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

Mariane Aparecida Pereira Silva

Papel de polimorfismos, instabilidade genética, resposta oxidativa e expressão gênica na exposição ocupacional aos anestésicos

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

Orientadora: Prof ª Dr ª Mariana Gobbo Braz

Botucatu
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1. Estresse oxidativo. 2. Exposição ocupacional. 3. Expressão gênica. 4. Testes de mutagenicidade. 5. Polimorfismo genético.

Palavras-chave: Estresse oxidativo; Exposição ocupacional; Expressão gênica; Mutagenicidade; Polimorfismos genéticos.

Dedicatória

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"A liberdade que Deus vos deu é de fato a maior e melhor prova do Seu amor."
Masanobu Taniguchi, Canto em Louvor ao Bodisatva que reflete os sons do mundo.

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"Se queres partir, ir embora.
Me olha da onde estiver
Que eu vou te mostrar que eu tô pronta
Me colha madura do pé"
Maria Gadú, Dona Cila

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"Vais encontrar o mundo, disse-me meu pai, à porta do Ateneu. Coragem para a luta".
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"O saber a gente aprende com os mestres e os livros. A sabedoria, se aprende com a vida e com os humildes." Cora Coralina

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"Não há satisfação maior do que aquela que sentimos quando proporcionamos alegria aos outros". Masaharu Taniguchi

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"Se consegui ver mais longe é porque estava aos ombros de gigantes".

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"Life is a study in contrasts between randomness and determinism"

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Louis Pasteur

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Epígrafe

"A felicidade não é algo que se persegue ou que acontece casualmente; ela deve ser descoberta no aqui-agora. É no aqui-agora que está a nossa vida."

Masaharu Taniguchi

Sumário

Resumo

Silva MAP. **Papel de polimorfismos, instabilidade genética, resposta oxidativa e expressão gênica na exposição ocupacional aos anestésicos [Dissertação]**. Botucatu: Faculdade de Medicina, Universidade Estadual Paulista - UNESP; 2021.72 f.

Profissionais que atuam em salas cirúrgicas (SC) podem estar diariamente expostos aos resíduos de gases anestésicos (RGA) devido ao uso de anestésicos inalatórios. De forma inédita, este estudo observacional monitorou a poluição anestésica dos RGA sevoflurano, isoflurano e óxido nitroso, avaliou biomarcadores de instabilidade genética em células bucais, incluindo micronúcleo (MN) e broto nuclear (BN), bem como os de estresse oxidativo por meio de lipoperoxidação (malondialdeído-MDA) e capacidade antioxidante plasmática (FRAP), a susceptibilidade genética por polimorfismos em genes de metabolismo de fase I e II (*CYP2E1*, *GSTP1*, *GSTM1* e *GSTT1*), danos genéticos/reparo (*OGG1*, *XRCC1* e *ATM*) e resposta antioxidante (*SOD2*), assim como a expressão dos genes *OGG1* e *SOD2*. Altas concentrações residuais de RGA foram observadas em SC, as quais não possuíam sistema de exaustão adequado. O grupo exposto, constituído por 100 profissionais, apresentou maior instabilidade genética (aumento de MN e BN), mas não estresse oxidativo, quando comparados aos 93 indivíduos não expostos aos RGA (grupo controle). Frequência elevada de MN e BN foi detectada em indivíduos expostos com idade superior a 30 anos em relação à mesma faixa etária no grupo controle; mulheres expostas apresentaram maior instabilidade genética que mulheres não expostas; e profissionais que tinham carga horária semanal (> 30 h/semana) maior em SC apresentaram aumento na formação de MN em relação aos indivíduos com menor carga horária semanal aos RGA. Quanto ao estresse oxidativo, aumento de MDA foi observado em indivíduos acima de 30 anos em ambos os grupos; os homens expostos apresentaram maior capacidade antioxidante que as mulheres expostas; e tanto aumento de MDA como diminuição de FRAP foram detectados em profissionais com maior tempo (> 9 anos) de exposição aos RGA. Não houve diferença significativa entre os grupos para expressão de ambos os genes avaliados. Nesse contexto, de acordo com a ocupação de trabalho, anestesiologistas apresentaram maior tempo médio de exposição (semanal/anual) aos RGA comparados aos cirurgiões/técnicos, resultando em quadro de estresse oxidativo, incluindo menor expressão de *SOD2*. Quanto aos polimorfismos avaliados, todos estavam em equilíbrio de Hardy-Weinberg e houve pouca influência nos marcadores analisados, exceto para os indivíduos com genótipo *GSTP1* (rs1695) AG/GG que apresentaram maior

valor de FRAP tanto no grupo exposto quanto no grupo controle comparados com o homozigoto selvagem; esse polimorfismo e *GSTT1* nulo foram associados a maior capacidade antioxidante em homens expostos. Observou-se aumento de expressão de *OGG1* em indivíduos genotipados como *OGG1* -/Cys (rs1052133) comparados àqueles genotipados com Ser/Ser somente no grupo controle, e diminuição da expressão desse gene em anestesiologistas -/Cys comparados aos cirurgiões/técnicos com o mesmo genótipo. Indivíduos *ATM* TT (rs600931) apresentaram maior FRAP comparados ao *ATM* CT, somente no grupo controle. Entre os expostos, os anestesiologistas *ATM* CC apresentaram maior frequência de MN enquanto os genotipados CC/CT, maior frequência de BN. Cirurgiões e técnicos *OGG1* -/Cys apresentaram aumento de MN em comparação aos indivíduos homozigotos; quanto ao FRAP, maiores valores foram observados para os *XRCC1* -/Gln (rs25487) e *ATM* TT. Concluindo, achados de instabilidade genética, estresse oxidativo e modulação da expressão gênica foram observados neste estudo, especialmente em relação às variáveis idade, sexo e tempo de exposição/tipo de ocupação; somente alguns polimorfismos parecem ter influenciado nos marcadores analisados. Ressalte-se a necessidade de minimizar a exposição ocupacional aos RGA para reduzir possíveis impactos à saúde dos profissionais.

Palavras-chave: exposição ocupacional; mutagenicidade; estresse oxidativo; expressão gênica; polimorfismos genéticos.

Abstract

Silva MAP. **Role of polymorphisms, genetic instability, oxidative response and gene expression facing occupational exposure to anesthetics [Master]**. Botucatu: Medical School, São Paulo State University - UNESP; 2021. 72 p.

Professionals who work in operating rooms (ORs) may be daily exposed to waste anesthetic gases (WAG) due to use of inhalational anesthetics. For the first time, this observational study monitored the anesthetic pollution of the WAG sevoflurane, isoflurane and nitrous oxide, evaluated buccal genetic instability (micronucleus-MN and nuclear bud-NBUD) and oxidative stress biomarkers through lipoperoxidation (malondialdehyde-MDA) and plasma antioxidant capacity (FRAP), genetic susceptibility by polymorphisms in genes of phase I and II metabolism (*CYP2E1*, *GSTP1*, *GSTM1* and *GSTT1*), genetic damage/repair (*OGG1*, *XRCC1* and *ATM*) and antioxidant response (*SOD2*) as well as *OGG1* and *SOD2* expressions. High pollution of WAG was observed in ORs, which did not have adequate scavenging systems. The exposed group, consisting of 100 professionals, showed greater genetic instability (increased MN and NBUD frequencies) but not oxidative stress, when compared to 93 volunteers (control group). Increased MN and NBUD frequencies were detected in exposed individuals aged over 30 years in relation to the same age in the control group; exposed females showed increased genetic instability than unexposed females; and professionals with greater weekly WAG exposure (> 30 h/week) exhibited increased formation of MN in relation to individuals with a shorter weekly WAG exposure. Increased MDA levels were observed in individuals over 30 years of age in both groups; exposed males had greater antioxidant capacity than exposed females; and oxidative stress was detected in professionals with greater years of exposure (> 9 years) to WAG. There was no significant difference between the groups for the expression of both genes evaluated. In this context, according to job occupation, anesthesiologists had greater duration of WAG exposure than surgeons/technicians, resulting in oxidative stress, including decreased *SOD2* expression. Regarding assessed polymorphisms, all of them were in Hardy-Weinberg equilibrium, and there was little influence on the analyzed markers, except for individuals from exposed and control groups carrying *GSTP1* (rs1695) AG/GG genotype, who exhibited higher FRAP values than the wild-type homozygote; this polymorphism and null *GSTT1* were associated with greater antioxidant capacity in exposed males. Increased *OGG1*

expression was observed among *OGG1* -/Cys (rs1052133) individuals compared to Ser/Ser only in the control group and decreased expression in -/Cys anesthesiologists compared to surgeons/technicians of the same genotype. *ATM* TT (rs600931) subjects showed higher FRAP values compared to *ATM* CT only in the control group. Anesthesiologists genotyped as *ATM* CC had higher MN frequency while those genotyped as CC/CT exhibited increased NBUD. Surgeons and technicians *OGG1* -/Cys showed increased MN compared to homozygous individuals; for FRAP, higher values were observed for *XRCC1* -/Gln (rs25487) and *ATM* TT. In conclusion, findings of genetic instability, oxidative stress and modulation of gene expression were observed in this study, especially in relation to the variables age, sex and time of exposure/type of occupation; only a few polymorphisms seem to have influenced the analyzed markers. The findings highlight the need to minimize WAG exposure to reduce the impacts observed on the healthcare workers.

Keywords: occupational exposure; mutagenicity; oxidative stress; gene expression; genetic polymorphisms.

Manuscrito 1

Evaluation of genetic instability, oxidative stress and metabolism gene polymorphisms in workers exposed to waste anesthetic gases*

Abstract

This cross-sectional study is the first to evaluate biomarkers of genetic instability, oxidative stress, and genetic susceptibility in professionals occupationally exposed to high trace amounts of halogenated (≥ 7 ppm) and nitrous oxide (165 ppm) anesthetics in operating rooms and in individuals not exposed to waste anesthetic gases - WAGs (control group). Elevated rates of buccal micronucleus (MN) and nuclear bud (NBUD) were found in the exposure group and in professionals exposed for more than 30 years. Exposed males showed higher antioxidant capacity (FRAP) than exposed females; exposed females had higher frequencies of MN and NBUD than nonexposed females. Genetic instability (MN) was observed in professionals with greater weekly WAG exposure, and those exposed for longer duration (> 9 years) exhibited oxidative stress (increased lipid peroxidation and decreased FRAP). No influence of the metabolism gene polymorphisms (*CYP2E1* and *GSTs*) were observed, except for *GSTP1* (rs1695) AG/GG on FRAP (both groups), and *GSTP1* AG/GG and *GSTT1* null, which were associated with greater FRAP values in exposed males. Minimizing WAG exposure is necessary to reduce impacts on healthcare workers.

