevoflurano em camundongos. A Comissão Examinadora foi constituida pelos seguintes membros: Profa. Dra. MARIANA GOBBO BRAZ (Orientador(a) - Participação Virtual) do(a) Depto. de Especialidades Cirúrgicas e Anestesiologia / FM/Botucatu - Unesp, Profa. Dra. LÍLIAN CRISTINA PEREIRA (Participação Virtual) do(a) Depto. de Bioprocessos e Biotecnologia / FCA/Botucatu - Unesp, Prof. Dr. DANIEL ARAKI RIBEIRO (Participação Virtual) do(a) Depto. de Biociências / Universidade Federal de São Paulo - Santos/SP. Após a exposição pela mestranda e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, a discente recebeu o conceito final APROVADA. Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Profa. Dra. MARIANA GOBBO BRAZ

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Dedicatória

Com profunda gratidão, dedico este trabalho a Deus, expressando minha apreciação por todas as pessoas e oportunidades que Ele colocou em meu caminho. Reconheço Sua orientação constante, que iluminou meus passos e me guiou em todas as situações.

“A mente que se abre a uma nova ideia jamais volta ao seu tamanho original”
Albert Einstein

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“De todo o amor que eu tenho
Metade foi tu que me deu
Salvando minha alma da vida
Sorrindo e fazendo o meu eu”
Maria Gadu, Dona Cila

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“Nunca alguém tão grande se fez tão pequeno para tornar grandes os pequenos”
Augusto Cury

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“A suprema felicidade da vida é a convicção de ser amado por aquilo que você é, ou melhor, apesar daquilo que você é”
Victor Hugo

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“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis.”

José de Alencar

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“A persistência é o caminho do êxito.”

Charles Chaplin

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"Foste um instrumento de nosso aprendizado? Foste apenas um objeto de experiência? Não!
Foste para nós, vítimas solicitadas pela ciência, para benefício da humanidade, porém, apesar do teu olhar mudo e de não teres a permissão da palavra, isso não nos impedirá de dizer-te sempre: Muito obrigado"

(Desconhecido)

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Epígrafe

“Não tente pular as fases que você tem vivido, mesmo que sejam difíceis e, às vezes, de apertarem o coração, porque são elas que irão ensinar lições importantes, são elas que irão te fortalecer.”

Laureane Antunes

Resumo

Destro, MV. Potencial genotóxico e mutagênico da exposição ao sevoflurano em camundongos [Dissertação]. Botucatu: Faculdade de Medicina, Universidade Estadual Paulista - UNESP; 2024. 34 f.

O sevoflurano, um anestésico inalatório halogenado, é amplamente utilizado na prática clínica para indução e/ou manutenção da anestesia geral. Contudo, pouco se sabe sobre seu potencial tóxico visto que em pesquisas clínicas as avaliações são realizadas sem que haja dissociação dos efeitos da anestesia em relação aos da cirurgia. Considerando esta importante lacuna existente na literatura e que até o momento nenhum estudo avaliou possíveis danos no ácido desoxirribonucleico (DNA) em roedores expostos ao sevoflurano seguindo as diretrizes da *The Organisation for Economic Co-operation and Development* (OECD), o presente estudo avaliou o efeito isolado da exposição aguda ao sevoflurano. Portanto, investigou-se, pela primeira vez, a genotoxicidade em células de sangue periférico e em órgãos-alvo (fígado, pulmão e rim) e a mutagenicidade em medula óssea de uma única exposição ao sevoflurano em três concentrações diferentes em camundongos monitorizados. Noventa camundongos *Swiss* machos foram distribuídos nos seguintes grupos: expostos ao sevoflurano a 3,3% (menor concentração), 4,5% (concentração intermediária) e 6,0% (concentração mais alta - máxima tolerada) com 40% de oxigênio (O_2) por 2 h, controle negativo (sem exposição), controle negativo com O_2 e controle positivo (etil metano sulfonato). Os animais expostos foram aquecidos, monitorizados quanto aos sinais vitais (temperatura, saturação de O_2, frequência cardíaca/pulso e frequência respiratória) e anestesiados por meio de um moderno sistema digital de baixo fluxo (tendência mundial em anestesiologia; $\leq$ 1 l/min). Os camundongos foram eutanasiados após 2 h e 24 h da exposição, para avaliação do teste do cometa (sangue, fígado, pulmão e rim) e teste do micronúcleo (MN) em células da medula óssea, respectivamente. Não houve indução de danos no DNA no grupo de 3,3% para todos os órgãos avaliados, e nenhum efeito genotóxico ou mutagênico foi observado, para todas as concentrações de sevoflurano, em células hepáticas ou do sangue periférico. No entanto, aumento significativo nos danos no DNA foi observado nos rins (4,5%), células pulmonares (6,0%) e na frequência de MN (4,5% e 6,0%). Não foram observadas citotoxicidade ou alterações histológicas. Em conclusão, concentrações mais elevadas de sevoflurano foram capazes de induzir danos no material genético enquanto a concentração equivalente à utilizada na prática clínica não demonstrou efeito genotóxico nem mutagênico, o que é relevante para a segurança do paciente durante a anestesia.

Palavras-chave: Anestesia geral, Ensaio Cometa, Teste de Micronúcleo

Sumário

Manuscrito

Genetic instability of a single exposure to sevoflurane at different concentrations in monitored mice*

Abstract

Sevoflurane is an inhalation anesthetic widely used for general anesthesia, but its genotoxic potential is controversial in clinical studies. It is unknown whether the effects are due to surgery or the anesthetic. Thus, for the first time, the present study investigated the genotoxicity in peripheral blood cells and in target organs (liver, lung and kidney) and mutagenicity in the bone marrow of a single exposure to sevoflurane at three different concentrations in monitored mice. Ninety Swiss mice were distributed into the following groups: exposure to sevoflurane at 3.3% (low), 4.5% (intermediate) and 6.0% (high) in 40% oxygen (O_2) for 2 h; negative control (no exposure); negative control with O_2; and positive control. Exposed animals were heated, monitored for vital signs (temperature, O_2 saturation, heart rate/pulse and respiratory rate) and anesthetized using a modern low-flow digital system. Mice were euthanized 2 h and 24 h after exposure for evaluation by the comet assay and micronucleus (MN) test, respectively. No DNA damage occurred in the 3.3% group for any of the organs evaluated, and no genotoxic or mutagenic effects were observed at any sevoflurane concentration in the peripheral blood or liver cells. However, a significant increase in DNA damage was observed at higher concentrations in kidney (4.5%) and lung cells (6.0%) and in the MN frequency (groups 4.5% and 6.0%). No cytotoxicity or histological alterations were observed. In conclusion, high concentrations of sevoflurane induce DNA damage, but concentrations equivalent to those used in clinical practice do not demonstrate genotoxic or mutagenic effects.