Keywords: occupational exposure; anesthetic gases; operating room; micronucleus assay; oxidative stress; genetic polymorphisms

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1. Introduction

The most commonly inhalational anesthetics used for general anesthesia include halogenated sevoflurane, isoflurane and desflurane, together with nitrous oxide (N_2O) gas. One important consequence of inhalational anesthesia is that operating room (OR) professionals can be exposed daily to waste anesthetic gases (WAGs) during a surgical procedure or postanesthesia recovery (NIOSH 2007). Such exposure can be worsened if professionals work in inadequate scavenged ORs, where high concentrations of WAGs are found, as demonstrated in previous studies (Braz et al. 2017; Figueiredo et al. 2021), that exceed the international recommendation threshold of up to 2 parts per million (ppm) for halogenated anesthetics (NIOSH 1992).

Human biomonitoring is an important tool for evaluating toxicological effects during/after exposure to chemicals (Esteban and Cataño 2009). However, controversial findings associating exposure to WAGs with DNA damage and oxidative stress (Malekirad et al. 2005; Wrońska-Nofer et al. 2012; Souza et al. 2016, 2021; Szyfter et al. 2016; Aun et al. 2018; Çakmak et al. 2019; Braz et al. 2020a; Hua et al. 2021) are found in the literature. Overall, controversial results related to genetic and oxidative damage can be caused by methodological differences, such as the population studied, type of anesthetic(s) and exposure time, whether professionals work in unscavenged or scavenged ORs, WAG exposure and concentration levels, tools used to evaluate oxidative markers and genetic instability, sample size, and also the genetic susceptibility of the population studied. Indeed, according to Kargar Shouroki et al. (2019), genetic polymorphisms can modulate the effect of WAG exposure on genetic lesions.

Despite having low biotransformation rates, isoflurane can be oxidized to inorganic trifluoroacetic acid and fluoride, and sevoflurane to inorganic fluoride and hexafluoroisopropanol, both by the phase I enzyme CYP2E1 (Torri 2010). Glutathione S-transferases (GSTs) are important enzymes of the phase II detoxification process owing to their catalytic action in conjugating reduced glutathione to electrophilic substrates and in helping to eliminate free radicals (Hayes et al. 2005; Board and Menon 2013). It is known that interindividual variability can affect the xenobiotic metabolism rate (Giacomini et al. 2007), with a large portion being attributed to the effects of polymorphisms.

Substitution of guanine (G) to cytosine (C) before the beginning of the transcription fragment (position 1293) in *CYP2E1* corresponds to a single nucleotide polymorphism (SNP), and the consequence of this change may vary according to the influence of the encoded enzyme on xenobiotic metabolism and detoxification (Neafsey et al. 2009). However, no study has yet

investigated this polymorphism with regard to occupational exposure to WAGs. In addition, exchange of adenine (A) to guanine (G) at position 313 of *GSTP1* results in substitution of amino acid isoleucine (Ile), expressed by the wild-type allele, for valine (Val), expressed by an alternative allele, which alters α-GST activity (Dusinská et al. 2001). Moreover, a reduction in metabolic activity due to polymorphism deletion of *GSTM1* and *GSTT1* has been reported (Neghab et al. 2018). Only a few recent studies have investigated some *GSTs* polymorphisms with regard to DNA damage (different endpoints) in professionals who work in European and Asian ORs (Santovito et al. 2015; Kargar Shouroki et al. 2019; Khisroon et al. 2020). Focusing on occupational exposure to WAGs, the role of the *CYP2E1* SNP was assessed for the first time in the current study; aiming to contribute to knowledge in this field, we evaluated important metabolic gene polymorphisms related to both phase I and II detoxification.

Hypotheses about possible mechanisms of the genotoxicity of halogenated anesthetics include interaction with DNA and/or oxidative metabolites (Jaloszyński et al. 1999). Alterations in the structure or function of metabolism/detoxification genes or polymorphisms may change the ability of a cell to protect against the harmful effects of oxidative stress (Yin et al. 2000). This imbalance between reactive oxygen species (ROS) and the body's antioxidant capacity can be detected by malondialdehyde (MDA), a gold-standard lipid peroxidation marker, and ferric reducing antioxidant power (FRAP). Additionally, cytogenetic damage markers, including micronucleus (MN) and nuclear bud (NBUD), can be detected in oral exfoliated cells, which comprise the first barrier to inhalational anesthetics; indeed, the buccal micronucleus cytome (BMCyt) assay has advantages of minimally invasive biological collection and not cell culture required (Bolognesi and Fenech 2019). Together, these markers can help in biomonitoring and understanding the possible link between occupational exposure to WAGs, oxidative status and genetic instability.

Considering the importance and relevance of genetic toxicology in exposed professionals, the novelty of our study lies in our assessment of two important genetic endpoints analyzed in buccal cells (target cell approach) and two markers of oxidative stress (oxidant and antioxidant markers) evaluated in plasma and the possible modulation of genetic susceptibility, as assessed by *CYP2E1*, *GSTP1, GSTM1* and *GSTT1* polymorphisms, in professionals occupationally exposed to WAGs. WAG exposure was also assessed, and associations of factors such as sex, age and time of exposure with biomarkers were evaluated.

2. Methods

2.1 Subjects

In total 193 subjects were involved in this cross-sectional study, including OR personnel who worked at two large public hospitals in southeastern Brazil mainly exposed to halogenated isoflurane and sevoflurane and to a lesser degree to N_2O for at least one year (exposed group); matched individuals who were not occupationally exposed to WAGs were included as the control group. The Human Research Ethics Committee of the Botucatu Medical School-UNESP, Brazil (30055120.7.0000.5411) approved the study before starting enrollment. All the participants signed an informed consent form and answered a detailed questionnaire regarding demographic and anthropometric data (age, sex, body mass index, etc.), medical history (vaccinations, medications, diseases, surgeries, etc.), lifestyle (physical exercise, alcohol and tobacco consumption, diet, etc.), and occupational exposure to WAGs.

To avoid possible confounding factors, individuals with infectious or malignant diseases, who were pregnant, used illicit drugs, took medications or antioxidant pills, were heavy drinkers (above nine doses/week for women and above 14 doses/week for men) and were recently exposed to anesthetic (for the control group) and/or X-ray for exams/surgery/dental procedures (for both groups) were not included in the current study.

2.2 Monitoring of WAG in the workplace

WAG exposure was assessed by OR air measurement. The assessed ORs where the professionals worked contained inadequate scavenging and ventilation systems (0-7 air changes/h). A real-time infrared spectrophotometer (InfraRan Four Gas Anesthetic Analyzer, Wilks, USA) was used to determine the concentrations of all WAGs monitored near the professionals' breathing area during surgeries under general inhalational anesthesia. The equipment was previously calibrated, and it was zeroed before each measurement, as recommended in the instruction manual. The concentrations were calculated, and the results are expressed as ppm (as time-weighted average - TWA for N_2O).

In all ORs, most of the anesthetic equipment used was modern (Fabius Plus and Fabius GS Premium, Dräger, Germany), and low to medium fresh gas flow (FGF) was employed during the maintenance of anesthesia, which contributed to less leakage compared to older equipment and high FGF. No individual protective equipment for inhalational anesthetics was used other than a surgical mask.

2.3 Biological sampling

Blood and buccal cell samples were concomitantly obtained from both groups during the same period (morning) to avoid possible bias. Before collecting oral cells, the participants performed three vigorous mouth rinses with water. Cells were obtained from both sides of the oral cavity (cheek) using a cytobrush, which was then placed in a tube containing cold phosphate-buffered saline. Blood and buccal samples were coded and immediately processed under yellow light.

Peripheral blood samples were collected by venipuncture into tubes containing ethylenediamine tetraacetic acid as an anticoagulant. Part of the blood samples was centrifuged at 4°C for 15 min at 3,500 rpm for the separation of plasma, and small aliquots were prepared. These aliquots and blood cells were frozen (-80°C) until oxidative stress marker evaluations and DNA extraction, respectively.

All experiments were performed in duplicate, with repetition for 10% of samples for genotyping. Samples from the control and exposure groups were always mixed in each batch of assays to avoid bias and were analyzed in a blinded manner.

2.4 Buccal micronuclei and nuclear buds (genetic instability markers)

The BMCyt assay was performed according to a previous protocol (Thomas et al. 2009), with slight modifications. Briefly, a Falcon tube containing fresh oral cells was vortexed and centrifuged, and the cells were fixed with 5 ml of methanol and acetic acid (3:1), followed by centrifugation and repetition of these steps two more times. Three drops of concentrated cells were delivered onto clean slides from 15 cm height, allowing the material to spread over the surface, and left to air-dry overnight. The slides were immersed in preheated hydrochloric acid (1 M) for cell hydrolysis and washed. The slides were stained using the Feulgen-Fast Green method, initially immersed in Feulgen (Schiff's reagent), left in the dark, immersed in distilled water, air-dried, and counterstained with cold Fast Green. After drying, the slides were covered with coverslips and analyzed at 1.000 x magnification under bright field-light microscopy (Olympus BX43, Japan). Using the criteria described by Thomas et al. (2009) for identifying MN and NBUD, 2,000 normal differentiated cells were scored per subject. Both parameters are expressed per thousand (‰).

2.5 Assessment of lipid peroxidation and antioxidant capacity (oxidative stress status)

The oxidative marker MDA was detected by high-performance liquid chromatography (HPLC) following the procedures described by Volpi and Tarugi (1998), with some

modifications. Briefly, plasma was mixed with 1% butylhydroxytoluene and 10 N sodium hydroxide, and the solution was incubated. Trichloroacetic acid (7.2%) was added, and the tubes were cooled and centrifuged. The supernatant was incubated with 0.6% 2-thiobarbituric acid and 10 µl 0.031 M iron chloride; the mixture was heated and then cooled and centrifuged. The supernatant was filtered and injected into an Agilent HPLC system consisting of a C18 column (5 µm, 150 × 4.6 mm) with a fluorescence detector. MDA was quantified based on standard curves constructed with known concentrations of 1,1,3,3-tetramethoxypropane, and the results are expressed as µM.

The FRAP assay was used to determine the reducing ability of plasma in a redox-linked colorimetric reaction using the method of Benzie and Strain (1996) with slight modifications (Berryman et al. 2013; Nogueira et al. 2018). This assay was used to measure total antioxidant capacity, because it is responsive to lipophilic and hydrophilic antioxidants. Briefly, plasma was incubated with FRAP cocktail solution (300 mM acetate buffer, 10 mM 2,4,6-tripyridyl-S-triazine in 40 mM hydrochloric acid and 20 mM iron chloride) at room temperature for 1 h, and the absorbance at 593 nm was recorded (Shimadzu UV-1800 spectrophotometer; Japan). Trolox was used as a reference to construct a standard curve to calculate the FRAP value of the samples. FRAP is expressed as µmol/l Trolox equivalents (TE).

2.6 Genotyping analysis

Genomic DNA was extracted from blood samples using the Illustra Blood GenomicPrep Mini Spin kit (GE Healthcare Life Sciences, USA) according to the manufacturer's instructions. The purity and concentration were assessed using a NanoDrop One spectrophotometer. *CYP2E1* (rs3813867, G>C) and *GSTP1* (rs1695, A>G; Ile/Val) polymorphisms were genotyped using TaqMan® SNP Genotyping Assays (Thermo Fisher, USA, ABI Assay C___2431875_10 and ABI Assay C___3237198_20, respectively) performed by real-time quantitative polymerase chain reaction (qPCR) using an ABI Prism 7500 FAST (Applied Biosystems, USA) following the manufacturer's guidelines.

GSTM1 and *GSTT1* polymorphism deletions were detected by PCR using *β-globin* as an internal control. The primers used were: *GSTM1*: F: 5-GAACTCCCTGAAAAGCTAAAGC-3 and R: 5-GGGCTCAAATATACGGTGG; *GSTT1*: F: 5-TTCCTTACTGGTCCTCACATCTC - 3 and R: 5 -TCACCGGATCATGGCCAGCA-3; *β-globin*: F: 5-CAACTTCATCCACGTTCACC-3 and R:5-GAAGAGCCAAGGACAGGTAC-3, all manufactured by Integrated DNA Technologies (IDT™, USA). Briefly, the reaction mixture of PCR consisted of 200 ng DNA, 2.5 µl PCR Buffer, 1.5 mM magnesium chloride, 0.5 mM of

each dNTP, 0.3 μM forward primer and 0.3 μM reverse primer for each target gene and endogenous gene and 2 U Platinum Taq DNA polymerase. The thermal cycler (MJ Research PTC-200 PCR Gradient DNA Engine Thermal Cycler, USA) conditions were as follows: *GSTM1*, 94°C for 10 min followed by 35 cycles and 94°C for 60 s for denaturation, annealing at 61°C for 60 s and extension at 72°C for 60 s followed by final extension at 72°C for 7 min; *GSTT1*, initial denaturation at 94°C for 10 min followed by 35 cycles consisting of denaturation at 94°C for 60 s, annealing at 60°C for 75 s and extension at 72°C for 60 s, followed by final extension at 72°C for 7 min (Singh et al. 2009). The amplified products were analyzed by 2.5% agarose gel electrophoresis. Genotypes were identified as present (+) or null (-) depending on the presence of at least one functional allele; homozygosity or heterozygosity could not be distinguished. The presence of a 219-bp band was indicative of *GSTM1* +, and the absence of this band indicated *GSTM1* -. The presence of 459-bp band was indicative of *GSTT1* +; and the absence of this band indicated *GSTT1* -. The endogenous control, producing a 268-bp band, was observed in all samples. A negative PCR control was also performed to assess probe contamination.

2.7 Statistical analyses

The sample size was determined using percentages provided in the literature (Kargar Shouroki et al. 2019) considering the variables MN and polymorphism (*GSTs*) with 80% test power, with at least 90 subjects per group.

For quantitative variables, a normality test (Shapiro-Wilk and Kolmogorov-Smirnov) was performed to verify the distribution of data. Student's t-test was applied to compare both groups in relation to age and body mass index. A generalized linear model with gamma distribution was adjusted for variables that presented an asymmetric distribution, as well as Poisson or negative binomial distribution for countable variables followed by multiple comparisons. Variables that presented a symmetrical distribution were fitted with a generalized linear model with a normal distribution, followed by multiple comparisons. These tests were performed for the following biomarkers: MN, NBUD, MDA and FRAP. Comparisons between frequencies for the variables sex, social alcohol intake, smoking status and physical activity were performed by the chi-square test. In addition, the aforementioned test was employed to compare allelic and genotypic frequencies and to evaluate Hardy-Weinberg equilibrium to check for differences between categories. The SAS program, version 9.4, was utilized to carry out the analyzes, and the figures were created in GraphPad Prism 9. Significance was considered if $p<0.05$.

3. Results

The characteristics of the studied population are presented in Table 1. No significant differences were observed between the groups. Professionals who worked in ORs had a mean exposure to WAGs of nine years. OR air monitoring concentrations were: 7 ± 5 ppm for isoflurane, 9 ± 7 ppm for sevoflurane and 165 ± 15 ppm for N_2O (TWA).

The results for genetic instability and oxidative stress markers are shown in Fig. 1. Increased frequency of buccal MN and NBUD was observed in the exposure group compared to the control group ($p<0.05$); there were no significant differences regarding MDA and FRAP ($p>0.05$).

Table 2 presents comparisons within and between groups regarding genetic instability and oxidative stress markers in relation to age, sex and time of exposure. The cutoff used for the variables age, weekly exposure and years of exposure was determined using values close to the mean: 30 years (age), 30 hours per week and nine years, respectively (Table 2). Higher MDA values were observed in individuals older than 30 years both in the control and in the exposure group. Moreover, higher frequencies of genetic instability markers (MN and NBUD) were detected in exposed professionals aged more than 30 years in comparison to the same age in the control group. Exposed females showed higher MN and NBUD frequencies than nonexposed females, and exposed males presented greater antioxidant capacity (FRAP) than exposed females. Regarding exposure to WAGs, professionals who spent more than 30 hours/week showed increased MN formation in relation to individuals with shorter weekly exposure. In addition, oxidative stress (increased MDA and decreased FRAP) was observed in professionals exposed for more than nine years compared to those with fewer years of exposure.

Table 3 summarizes the genotype and allele frequencies of the polymorphisms studied, which were similar in the control and exposed groups. The distribution frequencies of the genotyped polymorphisms were in agreement with Hardy-Weinberg equilibrium.

The effects of polymorphisms of phase I and II- metabolism genes at the level of genetic instability and oxidative stress markers evaluated in the nonexposed and exposed groups are shown in Table 4. Most results did not exhibit an influence of *CYP2E1*, *GSTP1*, *GSTM1* and *GSTT1* polymorphisms on the studied biomarkers, except for subjects who carried the *GSTP1* G allele (AG - Ile/Val or GG - Val/Val), with increased FRAP values in both groups ($p<0.05$). Although MN, NBUD and MDA values were slightly higher in exposed individuals carrying the *GSTP1* G allele, no statistical significance was found in relation to those harboring the *GSTP1* A allele. In addition, the evaluation of combined genotypes revealed no influence of genetic instability or oxidative stress markers (data not shown).

Considering FRAP values, analyses were performed according to sex and genotype/group, and only significant results are provided in Table 5. In relation to the *GSTP1* G allele (AG or GG), control females had higher FRAP values than those carrying *GSTP1* AA and exposed females harboring *GSTP1* G allele (AG or GG). Exposed males carrying the *GSTP1* G allele (AG or GG) showed higher FRAP values than those carrying the *GSTP1* AA and control males carrying the *GSTP1* G allele (AG or GG). Additionally, exposed males with *GSTT1* null presented higher FRAP values than *GSTT1* + males as well as higher values than control males with *GSTT1* null.

Table 1 - Characteristics of the studied population

Parameters	Control group (n=93)	Exposed group (n=100)	p value
Age (years) [a]	33.7 ± 12.4	34.2 ± 11.8	0.75
Sex (female/male) [b]	45 (48.4%)/48 (51.6%)	45 (45%)/55 (55%)	0.58
Body mass index (kg/m²) [a]	25.7 ± 4.6	25.5 ± 4.3	0.75
Social alcohol intake [b]	58 (62.4%)	70 (70%)	0.33
Current smoking [b] **(≤ 1 pack/week)**	5 (5.4%)	8 (8%)	0.66
Physical activity [b] **(aerobic/anaerobic)**	48 (52%)	65 (65%)	0.08

Data are expressed as the mean ± standard deviation or absolute number and percentage.
[a] Statistical analyzes were determined by Student's t-test
[b] Statistical analyzes were determined by chi-square test.