Keywords: General anesthetic, Comet assay, Micronucleus test

*manuscrito a ser enviado para o periódico *Environmental and Molecular Mutagenesis*

1. INTRODUCTION

Currently, approximately 260 million surgeries are performed annually worldwide, and sevoflurane is a widely used general inhalation anesthetic (Holmer et al., 2019). This halogenated anesthetic is characterized by low blood/gas and oil/gas partition coefficients, which determine rapid induction and recovery from anesthesia, and is particularly commonly used in children due to its pleasant nonirritating aroma (Apai et al., 2021, Huang et al., 2021).

The concentration stipulated for inhalational anesthetics is determined by the minimum alveolar concentration (MAC), defined as the concentration necessary for 50% of patients not to respond to a painful stimulus (Merkel and Eger, 1963). The MAC varies between inhalational anesthetics and between species. For sevoflurane, 1 MAC is 2.7% for mice (Dahan et al., 2001) and approximately 2% for humans (Suzuki et al., 1998).

In relation to clinical studies, controversial findings on genotoxicity have been described in surgical patients under sevoflurane anesthesia (Karabiyik et al., 2001; Kadioglu et al., 2009; Braz et al., 2011a; Chen et al., 2013; Orosz et al., 2014; Kesimci et al., 2017; Silva et al., 2023). However, many factors, such as the patient's physical state and age, possible comorbidities, degree of surgical invasiveness, duration of surgery and anesthesia, anesthetic protocol/MAC, and which the time biological sample was collected (Braz et al., 2011b), may influence the extent of DNA damage in patients who underwent surgery with sevoflurane anesthesia. In fact, considering surgical patients, it is impossible to determine whether the effects are due to the surgery, anesthetic or even other agents that are applied during general anesthesia. Therefore, the understanding of the pathophysiological mechanisms of sevoflurane in organisms is still limited (Huang et al., 2021; Odeh et al., 2022).

To determine the effect of sevoflurane on DNA damage, studies have been conducted in animal models. Indeed, only a few studies have evaluated exclusive exposure to sevoflurane and genetic damage. However, these studies involved repeated exposure to sevoflurane (only one concentration) for a few days in mice or rabbits (Kaymak et al., 2004; Brozović et al., 2010). Two studies performed the comet assay to compare one concentration at a single or multiple times of exposure to sevoflurane and another halogenated anesthetic, isoflurane, in mice or rats (Rocha et al., 2015; Brozović et al., 2017). Other papers, however, from the same research group, have evaluated the effects of exposure to sevoflurane (one concentration at a single time) and other inhalational anesthetics (modern and old anesthetics) in combination with exposure to ionizing radiation on DNA damage detected by the comet assay in different cells (Benković et al., 2021, 2022, 2023a, 2023b).

However, no study available in the literature has reported exposure to different concentrations of sevoflurane in relation to both comet and micronucleus (MN) assays. In addition, except for one study (Rocha et al., 2015) in which anesthetized animals were invasively monitored (heart rate and pressure), the other studies did not control/monitor vital signs of animals exposed to sevoflurane, revealing a relevant gap in the literature, since monitoring vital signs in clinical practice is needed.

Considering that the Organization for Economic Co-Operation and Development (OECD, 2016; protocols no. 474 and 489) recommends that *in vivo* exposure be conducted with at least three concentrations of the test substance to assess genetic damage, there is a clear lack of research that addresses the possible genotoxicity and mutagenicity effects of sevoflurane in experimental models. Aiming to contribute to this important gap in the literature by revealing the effect of anesthetics without surgical procedures, the originality of this study lies in evaluating the effects of single/acute exposure to three different concentrations of sevoflurane administered in a low-flow, which is a global trend in anesthesiology, on DNA damage in monitored mice, as detected by both comet assay in different cell types and MN test in bone marrow. In addition, cytotoxicity and histological evaluations were performed.

Thus, we tested the hypothesis that acute *in vivo* exposure to different concentrations of sevoflurane is linked to genotoxic and mutagenic effects, potentially exhibiting a dose-response effect.

2. MATERIALS AND METHODS

2.1 Chemicals

Sevoflurane was purchased from Baxter (USA); Triton-X, dimethyl sulfoxide (DMSO), ethyl methane sulfonate (EMS) and N-lauroyl sarcosinate were purchased from Sigma (USA); sodium hydroxide (NaOH), chlorohydric acid, methanol, Giemsa and Entellan® from Merck (Germany); sodium chloride (NaCl), xylene and alcohol from Dinamica (Brazil); trypan blue dye and fetal bovine serum from LGC Biotecnologia (Brazil); ethylenediaminetetraacetic acid (EDTA) and Tris from Ludwig Biotec (China); SYBR Gold, low melting point (LMP) agarose and normal melting point (NMP) agarose from Invitrogen (USA).

2.2 Animals and experimental design

This study was approved by the Ethics Committee for Animal Research (#1419/2022, UNESP-Botucatu, Brazil) prior to the start of the study, and followed the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (Percie et al., 2020). All experimental procedures were conducted in accordance with the ethical principles of animal research adopted by the Brazilian College of Animal Experimentation. Six-week-old male Swiss albino mice were acquired from the Animal Research and Production Center (CPPA/IBTEC, UNESP-Botucatu, Brazil). The 90 animals were housed at UNIPEX (UNESP, Botucatu, Brazil) in restricted-access rooms with a polysulfone individually ventilated caging system. Cages with up to four animals were kept under a controlled relative humidity (50-60%), temperature (23 °C ± 2 °C), a 12-h dark/light cycle, and free access to a standard pellet diet and water. The animals were marked with picric acid for identification, and weight, water and food consumption were monitored.

A pilot study was necessary to determine the concentrations and duration of sevoflurane inhalation based on vital signs and considering morbi-mortality and the effects described following the OECD (2018, guideline no. 433). The concentration was based on 1 MAC, a dose frequently used in human anesthesia practice. Thus, the lowest concentration to which mice were anesthetized was 1.2 MAC (3.3%), the intermediate concentration was 1.7 MAC (4.5%), and the highest concentration (deep anesthesia/maximum tolerated) was 2.2 MAC (6.0%). The duration was set at 2 h of sevoflurane exposure.