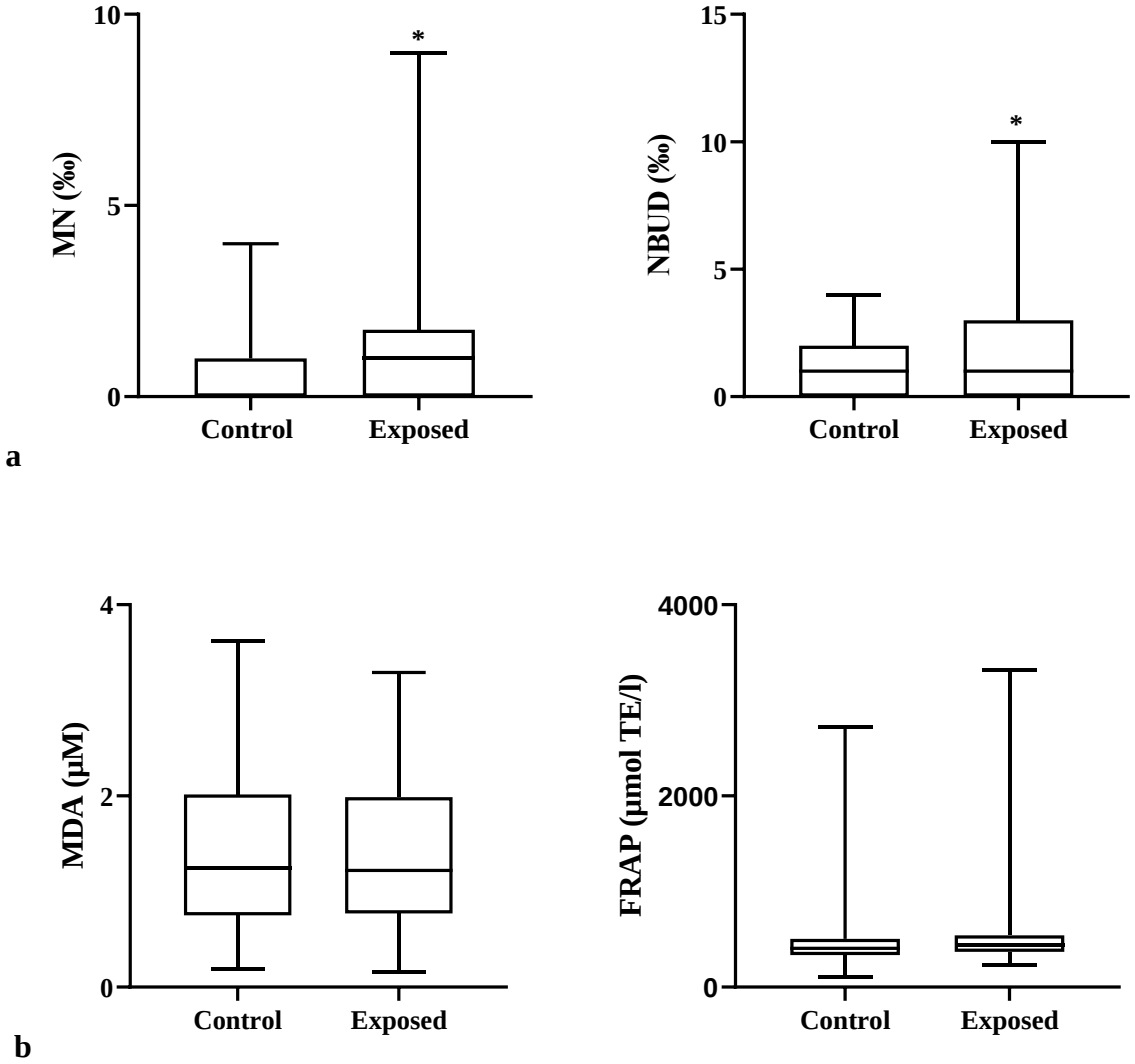

Fig. 1 a Buccal micronucleus (MN) and nuclear bud (NBUD) frequencies were significantly different between the groups (p=0.01 and p=0.007, respectively). **b** Evaluation of malondialdehyde (MDA) and ferric reducing antioxidant power (FRAP); there was no significant difference between the groups (p>0.05). Statistical analyzes were determined by generalized linear model with Poisson distribution for MN, negative binomial for NBUD, normal distribution for MDA and gamma distribution for FRAP. Data are presented as boxplots with maximum-minimum values.

Table 2 - Genetic instability and oxidative stress markers evaluated within and between groups in relation to sex, age and time of exposure

Parameters	MN (‰)	NBUD (‰)	MDA (µM)	FRAP (µmol TE/l)
Control group				
Age ≤ 30 years (n=58)	0.76 ± 1.06	1.38 ± 1.46	1.04 ± 0.63	499.79 ± 385.20
Age > 30 years (n=35)	0.49 ± 0.92	0.86 ± 1.06	1.96 ± 0.65 [a]	432.77 ± 136.06
Female (n=45)	0.62 ± 0.96	1.33 ± 1.14	1.33 ± 0.77	475.84 ± 413.53
Male (n=48)	0.69 ± 1.07	1.04 ± 1.27	1.44 ± 0.79	473.38 ± 187.98
Exposed group				
Age ≤ 30 years (n=59)	1.15 ± 1.47	2.08 ± 2.67	1.27 ± 0.81	554.51 ± 446.69
Age > 30 years (n=41)	1.02 ± 1.37 [b]	1.80 ± 2.17 [c]	1.61 ± 0.76 [d]	487.71 ± 379.61
Female (n=45)	1.36 ± 1.17 [e]	2.22 ± 2.51 [f]	1.41 ± 0.82	411.96 ± 140.01
Male (n=55)	0.89 ± 1.12	1.76 ± 2.43	1.41 ± 0.79	621.35 ± 535.71 [g]
Weekly exposure (≤ 30 h/week) (n=49)	0.84 ± 1.14	1.55 ± 2.20	1.40 ± 0.78	573.12 ± 530.78
Weekly exposure (> 30 h/week) (n=51)	1.35 ± 1.62 [h]	2.37 ± 2.66	1.42 ± 0.83	482.92 ± 272.71
Duration of exposure (≤ 9 years) (n=73)	1.15 ± 1.39	1.97 ± 2.51	1.22 ± 0.78	560.00 ± 481.74
Duration of exposure (> 9 years) (n=27)	0.96 ± 1.53	1.96 ± 2.39	1.91 ± 0.64 [i]	438.22 ± 130.53 [j]

MN: buccal micronucleus, NBUD: buccal nuclear bud, MDA: malondialdehyde, FRAP: ferric reducing ability of plasma; data are expressed as the mean ± standard deviation. Statistical analyzes were determined by generalized linear model.

[a] in relation to individuals ≤ 30 years from the control group (p<.0001)
[b] compared to individuals > 30 years from the control group (p=0.04)
[c] compared to individuals > 30 years from the control group (p=0.02)
[d] in relation to individuals ≤ 30 years from the exposed group (p=0.03)
[e] compared to control females (p=0.0006)
[f] compared to control females (p=0.04)
[g] in relation to exposed females (p<.0001)
[h] in relation to professionals exposed to ≤ 30 h/week (p=0.04)
[i] in relation to professionals exposed to ≤ 9 years (p<.0001)
[j] in relation to professionals exposed to ≤ 9 years (p=0.04)

Table 3 - Frequency distribution of *CYP2E1, GSTP1*, *GSTT1* and *GSTM1* genotypes and alleles

Genotype	Control group (n=93)	Exposed group (n=100)	Total (n=193)	p value
	n (%)	n (%)	n (%)	
CYP2E1				
GG	86 (92.47)	90 (90.00)	176 (91.19)	0.54
GC	7 (7.53)	10 (10.00)	17 (8.81)	
Allele				
G	93 (93.00)	100 (90.91)	193 (91.90)	0.58
C	7 (7.00)	10 (9.09)	17 (8.10)	
GSTP1				
AA (Ile/Ile)	40 (43.01)	54 (54.00)	94 (48.70)	0.23
AG (Ile/Val)	39 (41.94)	37 (37.00)	76 (39.38)	
GG (Val/Val)	14 (15.05)	9 (9.00)	23 (11.92)	
Allele				
A	79 (59.85)	91 (66.42)	170 (63.20)	0.26
G	53 (40.15)	46 (33.58)	99 (36.80)	
GSTM1				
+	32 (34.41)	36 (36.00)	68 (35.23)	0.82
-	61 (65.59)	64 (64.00)	125 (64.77)	
GSTT1				
+	75 (80.65)	78 (78.00)	153 (79.27)	0.65
-	18 (19.35)	22 (22.00)	40 (20.73)	

n: number of individuals. Statistical analyzes were determined by chi-square test.

Table 4 - Effect of individual detoxifying gene polymorphisms on genetic instability and oxidative stress biomarkers

Biomarkers	Genotypes	Control group (n)	Exposed group (n)
Buccal MN (‰)	*CYP2E1* GG	0.59 ± 0.97 (7)	1.11 ± 1.43 (10)
	CYP2E1 GC	1.00 ±1.15 (86)	1.00 ± 1.41 (90)
	p value	0.46	0.80
	GSTP1 AA (Ile/Ile)	0.58 ± 1.02 (40)	1.07 ± 1.59 (54)
	GSTP1 AG *(*Ile/Val) or GG (Val/Val)	0.70 ± 1.01 (53)	1.13 ± 1.22 (46)
	p value	0.66	0.83
	GSTM1 +	0.85 ± 1.13 (32)	1.22 ± 1.64 (36)
	GSTM1 -	0.53 ± 0.92 (61)	1.03 ± 1.30 (64)
	p value	0.16	0.49
	GSTT1 +	0.66 ± 0.99 (75)	1.04 ± 1.22 (78)
	GSTT1 -	1.22 ± 1.35 (18)	1.73 ± 2.14 (22)
	p value	0.32	0.39
Buccal NBUD (‰)	*CYP2E1* GG	1.16 ± 1.35 (7)	2.04 ± 2.54 (10)
	CYP2E1 GC	1.57 ± 1.27 (86)	1.30 ± 1.57 (90)
	p value	0.50	0.35
	*GSTP1 AA (*Ile/Ile)	1.07 ± 1.17 (40)	1.72 ± 2.14 (54)
	*GSTP1 AG (*Ile/Val) or GG (Val/Val)	1.26 ± 1.46 (53)	2.26 ± 2.80 (46)
	p value	0.54	0.32
	GSTM1 +	0.94 ± 1.30 (32)	2.00 ± 2.15 (36)
	GSTM1 -	1.27 ± 1.34 (61)	1.95 ± 2.64 (64)
	p value	0.30	0.93
	GSTT1 +	1.18 ± 1.34 (75)	2.04 ± 2.56 (78)

	GSTT1 -	1.22 ± 1.35 (18)	1.73 ± 2.14 (22)
	p value	0.90	0.62
MDA (µM)	*CYP2E1* GG	1.38 ± 0.79 (7)	1.38 ± 0.78 (10)
	CYP2E1 GC	1.48 ± 0.63 (86)	1.71 ± 0.99 (90)
	p value	0.74	0.20
	*GSTP1 AA (*Ile/Ile)	1.54 ± 0.84 (40)	1.37 ± 0.77 (54)
	*GSTP1 AG (*Ile/Val) or GG (Val/Val)	1.26 ± 0.71 (53)	1.46 ± 0.84 (46)
	p value	0.10	0.60
	GSTM1 +	1.24 ± 0.73 (32)	1.39 ± 0.84 (36)
	GSTM1 -	1.46 ± 0.80 (61)	1.42 ± 0.79 (64)
	p value	0.40	0.87
	GSTT1 +	1.43 ± 0.79 (75)	1.43 ± 0.81 (78)
	GSTT1 -	1.19 ± 0.71 (18)	1.33 ± 0.79 (22)
	p value	0.24	0.57
FRAP (µmol TE/l)	*CYP2E1* GG	465.72 ± 302.60 (7)	536.08 ± 439.82 (10)
	CYP2E1 GC	580.96 ± 468.45 (86)	444.55 ± 130.36 (90)
	p value	0.20	0.28
	*GSTP1 AA (*Ile/Ile)	404.64 ± 139.96 (40)	457.31 ± 134.85 (54)
	*GSTP1 AG (*Ile/Val) or GG (Val/Val)	527.05 ± 394.19 (53)	608.66 ± 594.64 (46)
	p value	**0.005**	**0.003**
	GSTM1 +	513.25 ± 288.45 (32)	566.84 ± 496.15 (36)
	GSTM1 -	454.01 ± 329.98 (61)	504.48 ± 372.45 (64)
	p value	0.23	0.24
	GSTT1 +	477.81 ± 343.93 (75)	508.77 ± 342.63 (78)
	GSTT1 -	460.16 ± 158.67 (18)	591.30 ± 628.24 (22)

p value	0.77	0.18

MN: buccal micronucleus, NBUD: buccal nuclear bud, MDA: malondialdehyde, FRAP: ferric reducing ability of plasma; data are expressed as the mean ± standard deviation. Statistical analyzes were determined by generalized linear model.

Table 5 - Effects of detoxifying gene polymorphisms according to sex and exposure on antioxidant capacity (FRAP) values (only statistically significant results are included)

Sex and genotypes	**FRAP (µmol TE/l)**		**p value**
	Control Group	**Exposed group**	
Female			
GSTP1			
AA (Ile/Ile)	382 ± 128 (n=20)	418 ± 106 (n=27)	0.50
AG (Ile/Val) or GG (Val/Val)	551 ± 536 (n=25)	403 ± 183 (n=18)	**0.03**
p value	**0.007**	0.80	
Male			
GSTP1			
AA (Ile/Ile)	427 ± 151 (n=20)	497 ± 150 (n=27)	0.25
AG (Ile/Val) or GG (Val/Val)	506 ± 207 (n=28)	741 ± 723 (n=28)	**0.0019**
p value	0.20	**0.0008**	
GSTT1			
+	468 ± 195 (n=37)	576 ± 422 (n=45)	0.06
-	490 ± 169 (n=11)	827 ± 892 (n=10)	**0.01**
p value	0.77	**0.02**	

Data are expressed as the mean ± standard deviation. Statistical analyzes were determined by generalized linear model with gamma distribution.

4. Discussion

Our study reports cytogenetic damage evaluated in oral exfoliated cells in professionals highly exposed to anesthetic pollution in the workplace. In the exposure group, greater weekly exposure to WAGs was associated with genetic instability (increased buccal MN), and greater years of exposure was associated with oxidative stress (increased lipid peroxidation and decreased antioxidant capacity). Additionally, factors such as age and sex influenced some of the biomarkers evaluated, though little influence of the polymorphisms of phase I and phase II-metabolism genes was observed on biomarkers. Among the studied polymorphisms, exposed males (*GSTP1* heterozygous Ile/Val or alternative homozygous Val/Val and *GSTT1* null) seemed to be the most susceptible to variations in antioxidant capacity.

We observed WAG values (both halogenated anesthetics and N_2O) far above the exposure limits recommended by international regulations (US NIOSH 1992; ACGIH 2019). The presence and efficiency of the scavenging system is an important factor to reduce anesthetic pollution in ORs and consequently into the environment, as WAGs are potent greenhouse gases and ozone-depleting agents (Varughese and Raza 2021). Modern central scavenging systems in ORs consist of a laminar flow system with at least 15 air changes/h without air recirculation (NIOSH 2007). However, considering that the assessed ORs contained inadequate scavenging and ventilation systems (0 to 7 air changes/h), this factor can certainly explain the high anesthetic pollution found in the current study.

Our findings showed genetic instability by two endpoints (MN and NBUD) in the exposure group when compared to the matched-control group. MN is a small round corpuscle containing chromatin present in the cytoplasm of cells, and NBUD is a corpuscle connected to the nucleus resulting from the elimination of amplified genes and repair complexes; both can occur due to a decrease or inefficiency in the ability to repair DNA damage (Fenech 2020). Interestingly, Wiesner et al. (2001) observed an increase in MN in lymphocytes in professionals exposed to high anesthetic pollution, but not in those exposed to concentrations within the exposure limit.

The first study carried out in Brazilian workers was performed by Souza et al. (2016), who observed an increased frequency of MN, but not NBUD, in buccal cells of anesthesiologists exposed a high WAGs (isoflurane, sevoflurane, desflurane and N_2O) for a mean of 16 years to when compared to physicians without exposure. Interestingly, a recent study from our research group detected increased MN and NBUD frequencies in the oral mucosa of professionals exposed to high WAG isoflurane in veterinary ORs (unpublished results; submitted). Additionally, in the current study, we were able to detect induction of both

biomarkers of effect (assessed in buccal cells) in exposed professionals older than 30 years compared with unexposed subjects in the same age range. Similarly, another study also found that professionals aged ≥ 40 years exposed to high WAG concentrations in the workplace demonstrated a significant increase in MN frequency when compared to unexposed professionals of the same age (Souza et al. 2016). Furthermore, Khisroon et al. (2020) observed the effect of age on comet assay values in Pakistani professionals exposed to WAGs, with higher genotoxicity values in individuals older than 25 years. It is possible that older individuals have longer exposure to WAG and consequent genotoxicity/genetic instability. Linear regression between individual age and buccal MN frequency was observed in different Brazilian populations (Rohr et al. 2020). In fact, Bonassi et al. (2011) demonstrated that age appears to be the most reliable predictor of buccal MN frequency.

Controversial findings are found regarding sex as a predictor of MN frequency facing WAG exposure (Pasquini et al. 2001; Chandrasekhar et al. 2006). However, similar to our results, some authors have reported a higher frequency of lymphocyte MN in WAG-exposed females (Rozgaj et al. 2001; Araujo et al. 2013). Although the influence of sex has not been demonstrated by the BMCyt assay in individuals with occupational exposure (Bonassi et al. 2011), our data suggest that sex can influence BMCyt biomarkers (MN and NBUD) in relation to occupational exposure to WAGs. Thus, we highlight that MN and NBUD assessed in oral exfoliated cells, which are in direct contact with WAGs and are collected via a less invasive method than lymphocytes, are sensitive and important cytogenetic markers of occupational exposure in OR professionals.

In the assessment of WAG exposure, professionals exposed for more than 30 hours per week had higher frequencies of buccal MN than those exposed for ≤ 30 h/week. We therefore suggest that weekly workload is may be more relevant than years of exposure because weekly workload represents effective exposure to WAGs. Indeed, medical residents in anesthesiology (who spend approximately 60 h/week in OR; high-hours exposure) have a higher MN frequency than unexposed physicians, and the results from medical residents in surgery (18 h/week) are between those of highly exposed and nonexposed groups (Braz et al. 2018). On the other hand, we did not observe years of exposure to be a relevant factor among exposed professionals. Supporting our findings, another Brazilian study did not find a difference between buccal MN frequency in anesthesiologists exposed when a cutoff of a 15-year exposure was applied (Souza et al. 2016). In contrast, increased MN frequency was reported with time of exposure to WAGs only in females (Araujo et al. 2013) and in professionals occupationally exposed for more than

ten years to a mixture of WAGs including old-fashioned and modern halogenated WAGs (Chandrasekhar et al. 2006).

In our study, MDA and FRAP levels did not show significant differences between the control and exposure groups. In line with our findings, two other Brazilian studies (Braz et al. 2020a; Souza et al. 2021) also did not detect changes in oxidative markers/antioxidants in professionals exposed to WAGs. Moreover, despite lipoperoxidation in the exposure group, no change in FRAP parameters was found in the study of Malekirad et al. (2005) of Iranian professionals exposed to nine years of unmeasured concentrations of halothane (an older halogenated anesthetic) and N_2O.

In contrast, other Brazilian studies performed in young physicians with fewer years of exposure to high anesthetic pollution in unscavenged ORs but with an intense weekly workload showed oxidative stress and alteration of antioxidant enzymes (catalase - CAT and glutathione peroxidase - GPX) (Costa Paes et al. 2014; Braz et al. 2020b). Additionally, Neghab et al. (2020) observed a significant reduction in antioxidant capacity and an increase in MDA in Iranian professionals exposed to high anesthetic pollution (isoflurane and N_2O) for 8 hours daily in unscavenged ORs compared to unexposed nurses. Among Chinese professionals, Hua et al. (2021) observed induction of oxidative stress markers and a decrease in plasma antioxidants even in professionals occupationally exposed to low traces of sevoflurane compared to those not exposed to WAGs. The aforementioned studies differ from ours regarding the time of exposure to WAGs, presence or absence of scavenging systems/WAG exposure assessment, and type(s) of anesthetic and methods used, contributing to controversial findings in the literature concerning oxidative stress with occupational exposure to WAGs.

It is postulated that oxidative damage may correlate with the aging process (López-Otín et al. 2013; Luo et al. 2020). In the present research, we observed higher MDA levels in individuals over 30 years of age in both the control and exposure groups. An increase in antioxidant capacity, as based on FRAP levels, in exposed males when compared to exposed females was not found for the control group, may be associated with exposure to WAGs and possible genetic susceptibility. Interestingly, when we considered WAG exposure time, professionals exposed for more than nine years to high anesthetic pollution presented increased MDA and decreased FRAP compared to individuals exposed for fewer years, showing the trend of increasing oxidative stress. However, the association with MDA values needs to be analyzed with caution, as the level of this biomarker significantly increased with age, in both the control and exposed groups. Unfortunately, studies in this field have not analyzed oxidative stress parameters while taking into consideration possible variables, such as sex, age and time of

exposure. Therefore, our study contributes to demonstrating an association between year-WAG exposure with oxidative stress (oxidative and antioxidative markers), and weekly hours of exposure with regard to genetic instability in healthcare personnel who work in ORs with inadequate scavenging-ventilation systems.

This study also contributes to knowledge by evaluating metabolism gene polymorphisms (phases I and II) in relation to buccal MN and NBUD and systemic MDA and FRAP markers in individuals exposed to WAGs. According to Kargar Shouroki et al. (2019), studies on the role of polymorphic *CYP* genes are necessary when investigating exposure to WAGs. Our findings are the first to suggest a lack of influence of *CYP2E1* polymorphism on biomarkers in professionals occupationally exposed to WAGs. However, considering that the frequency of *CYP2E1* heterozygosity was proportionally lower than that of wild type, a conclusion cannot be stated. Indeed, there are a limited number of studies on the effect of *CYP* polymorphism on chromosome damage, and unclear associations between *CYP* genotype and phenotype as well as the rarity of variant alleles complicate their interpretation (Norppa 2004).

Our study reveals no influence of *GSTP1* Ile105Val, *GSTM1* null or *GSTT1* null polymorphisms on cytogenetic damage biomarkers. In line with our findings, Kargar Shouroki et al. (2019) did not observe an influence regarding individual *GSTP1*, *GSTM1* and *GSTT1* genotypes on MN frequency in lymphocytes among 60 OR personnel exposed to a mixture of WAGs. In contrast, a higher MN frequency in exposed individuals carrying the combined genotype of *GSTT1* (−), *GSTM1* (−) and *GSTP1* AG compared with subjects carrying a combination of *GSTT1* (+), *GSTM1* (+), and *GSTP1* AA has been reported (Kargar Shouroki et al. 2019). A parallel to studies on occupational exposure to other agents, no influence of individual genotypes (*GSTP1*, *GSTM1* and *GSTT1*) on genetic instability (MN) in Brazilian subjects was found (da Silva et al. 2008; de Souza et al. 2020).