After an acclimation period of two weeks, the mice (35 g ± 5 g) were allocated into six groups as follows (Figure 1): 1, negative control (without exposure); 2, negative control not exposed to anesthetic but exposed to 40% oxygen (O_2 - vehicle); 3, exposure to 3.3% sevoflurane; 4, exposure to 4.5% sevoflurane; 5, exposure to 6.0% sevoflurane; and 6, positive control (a single dose of EMS - 300 mg/kg b.w. I.P., according to Recio et al., 2010, and OECD protocols 489 and 474, 2016).

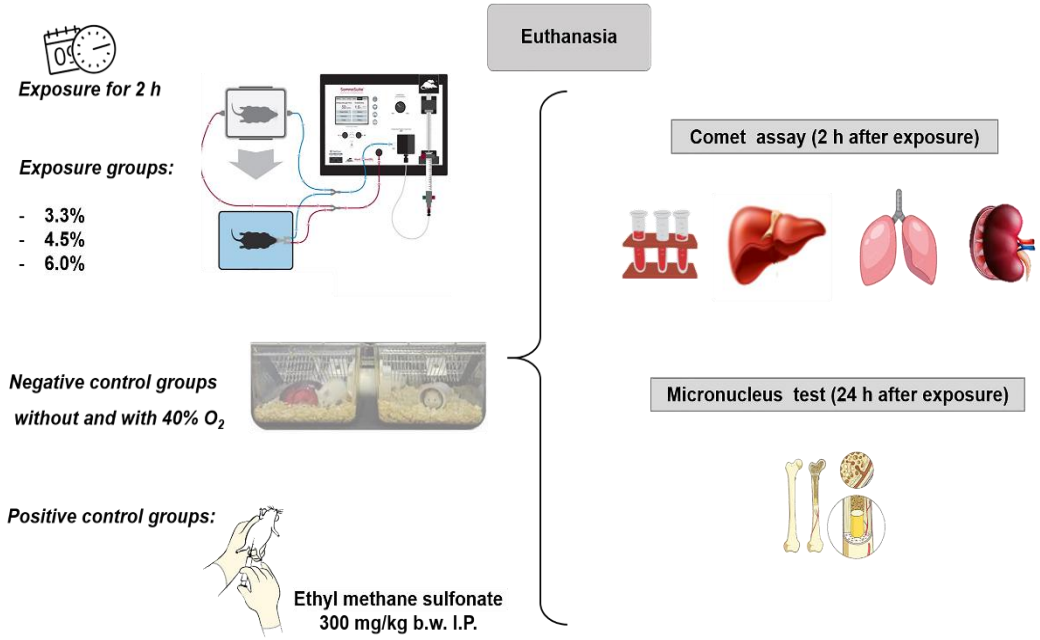

Figure 1 - Experimental design of the current study.

This study aimed to ensure animal immobility during anesthesia maintenance and to monitor vital signs and recovery. The animals in the exposed groups were individually exposed to sevoflurane inhalation using a modern low-flow digital inhalation anesthesia system (SomnoSuite, Kent Scientific, USA; Figure 2), which has an activated charcoal absorption filter that adsorbs the waste anesthetic gas released by the animal, avoiding anesthetic pollution in the workplace/laboratory. This rodent anesthesia equipment allows the use of both room air and O_2 for inhalation anesthesia. However, during the pilot study, the use of room air was inadequate for anesthetic exposure, resulting in severe hypoxia in the animals. Thus, a supply of 40% O_2 to the rodent anesthesia equipment was necessary using a traditional anesthesia device that supplied both O_2 (0.3 l/min) and compressed air (1 l/min), mimicking clinical practice conditions.

To achieve the desired concentration of sevoflurane (Baxter, USA), the anesthetic in liquid form was introduced into the digital vaporizer through an electronically controlled syringe in the rodent anesthesia equipment. Anesthesia was induced in the induction chamber (step 1 of Figure 2) with exposure to 7% sevoflurane at a flow rate of 0.5 l/min, supplied with O_2, until the animal's righting reflex was lost. Before opening the lid of the induction box and removing the animal, the rodent anesthesia equipment was flushed to remove the sevoflurane from the device's activated carbon filter.

Subsequently, the animal was positioned on a pad with an infrared heated plate (step 2 in Figure 2). The animal's snout was connected to a conical inhalation mask specifically

designed for mice, ensuring adequate coupling, with a flow of 40% O_2 at 0.05 l/min and one of the three previously defined concentrations of sevoflurane for 2 h. The pad was connected to the MouseSTAT module (Figure 2), which monitored vital signs (O_2 saturation - SpO_2, heart rate/pulse - HR and respiratory rate - RR) through sensors positioned on the animal's hind paw. The core temperature was also monitored using a rectal probe. All these parameters were recorded every 15 min during sevoflurane inhalation. Additionally, ophthalmic gel was carefully applied to the eyes to protect against drying of the animals' corneas before anesthetic exposure.

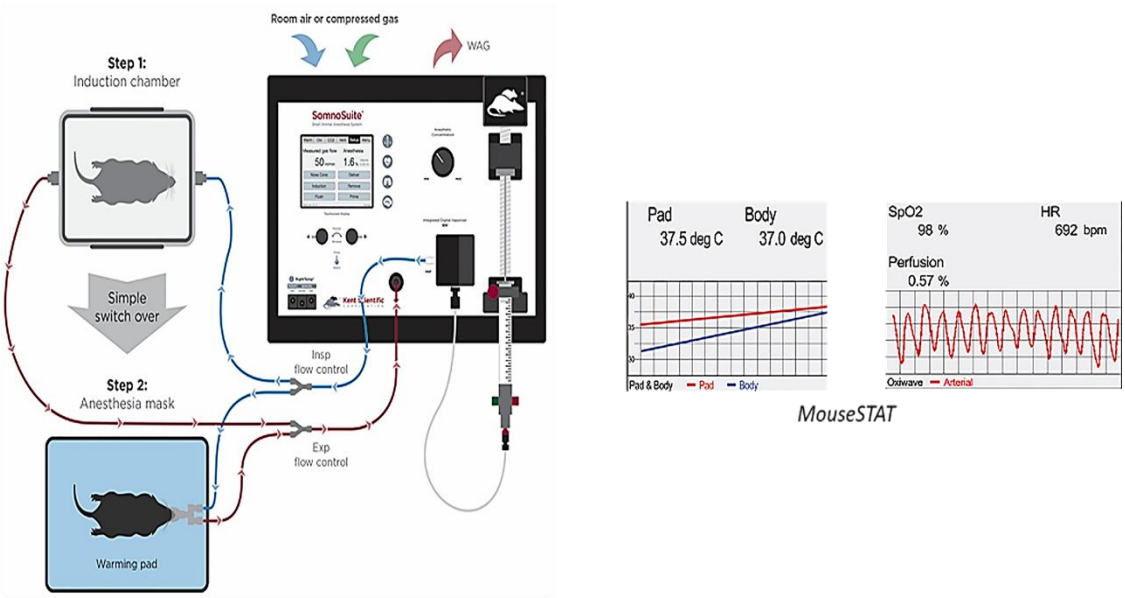

Figure 2 - Low-flow digital inhalation anesthesia equipment specifically designed for laboratory rodents (SomnoSuite, Kent Scientific), which monitors core temperature, oxygen saturation, heart/pulse rate (MouseSTAT module, Kent Scientific), and respiratory rate (included in the MouseSTAT module). Adapted from https://www.kentscientific.com/