GSTs are isoenzymes encoded by a large family of polymorphic genes. In addition to their detoxifying role, they act to regulate the intracellular concentration of lipoperoxidation compounds, modulating redox homeostasis and apoptosis pathways (Ketterer et al. 1998; Laborde et al. 2010). Previous studies have demonstrated reduced α-GST enzymatic activity in *GSTP1* Val/Val genotype subjects compared to *GSTP1* AA (Ile/Ile) genotype subjects (Ryberg et al. 1997; Dusinská et al. 2001). Although we did not assess serum α-GST levels, heterozygous AG (Ile/Val) or alternative homozygous GG (Val/Val) individuals exhibited higher levels of antioxidant capacity by FRAP than homozygous AA (Ile /Ile) in both groups.

In the current study, the allelic variant G was related to greater antioxidant capacity in control females and exposed males. Additionally, only exposed males lacking *GSTT1* enzyme

presented increased FRAP values. Decreased glutathione enzymatic activity results in increased ROS, such as hydroxyl and superoxide radicals (Dumaswala et al. 1999). The enzymatic activity of GSTT1 is known for its affinity toward lower molecular weight substrates, aiding in conjugation with glutathione (van Bladeren 2000). Taking into consideration that WAGs are relatively small lipophilic molecules, it appears that such polymorphisms result in greater susceptibility to the interaction of these compounds with the body, leading to increased antioxidant capacity (Ketterer, 1998). Khisroon et al. (2020) also associated the *GSTT1* null polymorphism, together with *GSTM1* null, with increased genotoxicity (by the comet assay) in 50 male Pakistani professionals exposed to unmeasured concentrations of WAGs.

As the antioxidant system is formed by several compounds, we propose that other enzymes, including CAT and superoxide dismutase (SOD), of other antioxidant pathways, may be involved, which resulted in the observed FRAP values. Interestingly, a study conducted among medical staff professionally exposed to ionizing radiation reported higher activities of CAT and SOD, but not of GPX, in exposed subjects carrying the genotypes *GSTP1* low active and *GSTT1/GSTM1* null compared to controls with the same genotypes (Doukali et al. 2017). Thus, the aforementioned authors suggest that antioxidant enzymes act as an adoptive phenomenon indicative of a possible genetic predisposition toward a worse oxidative phenotype. In addition, lack of or low-activity *GST* variants might contribute to increased susceptibility to oxidative stress (Ercegovac et al. 2015). These previous studies support our findings in relation to increased antioxidant capacity observed in exposed subjects (mainly males).

We observed higher FRAP values for *GSTP1* AG (Ile/Val) or GG (Val/Val) females than for AA (Ile/Ile) females only in the control group, and the same results were found in exposed males. The fact that we did not find differences in the antioxidant capacity of females in the exposed group may be due to different susceptibilities to exposure to WAGs and other factors that should be further investigated.

5. Strengths and limitations

To our knowledge, this is the largest study to enroll matched-subjects, monitor anesthetic pollution in OR air, evaluate polymorphisms of phase I and phase II-metabolism genes, assess two genetic instability markers and one oxidative marker and one antioxidant marker (oxidative stress) and associate endpoints with occupational exposure to WAGs. Nevertheless, potential limitations to our study should be noted. First, all assessed subjects were from the Brazilian population, and different findings might be found in other ethnic populations. We also highlight

the need for future studies that assess other oxidative stress biomarkers, whether possible in target cells, as well as GST enzyme activity and other antioxidant enzymes (SOD, CAT and GPX), in an attempt to establish possible associations between metabolism polymorphisms and antioxidant defense with WAG exposure.

6. Conclusion

Under the conditions of this study, we found elevated rates of MN and NBUD detected in buccal cells of professionals occupationally exposed to high anesthetic pollution. Exposed professionals aged over 30 years presented genetic instability, exposed females showed higher frequencies of MN and NBUD compared to nonexposed females, and exposed males, but not exposed females, showed increased antioxidant capacity. In addition, genetic instability was observed in professionals with greater exposure to WAGs per week, and those exposed for longer years showed oxidative stress. *GSTP1* AG/GG and *GSTT1* null were associated with greater antioxidant capacity in exposed males. Further studies are needed to provide new insight into the identification of responsive genes and genotypes more susceptible to WAG exposure. Reducing occupational exposure is relevant to minimize the impact observed in exposed health workers.

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Manuscrito 2

Modulation of gene expression and influence of gene polymorphisms related to genotoxicity and redox status on occupational exposure to inhaled anesthetics*

Abstract

Influence of genetic susceptibility on DNA damage and oxidative stress and possible modulation of gene expression has not yet been investigated facing occupational exposure to inhaled anesthetics (WAGs). This study assessed genes expression related to oxidized DNA repair and antioxidant capacity and the influence of their polymorphisms (*OGG1* rs1052133 and *SOD2* rs4880) in 100 professionals highly exposed to WAGs and 93 volunteers (control group). Additionally, other gene polymorphisms (*XRCC1* rs1799782 and rs25487, and *ATM* rs600931) were assessed as well as genetic instability (micronucleus-MN and nuclear bud-NBUD) and oxidative stress (malondialdehyde-MDA and ferric reducing capacity-FRAP) biomarkers in groups and according to job occupation. There was no significant difference between groups for the expression of both genes. However, among exposed professionals, anesthesiologists had greater duration of WAG exposure than surgeons/technicians, resulting in increased MDA, and decreased FRAP and *SOD2* expression (redox status). Regarding polymorphisms, increased *OGG1* expression was observed among *OGG1* -/Cys individuals compared to Ser/Ser only in the control group and decreased expression in -/Cys anesthesiologists compared to surgeons/technicians of the same genotype. Increased antioxidant capacity was noted in *ATM* TT in the control group as well as in the surgeons/technicians. Anesthesiologists with *ATM* CC exhibited increased MN and those with CC/CT genotype, increased NBUD. In surgeons/technicians, increased MN was influenced by *OGG1* -/Cys and increased antioxidant capacity was observed in those carrying *XRCC1* -/Gln. These findings contribute to advancing knowledge on genetic susceptibility/gene expression/genetic instability/oxidative stress, including the differences of job occupation, in response to occupational exposure to WAGs.

Keywords: biomonitoring; gene expression; genetic polymorphisms; genomic instability; oxidative stress; inhalation anesthetics

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1 INTRODUCTION

Operating room professionals are constantly exposed to waste anesthetic gases (WAGs) due to inhalational anesthetics use [NIOSH, 2007]. Consequently, WAG stand out for their potential and controversial damage to genetic material and generation of reactive oxygen species (ROS), leading to cellular oxidative stress in occupationally exposed individuals, which may represent risks to the health of professionals [Costa Paes et al, 2014; Souza et al., 2016; Szyfter et al., 2016; Aun et al., 2018; Braz et al., 2018; Çakmak et al., 2019; Braz et al., 2020a; Neghab et al., 2020; Hua et al., 2021].

Application of molecular biology techniques to identify sensitive changes induced by exposure to chemical agents is a widely used tool in toxicology [David, 2020]. Gene expression analysis allows early detection of changes in the profile of transcripts, helping to understand the cellular mechanisms used in response to exposure [Joseph, 2017]. Making a parallel to clinical studies, expression of important genes has already been studied in surgical patients under inhalation anesthetics [Braz et al., 2011; Freire et al., 2018; Jafarzadeh et al., 2020; Arruda et al., 2021]. However, so far, only one study evaluated the expression of some genes in professionals occupationally exposed to WAGs [Souza et al., 2021], which is a topic that has been little explored.

One of the great challenges in risk assessment facing occupational exposure is the genetic variability and consequent individual sensitivity of response during/after interaction with chemical agent [Dourson et al., 2013]. In this context, only a few studies have reported associations of single nucleotide polymorphisms (SNP) in metabolism genes with increased DNA damage in professionals exposed to WAGs [Mušák et al. 2009; Santovito et al., 2015; Kargar Shouroki et al., 2019; Khisroon et al., 2020]. However, no study yet has evaluated polymorphisms in key genes related to oxidative and DNA damage repair and antioxidative

status in professionals occupationally exposed to WAGs, showing a gap of knowledge in this field.

When lesions occur in genome, repair pathways are activated for the recognition and excision of DNA strand breaks, oxidized bases and by-products of methylation agents [Beaver et al., 2018]. If there is inefficiency in the repair system, the structure and function of DNA can be affected, leading to genetic instability (micronucleus - MN and nuclear bud - NBUD as biomarkers of DNA damage) that can be detected in exfoliated oral cells, which are in direct contact of WAGs [Sommer et al., 2020]. It is well established the relation between oxidative stress and DNA damage; thus, evaluation of oxidative stress (oxidant and antioxidant markers) is important as well as to assess the impact of SNP and gene expression related to repair of DNA damage and antioxidant status on these biomarkers concerning WAG exposure.

Therefore, this is the first study to evaluate possible pathways of WAG interaction in genes related to repair/antioxidant response (*OGG1*/*SOD2*) through the analysis of both genes expression and genetic susceptibility. In addition, other important gene polymorphisms (*XRCC1* and *ATM*) were also assessed; the influence of genetic susceptibility on cytogenetic and oxidative stress markers was performed as well as according to job occupation (duration of exposure to WAGs). Taking into account the lack of knowledge in the genetic toxicology area concerning occupational exposure to WAGs, our study tried to contribute to better understand the possible association between genetic susceptibility and gene expression with genetic instability and oxidative stress markers.

2 MATERIAL AND METHODS

2.1 Study population

Before the study was conducted, it was approved by the Research Ethics Committee from Botucatu Medical School-UNESP, Brazil (protocol number # 4063468). All participants signed

an informed consent form and completed a detailed questionnaire on demographic and anthropometric data, medical history, lifestyle, and additional data on occupational exposure to inhaled anesthetics (exposed group). Individuals who used illicit drugs, medications and/or vitamin supplementation, patients with infectious, malignant or inflammatory diseases, pregnant women, exposed to anesthetics (control group) and/or X-ray examinations, surgical or dental procedures (both groups), and individuals considered heavy drinkers [NIAAA, 2020] were excluded from the study.

This observational study involved 100 professionals who worked in operating rooms from university hospitals (anesthesiologists, surgeons and technicians), and who were occupationally exposed to halogenated anesthetics, especially isoflurane and sevoflurane, and to a lesser degree to nitrous oxide - N_2O (exposed group; 34 ± 12 years; 45% of female population; 33 ± 21 h worked/week and 9 ± 11 years of exposure to WAGs), and 93 volunteers who were not exposed to WAGs (control group; 34 ± 12 years; 48% of female population). Monitoring of anesthetic pollution in the workplace was carried out and is described in a recent study from our research group (Silva et al. unpublished results; submitted). The values of WAGs (≥ 7 ppm for halogenated anesthetics and ≥ 160 ppm for N_2O) revealed that exposed professionals showed high exposure, which was greater than the exposure limit values recommended (2 ppm for halogenated anesthetics and 25 ppm for N_2O) by the US National Institute for Occupational Safety and Health [NIOSH, 1992].