When the exposure was finished, the ophthalmic gel was removed, and the mice were removed from the mask by flushing and monitored until complete recovery. Soon after, the animal was placed in an individual cage (with water and a pellet diet) with environmental enrichment until euthanasia (2 h or 24 h after exposure). The animals were monitored for weight and possible abnormal behavior, such as weakness, lethargy and the presence of bristly hair.

In relation to animals in the (negative) control groups, mice were housed in a separate room from the exposed groups to avoid possible traces of sevoflurane, which could introduce bias. A cage adapted to receive 40% O_2 was used by two conscious animals at a time for 2 h.

The same animals from the negative control group (without O_2, n = 7 animals) were used to perform the comet assay and MN test.

2.3 Sampling and biological collection

Euthanasia was performed by cervical dislocation by an experienced professional 2 h after the end of sevoflurane exposure to collect blood, lung, liver and kidney for assessing the comet assay. For the MN test, euthanasia was performed by cervical dislocation 24 h after the end of the 2-h sevoflurane exposure, and femurs were collected. The organs analyzed (liver, kidney and lung) were immediately cleaned and weighed, under yellow light.

2.4 Comet assay

The comet assay was performed according to the protocol described by Tice et al. (2000), with some modifications from Rodrigues Tanamachi et al. (2021). All procedures were performed in accordance with OECD guidelines (no. 489, 2016) and the Minimum Information for Reporting on the Comet Assay (Møller et al., 2020) under yellow light.

Fresh liver, kidney and lung tissues were washed in ice-cold saline solution (PBS) and small fragments of each organ were chopped into 500 µl of PBS. A volume of 10 µl of the homogenized cell suspension from each organ was combined with 10 µl of trypan blue dye for the evaluation of cell viability, which was considered ≥ 70%, using an automated counter (Countess™ Automated Cell Counter, Invitrogen, USA). Subsequently, 50 µl of cell suspension was mixed with 150 µl of 0.5% LMP at 37 ºC and distributed on each of the two coded microscopy slides/tissues previously precoated with 1.5% NMP. A similar procedure was used for blood samples (20 µl of blood was mixed with 180 µl of LMP on coded slides).

After the slides solidified, they were immersed overnight in chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris - pH 10; 1% Triton-X and 10% DMSO were added fresh). After lysis, all slides were washed with PBS for 5 min and transferred to a horizontal acrylic vat containing a freshly prepared electrophoresis solution maintained at 4 ºC (composed of 1 mM EDTA and 300 mM NaOH with pH > 13) for 20 min. Electrophoresis (Bio-Rad, USA) was performed at 25 V (0.8 V/cm) and 0.3 A for 20 min. Negative and positive controls were used in each electrophoresis run. The slides were then placed in neutralization solution (0.4 M Tris, pH 7.5) for 15 min and subsequently immersed in absolute alcohol for fixation. For analysis, the slides were stained with 75 µl of SYBR Gold diluted 1:9999, and the nucleoids were visualized under a fluorescence microscope coupled to an image analysis system (Comet

Assay IV - Perceptive Instruments, UK). Each slide (75 nucleoids) was analyzed at 400x magnification to assess DNA damage using the tail intensity (TI) descriptor (% DNA in tail). Thus, 150 nucleoids (two slides) for each tissue/animal were analyzed (for a total of eight slides per animal).

2.5 Micronucleus test

The MN test was carried out according to the procedures described in guideline no. 474 (OECD, 2016) and following the protocol described by MacGregor et al. (1987) with minor modifications (Fernandes et al., 2018). After the mice were euthanized (24 h after exposure), both femurs were collected, and the bone marrow was transferred to a tube using the flushing technique with 2 ml of fetal bovine serum using a 1 ml syringe. The cell suspension was centrifuged at 1000 rpm for 5 min; approximately 1.5 ml of the supernatant was discarded. Subsequently, the remaining content was homogenized, and with a Pasteur pipette, two drops of this material were placed on the edge of two coded slides, and the smear technique was performed on each of them.

After drying at room temperature for 24 h in a vertical position, the slides were immersed in methanol for 5 min, stained with undiluted Giemsa for 3 min, and then submerged in diluted Giemsa (1:5 distilled water) for 15 min. After the slides were lightly rinsed with running water, they were immersed in distilled water for approximately 15 min. The slides were then allowed to dry at ambient temperature for 24 h. The next day, the slides were quickly submerged in xylene, 2 drops of Entellan® were applied, and cover slips were added for preservation until analysis. Cell analysis was performed under an optical light microscope at 1000x magnification with immersion oil. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) was scored in 4000 polychromatic erythrocytes (PCEs) per animal, and the cytotoxic potential, based on the nuclear division index (NDI), was calculated using the formula NDI = PCE/PCE + NCE of 500 cells per animal, where NCE indicates normochromic erythrocyte.

2.6 Histopathological evaluation

The collected organ fragments were fixed for 24 h in 10% phosphate-buffered formalin, followed by 70% alcohol until processing. The fragments were then embedded in paraffin, cut into 4 µm sections and stained with hematoxylin-eosin. Transverse sections of the liver and kidneys were analyzed under a microscope (Carl Zeiss, Camera Opticam, 2k, Germany) at

200x and 400x magnification according to the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND Project) criteria (Thoolen et al., 2010; Frazier et al., 2012).

2.7 Statistical analysis

The distribution of the data was verified with the Ryan-Joiner test. Then, one-way analysis of variance (ANOVA) was used followed by the Tukey test for the comet assay and weight data. MN test data were analyzed using generalized linear models with the Poisson test. For vital sign variables, profile analysis (repeated-measures ANOVA) was performed considering time points and exposure groups. Statistical significance was set at $p < 0.05$.

3. RESULTS

During the experiment, no behavioral changes, weight loss or macroscopic tissue injuries were observed in the groups exposed to sevoflurane; there were no differences between the groups at either 2 h or 24 h and the control groups. Figure 3 shows vital signs during exposure to different concentrations of sevoflurane. All the groups had similar SpO_2 values, except for at the last two time points (105 min and 120 min), which decreased in the 6.0% group. RR differed among the groups; it was greater in the 3.3% group and lower in the 6.0% group. The mean values were within the physiological values for the 3.3% group, near the range for the 4.5% group and lower for the 6.0% group. HR/pulse was similar for the 3.3% and 4.5% groups but was greater in the 6.0% group than in the lower concentration groups; however, all the mean values were within the normal range for the three groups. In terms of body temperature, all the groups were very similar, as expected, considering that the animals were heated.

The results obtained in the comet assay in blood, liver, kidney and lung cells are shown in Figure 4. No increase in DNA damage was detected in leukocytes or hepatocytes. Compared with those in the negative controls, significant increases in DNA damage were detected in the kidney cells of animals exposed to 4.5% sevoflurane and in the lung cells of animals exposed to 6.0% sevoflurane ($p < 0.05$). No significant differences were detected among the sevoflurane concentrations or between the negative controls. As expected, the positive control significantly induced DNA damage in comparison with the negative controls.

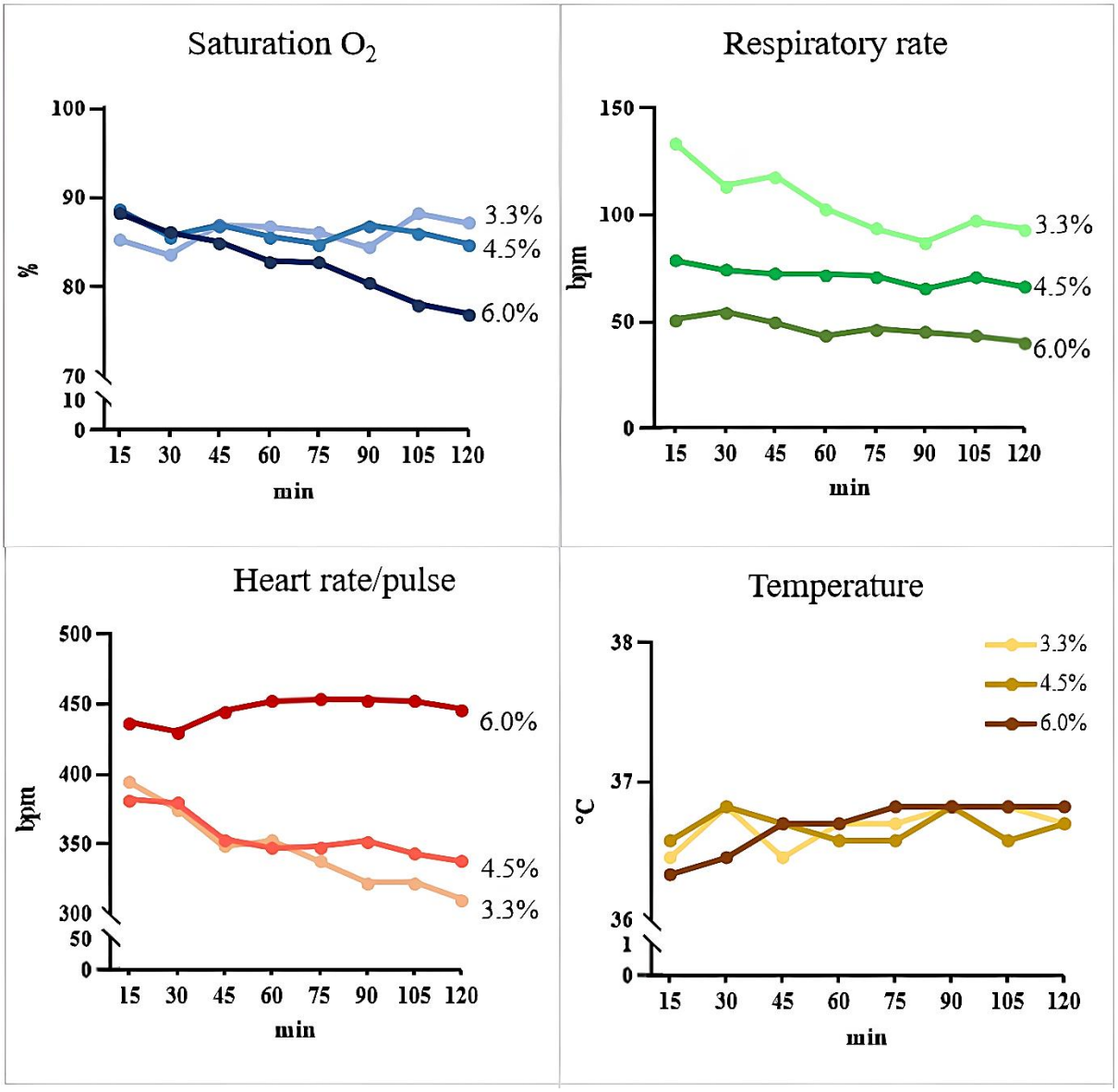

Figure 3 - Vital signs of all exposed mice were recorded every 15 min during 2 h of exposure to sevoflurane.

In addition, there was a significant increase ($p < 0.05$) in the frequency of MNPCEs in the bone marrow of animals anesthetized with intermediate or the highest concentrations (4.5% and 6.0%) (Table 1). No significant differences were observed among the concentrations. No significant difference in the PCE/(PCE + NCE) ratio was detected. As expected, compared with the negative controls, the positive control significantly induced MNPCEs.

No histopathological damage was detected in the liver or kidney of mice exposed to three different concentrations of sevoflurane (data not shown).