2.2 Collection of biological samples

Peripheral blood and oral cells were collected from all participants after 8 h of fasting. Venous blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and in PAXgene Blood as RNA stabilizer (Qiagen/PreAnalytiX, Switzerland). An aliquot of blood was centrifuged at 4°C for 15 min at 3500 rpm to obtain

plasma that was stored at -80°C for analysis of redox status parameters while other aliquot of blood was frozen until DNA isolation.

All individuals performed mouth rinses with water before collecting exfoliated oral cells, which were obtained from both cheeks with a cytobrush and was placed in a 15 ml tube containing cold saline solution.

All samples were coded and immediately processed under yellow light. The evaluation of gene expression was performed in triplicate while cytogenetic and oxidative stress markers and genotyping in duplicates. Quality control samples were used for gene expression and oxidative stress markers. Samples from the control and exposed groups were randomly evaluated and analyzed in a blinded manner.

2.3 Gene expression assessment

Blood samples collected in PAXgene Blood RNA Tubes were frozen at -80°C. Then, they were thawed at room temperature for further RNA isolation according to the manufacturer's protocol (PAXgene Blood RNA Kit; Qiagen/PreAnalytiX, Switzerland). The purity ratio and RNA concentration were determined by a Nano Drop One spectrophotometer (Thermo Fisher Scientific, USA) with values between 1.9 to 2.1. Sample integrity was assessed by a 1.5% agarose gel using Tris/borate /EDTA buffer. Transcription from RNA to complementary DNA (cDNA) was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems - ABI, USA), following the manufacturer's recommendations.

Gene expression was performed by real-time polymerase chain reaction (qPCR) using the TaqMan system for the *OGG1* (Hs00213454_m1) and *SOD2* (Hs00167309_m1), in addition to the endogenous housekeeping genes *ACTB* (Hs99999903_m1) and *HMBS* (Hs00609297_m1), which were selected and analyzed to the study. Thermal cycling and real-time fluorescence detection were performed in an ABI Prism 7500 FAST (USA), following the amplification

parameters: denaturation at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 60 s. In each 96-well plate protected from light, a negative control (without cDNA) was added to ensure the absence of contamination [Braz et al., 2012]. The relative quantification of gene expression was determined by the formula $2^{-\Delta\Delta Ct}$ [Livak and Schmittgen, 2001].

2.4 Genotyping analyses

DNA was isolated from blood cells according to the manufacturer's guidelines (DNA Illustra Blood GenomicPrep Mini Spin Kit; GE, Sweden). The concentration and purity of the samples were determined by a Nano Drop One spectrophotometer (Thermo Fisher Scientific, USA) and kept at -20°C until genotyping. The *OGG1* (rs1052133, C>G, Ser/Cys), *XRCC1* (rs1799782, C>T, Arg/Trp and rs25487, G>A, Arg/Gln), *ATM* (rs600931, C>T) and *SOD2* (rs4880, A>G, Val/Ala) polymorphisms were performed on DNA samples diluted to concentration of 3 ng/µl. The evaluation was performed by qPCR by TaqMan® SNP Genotyping Assays (Thermo Fisher, USA, ABI Assays C___3095552_1_; C__11463404_10; C____622564_10; C___2609420_10; C___8709053_10, respectively) in an ABI Prism 7500 FAST (USA) equipment following the manufacturer's recommendations. A negative control (PCR mix) was added to ensure the absence of contamination.

2.5 DNA damage markers in buccal cells

The buccal micronucleus cytome assay was performed following the protocol described by Thomas et al. [2009], with slight modifications. Falcon tubes containing buccal cells were centrifuged, the supernatant was removed and the cells were fixed with methanol and acetic acid in a 3:1 ratio. A new centrifugation was performed, and the process was repeated 2 more times. Three drops of concentrated cell samples were dropped on previously cleaned slides, with the material drying at room temperature after 24 h. Subsequently, the slides were immersed

in hydrochloric acid for hydrolysis and after washed. This process favors the staining of the nucleus by the Feulgen dye (Schiff reactive). After 90 min in the dark, slides were washed in distilled water to remove excess staining, followed by drying for at least 12 h. Counterstaining was performed by ice-cold Fast Green for cytoplasm differentiation. After drying, the slides were covered with coverslips and analyzed by an experienced investigator under a light microscope (Olympus BX43, Japan) at 1000 x magnification to identify MN (oval or round corpuscle of size 1/3 to 1/16 of the main nucleus) and NBUD (similar to MN but with a narrow constriction linked to the nucleus) [Thomas et al., 2009]; both parameters were confirmed by fluorescence with propidium iodide when necessary and were scored in 2000 differentiated cells from each participant.

2.6 Oxidative stress marker

Lipid peroxidation marker (malondialdehyde - MDA) was quantified by high-performance liquid chromatography (HPLC) following a protocol previously described by Volpi and Tarugi [1998], with minor modifications [Braz et al., 2020a]. Briefly, 100 µl of plasma were mixed with butylhydroxytoluene and sodium hydroxide, and the solution was incubated. For acid deproteinization, trichloroacetic acid was added, followed by ice-cooling the solution and subsequent centrifugation to collect the supernatant, which was incubated with 2-thiobarbituric acid and iron chloride. The mixture was heated, ice-cooled and centrifuged. The supernatant was filtered and injected into the Agilent HPLC system (USA). The mobile phase was composed of phosphate buffer (KH_2PO_4) and methanol (60:40), with 0.5 ml/min flow in C18 column system (5 µm, 150 x 4.6 mm) and fluorescence detector at 532 nm. MDA was quantified with a standard curve of tetramethoxypropane.

2.7 Antioxidant capacity status

The plasma antioxidant capacity was evaluated by ferric reducing antioxidant power (FRAP), which consists of the colorimetric measurement method of the antioxidant capacity to reduce the iron complex and tripyridyltriazine reagent (TPTZ) to the ferrous form, following a protocol previously described by Benzie and Stain [1996], with some modifications [Nogueira et al., 2018]. Briefly, aliquots of plasma diluted in water were incubated with the FRAP cocktail solution (acetate buffer, TPTZ in hydrochloric acid, and iron chloride) in specific cuvettes for 1 h at room temperature (protected from light). After this interval, the absorbance was measured at 593 nm (Shimadzu UV-1800; Japan). Trolox diluted in methanol at concentrations of 1000 µM, 500 µM, 250 µM, 125 µM, 62.5 µM and 31.25 µM was used as a standard curve to calculate the FRAP values of the samples.

2.8 Statistical analyses

Statistical analyses were performed by SAS program, version 9.4. The normality of variables was evaluated by Shapiro-Wilk and Kolmogorov-Smirnov to verify data distribution. For comparisons of age, time of exposure (h/week and years) and gene expression, the Student's t-test was used. The chi-square (χ^2) test was used for comparing the sex. A generalized linear model with gamma distribution was adjusted for variable that presented an asymmetric distribution (FRAP) and symmetrical distribution were fitted with normal distribution (MDA) as well as Poisson (MN) or negative binomial distribution (NBUD) for countable variables, which were followed by multiple comparisons. To compare genotypic and allelic frequencies, χ^2 test or proportion test was performed to verify whether they were in Hardy-Weinberg equilibrium. A significance level of less than 5% was considered significant.

3 RESULTS

The relative expression of genes related to repair of oxidized DNA damage (*OGG1*) and antioxidant capacity (*SOD2*) is represented in Figure 1. No significant difference was observed between the control and exposed groups ($p>0.05$).

Table 1 shows comparisons between anesthesiologists and surgeons/technicians by years, sex, and time of exposure to WAGs. Anesthesiologists had longer exposure time (h/week and years; $p<0.05$) than surgeons/technicians. Among those exposed, there were no differences in the *OGG1* expression (Figure 2); however, a down-regulation of *SOD2* ($p=0.004$) together with increased lipid peroxidation ($p=0.0001$) and reduced antioxidant capacity ($p<0.0001$) was observed in anesthesiologists compared to surgeons/technicians (Figure 2). In addition, no significant differences were observed in DNA damage biomarkers comparing the professionals evaluated.

Genotype and allele frequencies of polymorphisms (*OGG1*, *XRCC1*, *ATM* and *SOD2*) are shown in Table 2; there were no significant differences between the groups ($p>0.05$). The distribution frequencies of the genotyped polymorphisms were in agreement with Hardy-Weinberg equilibrium.

The expressions of *OGG1* and *SOD2* according to their respective polymorphisms were evaluated for control and exposed groups (Figure 3). The *OGG1* Cys allele (-/Cys) was associated with increased *OGG1* expression compared to the wild-type homozygote (Ser/Ser) only in the control group ($p=0.005$). No significant changes in *SOD2* expression were observed according to its polymorphisms in both groups.

Table 3 shows the individual effect of assessed polymorphisms on genetic instability and oxidative stress biomarkers. The results did not exhibit an influence of *OGG1*, *XRCC1*, *ATM* and *SOD2* polymorphisms on MN, NBUD, MDA and FRAP in each group, except for *ATM* TT genotyped controls who exhibited higher antioxidant capacity than *ATM* CT controls.

The effect of polymorphisms in different biomarkers was evaluated according to job occupation and only significant results are provided in Table 4. *ATM* CC anesthesiologists showed higher MN frequency compared to heterozygotes and alternative homozygote, and the C allele of *ATM* polymorphism was associated with higher values found for NBUD. In relation to surgeons/technicians, *OGG1* Ser/Cys or Cys/Cys exhibited higher MN frequency than those carrying Ser/Ser, and higher antioxidant capacity was associated with *XRCC1* (codon 399) -/Gln genotype and *ATM* TT (T allele).

The expression of *OGG1* according to its respective polymorphism by job occupation is shown in Figure 4. Higher expression of *OGG1* was observed in Ser/Cys or Cys/Cys surgeons/technicians than in anesthesiologists with the same genotype. Genotypic comparisons for *SOD2* expression were not possible due to insufficient number of surgeons/technicians carrying -/Ala.