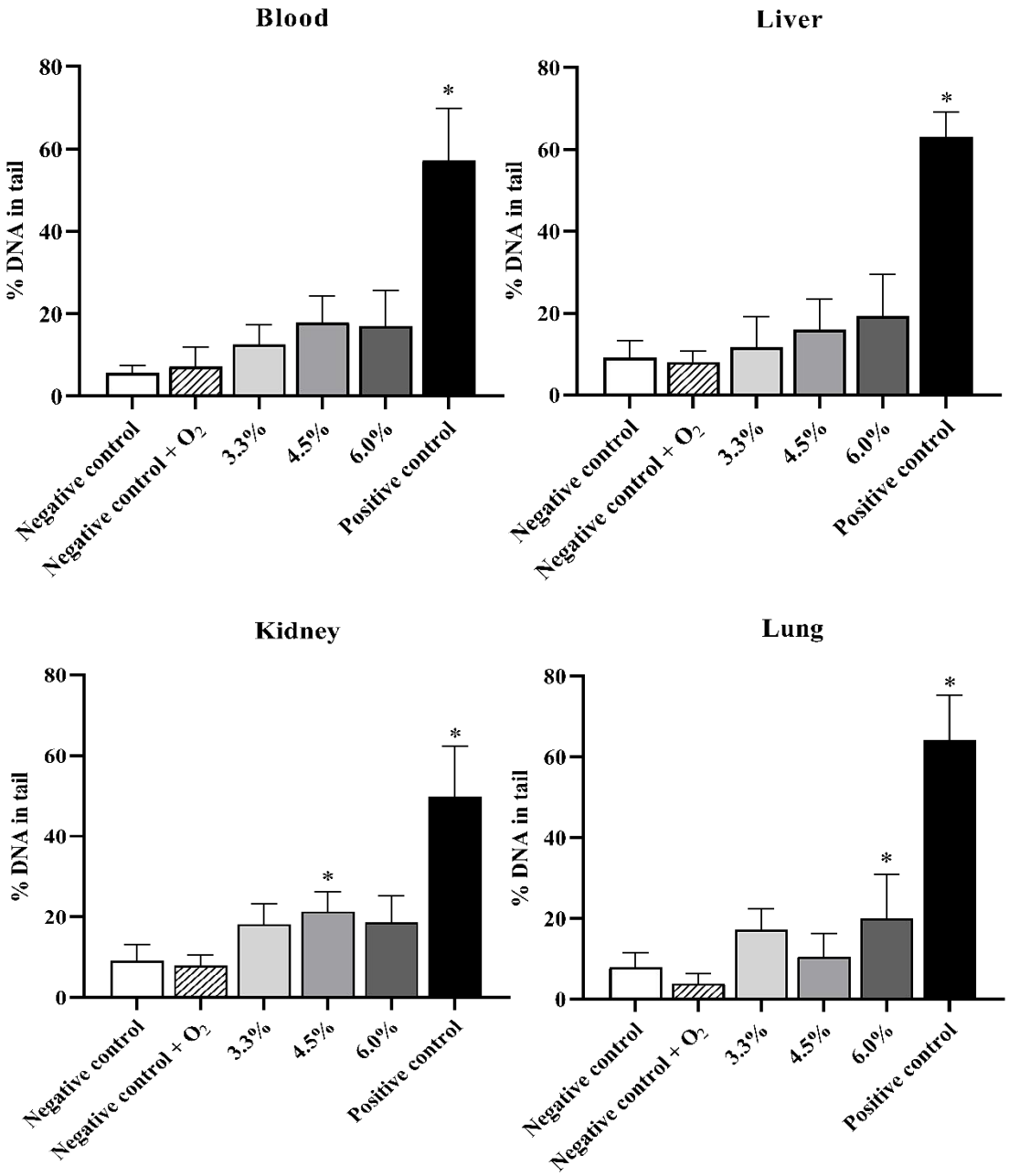

Figure 4 - DNA damage evaluated in the blood, liver, kidney and lung of mice exposed to sevoflurane (n = 10 animals/group). Positive control - ethyl methane sulfonate (300 mg/kg b.w. I.P.; n = 8 animals); negative controls (with or without 40% oxygen gas; n = 5 animals and n = 7 animals, respectively). The results are expressed as the X ± SD; * $p < 0.0001$ in relation to the negative controls.

Table 1 - Frequency of micronucleated polychromatic erythrocytes (MNPCEs) in the bone marrow of mice exposed to different concentrations of sevoflurane

Groups	No. of animals	No. of analyzed cells	MNPCEs			PCE/(PCE+NCE) (Mean ± SD)
			n	%	Mean ± SD/4000[b]	
Negative control	7	28,000	16	0.06	2.0 ± 1.1	0.53 ± 0.03
Negative control + O_2	5	20,000	24	0.12	4.5 ± 1.9	0.56 ± 0.06
3.3%	8	32,000	45	0.14	5.6 ± 1.6	0.54 ± 0.03
4.5%	10	40,000	90	0.23*	9.0 ± 2.6	0.52 ± 0.07
6.0%	9	36,000	106	0.29*	11.8 ± 3.0	0.57 ± 0.03
Positive control[a]	8	32,000	365	1.14*	45.6 ± 8.3	0.55 ± 0.05

PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes

[a]Ethyl methane sulfonate (300 mg/kg b.w. I.P.)

[b]4000 PCEs/ animal

* $p < 0.0001$ in relation to the negative controls

4. DISCUSSION

The results of this study contribute to a better understanding of the effects of a single exposure to sevoflurane at different concentrations delivered at a low flow rate on DNA damage in mice monitored for vital signs. Evidence of genotoxicity and mutagenicity was found in some of the target organs analyzed (lung, kidney and bone marrow) after exposure to sevoflurane at 4.5% and 6.0%, but not at the low concentration (3.3%), which is equivalent to that observed in human clinical practice (1.2 MAC). No cytotoxicity or histological damage was observed in the analyzed organs.

There are some hypotheses regarding the possible mechanisms of the genotoxicity of halogenated anesthetics, including reacting directly with DNA (alkylation at the N7 position of purines) or by forming reactive metabolites and/or by inhibiting the mitochondrial respiratory chain (Jaloszyński et al., 1999; Alleva et al., 2003; Zimin et al., 2018).

In parallel with studies in which mice were exposed to sevoflurane once for 2 h and a comet assay was performed 2 h after exposure (Benković et al., 2021, 2022, 2023a), the authors applied 2.4% sevoflurane, which represents less than 1 MAC, in 50% O_2 at a high flow rate (3 l/min). These authors reported increased DNA damage compared with the control when only evaluating sevoflurane without radiation or comparing the results with other

anesthetics; in blood (white cells) considering TI, tail moment (TM) and tail length (TL) parameters (Benković et al., 2021); in kidney cells considering TI and TL (Benković et al., 2022); and in liver cells when TL was considered since the TI and TM values were similar to those in nonexposed animals (Benković et al., 2023a). Thus, our findings differ from those studies when comparing them at the lowest concentration (3.3%) in peripheral blood cells and the kidney. Our results are similar to those reported in the liver, in which genotoxicity was not reported at low concentrations of sevoflurane (or even at relatively high concentrations), considering the same parameter we used (TI).

Unlike in our study, there was no monitoring of the animals' vital signs in the mentioned studies, which could introduce bias into the experiments, and the authors considered anesthesia achieved when the mice were sleeping calmly, breathing spontaneously, and without tail movement. In fact, in our pilot study, 1 MAC was not sufficient to maintain anesthesia for the necessary time, and a concentration less than 3.3% proved to be very superficial, with animals recovering from anesthesia, which was not ideal. In addition to the similar concentration used in human practice (around 1 MAC), we aimed to guarantee total immobility of the animals during the maintenance of anesthesia, and we warmed the animals since anesthetics, such as sevoflurane, decrease the core body temperature. Additionally, we monitored three vital signs, as occurs in clinical practice, while guaranteeing the recovery of the mice after anesthesia. We also highlight other notable differences between our study and those mentioned previously, such as the anesthetic concentration used, flow rate (low [≤ 1 l/min] *versus* high [≥ 3 l/min]), different objectives and experimental design in addition to the use of a positive control group. Previous studies only used the negative control (animals without sevoflurane exposure) and did not evaluate the vehicle (O_2); thus, our study is the first to show a lack of significant differences between the negative control and negative control with O_2 for the comet and MN assay results. Additionally, we used one parameter (TI or % DNA in tail), which is the recommended descriptor to be applied instead of several parameters for the comet assay (Brunborg and Collins, 2020).

No genotoxicity in peripheral blood or liver cells was found regardless of the sevoflurane concentration. The absence of genotoxic effects (including also oxidized DNA damage) in peripheral blood cells is supported by studies conducted in patients without comorbidities who underwent minimally invasive surgeries using 1.0-1.3 MAC sevoflurane anesthesia in 40% O_2 for 2 h (Orosz et al., 2014; Silva et al., 2023; Braz et al., 2024), although not only anesthetics, but also other drugs are applied during general anesthesia. In these cited studies, blood samples were collected immediately or 24 h after sevoflurane

anesthesia, but even at higher concentrations and samples collected 2 h after 2 h of exposure, no DNA damage was induced in white blood cells. Even when patients underwent major colorectal surgery for more than 3 h, no genotoxic effect (TI parameter) was observed in patients who received sevoflurane with 30% or 80% O_2 (Chen et al., 2013). In contrast, patients who underwent invasive lower abdominal surgery with sevoflurane administered at a high flow rate of 6 l/min presented an increase in DNA damage in blood lymphocytes during anesthesia up to the first postoperative day; the DNA damage evaluated on the third day of anesthesia was similar to that before anesthesia, suggesting that the DNA was repaired (Karabiyik et al., 2001). Despite the different study designs, Croatian authors also showed a lack of genotoxicity when mice were repeatedly exposed to very low concentrations of sevoflurane (2.4% for 2 h daily, for 3 days) in blood cells, when they were collected 2 h after exposure when TI, TM and TL were considered (Brozović et al., 2010).

In addition to the kidneys and lungs, the liver contributes to the metabolism of inhaled halogenated anesthetics (Forman and Ishizawa, 2015); the metabolism of sevoflurane is 5%, which is greater than that of other commonly applied halogenated anesthetics (Forman and Ishizawa, 2015) but lower than that of the older halothane (25%). Sevoflurane is rapidly metabolized by cytochrome P450 - CYP 2E1 (Kharasch et al., 1995), giving rise to fluoride and hexafluoroisopropanol (HFIP) in phase 1 and glucuronidation in phase 2, which is rapidly transformed into HFIP-glucuronide, the main oxidative metabolite (Orhan et al., 2004) that is excreted in urine (Park et al., 2009).

Despite its important role in metabolism, we could not detect genotoxicity of sevoflurane/metabolites in liver cells considering threat any of the tested sevoflurane concentrations and collection time. In addition, we did not observe histopathological alterations regardless of the sevoflurane concentration. Despite different study designs, some authors have also shown a lack of genotoxicity when mice are repeatedly exposed to very low concentrations of sevoflurane (2.4% for 2 h daily, for 3 days) in liver cells when they are collected 2 h after exposure when TI, TM and TL are considered (Brozović et al., 2010). This study revealed greater DNA damage in livers collected 6 h after 2 h of exposure to sevoflurane than in those collected from the control group; however, no genotoxicity was observed immediately or 24 h after anesthetic exposure. We highlight that the OECD guidelines (no. 489, 2016) recommend a sampling time of 2 h to 6 h for the assessment of genotoxicity by the comet assay. This is consistent with the divergence of observed results; although we did not find DNA damage in the liver collected after 2 h of 2 h of exposure to sevoflurane, Brozović et al. (2010) reported damage in the liver collected after 6 h of 2 h of

exposure to sevoflurane, despite differences noted between the studies. Interestingly, when halothane was compared with sevoflurane, DNA damage was induced in livers of mice when the cells were analyzed immediately and after 2 h, 6 h and 24 h after exposure, which is different from the results obtained for sevoflurane (Benković et al., 2023a), suggesting that greater biotransformation and greater toxic metabolites are formed and are toxic to DNA.

Respiration is the main route of input and elimination for volatile agents (Kharasch et al., 1995). Our study is the first to analyze DNA damage in the lung cells (important target organ) of mice exposed to sevoflurane; the findings showed an absence of genotoxicity at lower concentrations, but DNA damage was induced at the highest concentration. In fact, in the 6.0% group, the SpO_2 and RR were lower than those in the other exposure groups, which certainly contributed to our findings since these parameters are related to airway management. Considering that inhalational anesthetics, including sevoflurane, depress the respiratory center at high concentrations, a decrease in both the SpO_2 and RR was expected in the 6.0% group because of the more pronounced anesthetic effect. In addition, because sevoflurane is a powerful vasodilator at high concentrations, it decreases venous return and stroke volume and consequently increases HR/pulse to maintain adequate cardiac output (Conzen et al., 1992; Rooke et al., 1997). Indeed, when mice were anesthetized with 5% sevoflurane, a mean HR of 401 bpm, a RR of 39 bpm and a SpO_2 less than 90% were reported (Oliveira et al., 2022), and these values were similar to ours in relation to higher concentrations, especially the 6.0% group. Considering the vital signs, animals in the 4.5% group presented better vital signs than did those in the 6.0% group (deep anesthesia) and 3.3% group; despite vital signs being within the normal range, the animals in the 3.3% group were more superficially anesthetized than those in the 4.5% group.

Wei et al. (2014) demonstrated that 5% sevoflurane induced apoptosis, decreased cell viability and compromised DNA integrity in A549 lung alveolar epithelial cells, corroborating the genotoxic effect observed in the 6.0% sevoflurane group. The A549 cell line is considered a suitable system for evaluating the metabolic and toxicological effects of drugs, because it has metabolic and transport properties consistent with those of type II lung epithelial cells *in vivo* (Foster et al., 1998).

The kidneys are large organs that receive high blood flow and clear most of the hydrophilic metabolites resulting from the biotransformation of sevoflurane; kidneys also contain CYP enzymes that catalyze both phase 1 and phase 2 reactions (Forman and Ishizawa, 2015). Thus, kidneys together with the liver are exposed to the highest concentrations of sevoflurane metabolites and thus are most susceptible to damage from toxic metabolites. In

fact, fluoride (one of the metabolites of sevoflurane) has already been associated with renal injury, especially with anesthetics that undergo extensive biotransformation and are no longer used in clinical practice (methoxyflurane). Inorganic fluoride likely causes renal injury, but clinical studies have demonstrated no significant nephrotoxicity after the administration of sevoflurane (Forman and Ishizawa, 2015).

The evidence of nephrotoxicity in rodents compared with humans suggests that the mechanisms of sevoflurane metabolism and toxicity can differ between these species (Forman and Ishizawa, 2015). Although no genotoxic hazard was detected in the 3.3% and 6.0% sevoflurane exposure groups, DNA damage was induced in the 4.5% group. In parallel with studies that evaluated DNA damage in kidney cells, as evaluated by the comet assay (% DNA in tail), in Swiss mice exposed to 2.4% sevoflurane for 2 h, the authors reported genotoxicity at this low concentration (repeated exposure) only after 24 h after exposure but not from 0 h to 6 h (Brozović et al., 2010); at 6 h and 24 h after the exposure, but not immediately or 2 h after the last exposure (Brozović et al., 2017); and with a single anesthetic exposure evaluated from 0 h to 24 h after exposure (Benković et al., 2022). Thus, our finding (lowest concentration - 3.3%) is in line with those of Brozović's studies that showed an absence of genotoxicity in the kidney when samples were collected 2 h after exposure. The descriptive histological analysis corroborated the results obtained for cell viability, confirming that there was no cyto- or tissue toxicity in the kidneys.

According to Brozović et al. (2010), kidney cells are the most sensitive cell type to DNA damage caused by sevoflurane. This anesthetic is also associated with the formation of oxidative reactive products, which could contribute to the DNA damage observed in the intermediate group (4.5%). It is possible that the highest concentration (6.0%) caused some injury or toxicity; however, the comet assay is not an adequate test for evaluating cytotoxicity or apoptosis. In fact, 3.0% of sevoflurane induces apoptosis in different human cell lines (Kvolik et al., 2005).

Consistent with the increased DNA damage detected in the kidney (4.5%) and lung (6.0%), the mutagenicity test showed a significant increase in the MN frequency (24 h after exposure) at relatively high concentrations of 4.5% and 6.0% but not in the 3.3% group in comparison to the negative control groups. In addition, although no change was observed in the mean NDI values (cytotoxic ratio), it is possible that the higher concentrations demonstrated injury or toxicity, but perhaps this ratio was not sufficiently sensitive enough to detect such effects.

It should be noted that there are no studies of MN detected in the bone marrow of mice

exposed to sevoflurane. The only study available in the literature showed an increase in the MN frequency detected in peripheral blood reticulocytes of mice repeatedly exposed to sevoflurane (2.4%) in samples collected from 0 h to 48 h after exposure (Brozović et al., 2010). In parallel with the findings of a clinical study, an increased frequency of MN in nasal cells of adult patients who underwent ear and throat surgeries under sevoflurane anesthesia (8% induction in 6 l/min de O_2) and maintenance at 0.5-1.5 MAC was reported in the postoperative period and 21 days after the surgical procedure when compared to the values observed before surgery (Kesimci et al., 2017), supporting our data.

In conclusion, under the conditions of the present study, a single 2 h exposure to relatively high concentrations of sevoflurane administered under low-flow conditions induce genotoxicity in the kidney (4.5%) and lung (6.0%) and mutagenicity in the bone marrow (4.5% and 6.0%), but none of the concentrations induce DNA damage in the blood or liver cells of the monitored mice. In contrast, sevoflurane has no genotoxic or mutagenic effects on any of the target organs evaluated at the lowest concentration, which is equivalent (1.2 MAC) to that used in human practice and is relevant for anesthetized patients. Future studies should investigate further toxicogenetic and cytotoxic effects after repeated exposure to sevoflurane at concentrations equivalent to those used in clinical practice to mimic patients who undergo multiple surgeries under sevoflurane inhalation anesthesia.

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Anexo

Aprovação do Projeto pela Comissão de Ética no Uso de Animais

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

Comissão de Ética no Uso de Animais

Criada pela da Portaria DFM nº 611 de 13/12/2012

CERTIFICADO Nº 1419/2022 – CEUA

Certificamos que o projeto intitulado: "**Potencial genotóxico e mutagênico da exposição ao sevoflurano em camundongos"-** conduzido pela Pesquisadora: **Maria Vitória Destro– Orientadora:**Profa. Dra.Mariana Gobbo Braz – **Coorientador:** Prof. Ass. Leandro Gobbo Braz – **Colaboradores:**Mariane Aparecida Pereira Silva, registrado com o **nº 1419/2022**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei n. 11.794, de 08 de outubro de 2008, do Decreto n. 6.899, de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi APROVADO pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Botucatu, em reunião ordinária de 25 de outubro de 2022.

Finalidade	( ) Ensino (**X**) Pesquisa Científica
Vigência da Autorização	25/10/2024
Espécie/Linhagem/Raça	Camundongo Heterogênico Swiss
Nº Total de animais	90
Idade/Peso	08 Semanas – 35 Gramas
Sexo	Machos

Profa. Associada Bertha Furlan Polegato
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina de Botucatu/UNESP

Sara Rosa Stanley Sampaio
Secretária da Comissão de Ética no Uso de Animais
Faculdade de Medicina de Botucatu/UNESP

The research project registered by the number 1419/2022 is in agreement with the Ethical Principles for Animal Research established by the National Council for Control of Animal Experimentation (CONCEA) and was approved by the Committee on Ethics in the Use of Animals of Botucatu Medical School – Sao Paulo State University (UNESP) on 25/October/2022.