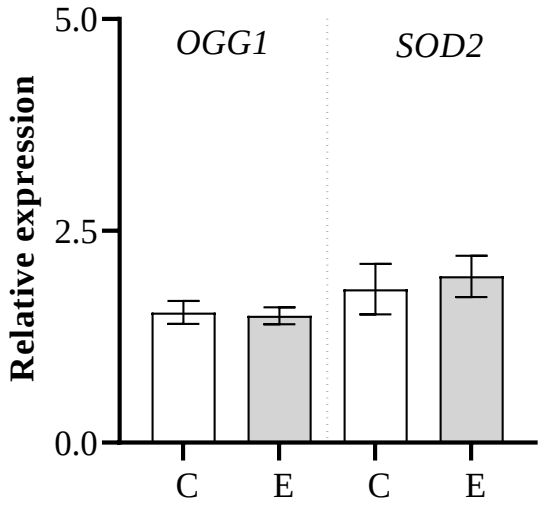
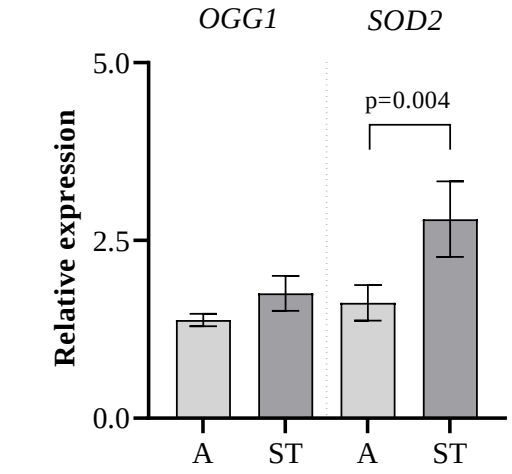


Figure 1 Expression of gene related to DNA damage repair (*OGG1*) and antioxidant defense (*SOD2*) according to group (C = control and E = exposed). Data are expressed as X ± SEM. $p>0.05$.

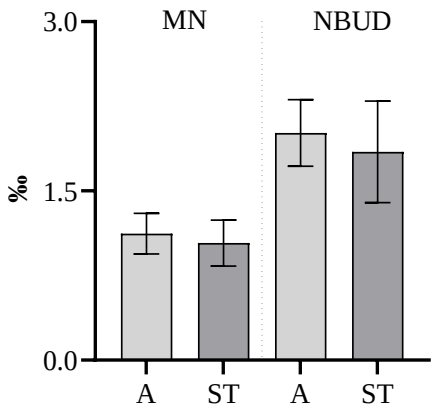
Table 1- Characteristics of the exposed group according to job occupation

Job occupation	Age (years)[a]	Sex[b]		Time of exposure[a]	
		Male (n)	Female (n)	Hours/week	Years
Anesthesiologists (n=74)	35 ± 13	39 (53%)	35 (47%)	37 ± 22[*]	11 ± 12[**]
Surgeons and technicians (n=26)	31 ± 7	16 (62%)	10 (38%)	21 ± 13	4 ± 3

Data are expressed as X ± SD[a], absolute number and percentage[b]; [*]p=0.001, [**]p=0.0009.



a



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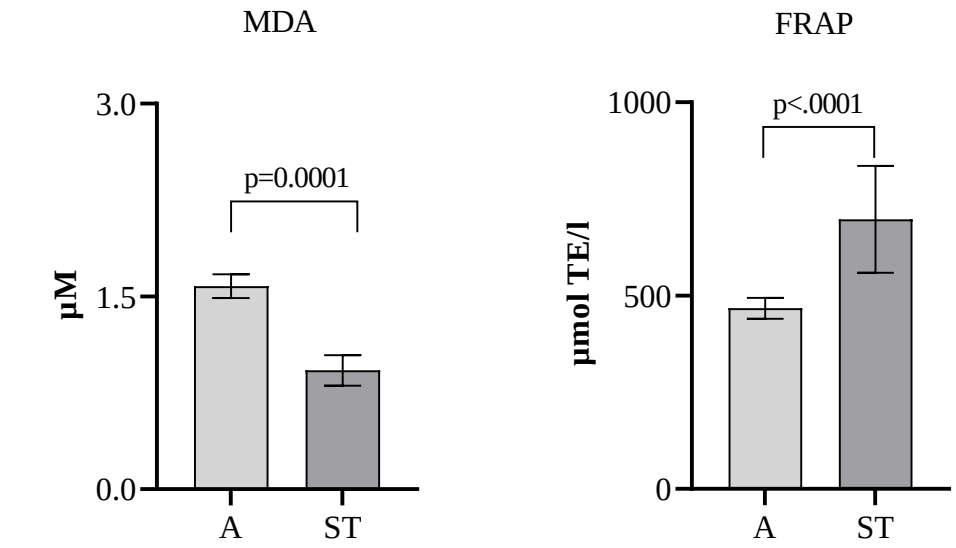

c

Figure 2 Comparison of *OGG1* and *SOD2* expression (**a**), buccal micronucleus - MN and nuclear bud - NBUD (**b**), and lipid peroxidation marker (MDA) and antioxidant capacity (FRAP) between job occupations - anesthesiologists (A) and surgeons/technicians (ST). Data are expressed as X ± SEM.

Table 2- Frequency distribution of *OGG1*, *XRCC1*, *ATM* and *SOD2* genotypes in the groups

Genotype	Control group (n=93)	Exposed group (n=100)	Total (n=193)
	n (%)	n (%)	n (%)
***OGG1* (rs1052133)**			
Ser/Ser (CC)	55 (59)	53 (53)	108 (56)
Ser/Cys (CG)	35 (38)	38 (38)	73 (38)
Cys/Cys (GG)	3 (3)	9 (9)	12 (6)
Allele			
C	90 (70)	91 (66)	181 (68)
G	38 (30)	47 (34)	85 (32)
***XRCC1* (rs1799782)**			
Arg/Arg (CC)	77 (83)	88 (88)	165 (86)
Arg/Trp (CT)	14 (15)	10 (10)	24 (12)
Trp/Trp (TT)	2 (2)	2 (2)	4 (2)
Allele			
C	91 (85)	98 (89)	189 (87)
T	16 (15)	12 (11)	28 (13)
***XRCC1* (rs25487)**			
Arg/Arg (GG)	34 (36)	39 (39)	73 (38)
Arg/Gln (AG)	51 (55)	53 (53)	104 (54)
Gln/Gln (AA)	8 (9)	8 (8)	16 (8)
Allele			
G	85 (59)	92 (60)	177 (60)
A	59 (41)	61 (40)	120 (40)
***ATM* (rs600931)**			
CC	16 (17)	14 (14)	30 (16)
CT	41 (44)	42 (42)	83 (43)
TT	36 (39)	44 (44)	80 (41)
Allele			
C	57 (42)	56 (39)	113 (41)
T	77 (58)	86 (61)	163 (59)

***SOD2* (rs4880)**			
Val/Val (AA)	26 (28)	20 (20)	46 (24)
Val/Ala (AG)	49 (53)	56 (56)	105 (54)
Ala/Ala (GG)	18 (19)	24 (24)	42 (22)
Allele			
A	75 (53)	76 (49)	151 (51)
G	67 (47)	80 (51)	147 (49)

n=number of individuals; $p>0.05$.

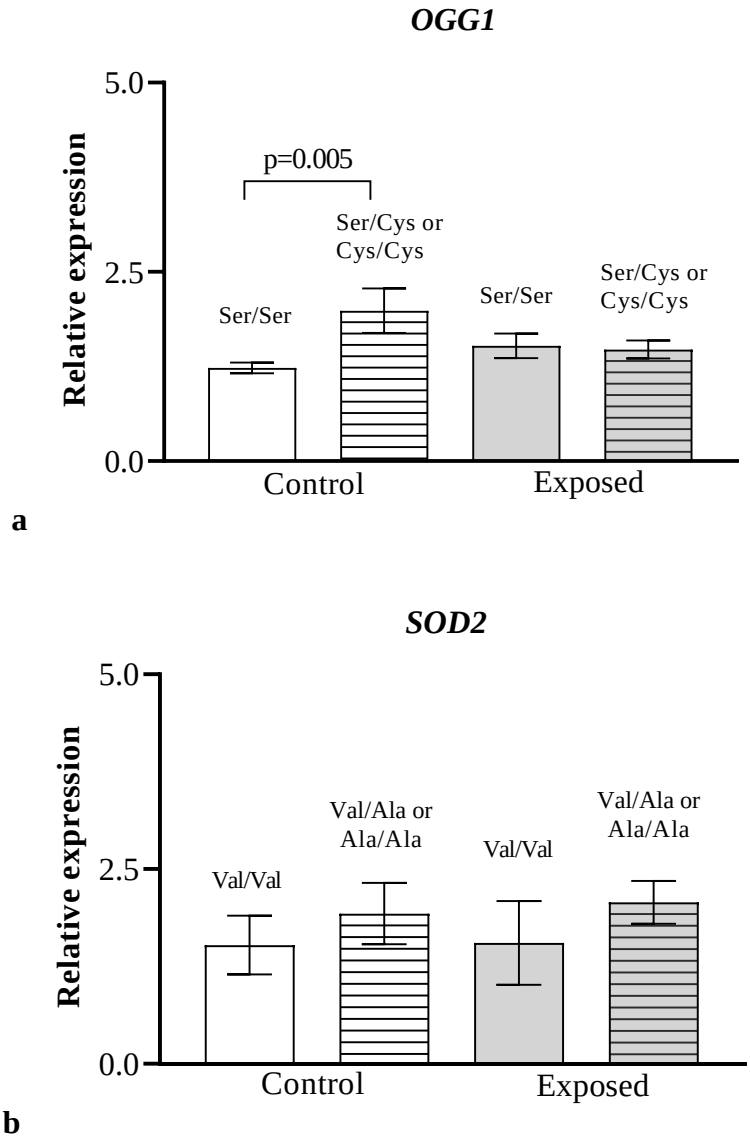

Figure 3 (a) *OGG1* expression according to *OGG1* polymorphism genotypes (rs1052133) in both groups. **(b)** *SOD2* expression according to *SOD2* polymorphism genotypes (rs4880) in both groups. Data are expressed as X ± SEM.

Table 3- Effect of individual genetic susceptibility on biomarkers of genotoxicity and oxidative stress

Genotype	MN (‰) (n)	NBUD (‰) (n)	MDA (µM) (n)	FRAP (µmol TE/l) (n)
Control				
***OGG1* (rs1052133)**				
Ser/Ser	0.6 ± 1.0 (55)	1.1 ± 1.2 (55)	1.4 ± 0.8 (55)	451 ± 160 (55)
Ser/Cys or Cys/Cys	0.8 ± 1.2 (38)	0.9 ± 1.4 (38)	1.3 ± 0.7 (38)	508 ± 457 (38)
***XRCC1* (rs1799782)**				
Arg/Arg	0.7 ± 1.0 (77)	1.2 ± 1.3 (77)	1.3 ± 0.7 (77)	483 ± 343 (77)
Arg/Trp or Trp/Trp	0.6 ± 1.0 (16)	1.2 ± 1.3 (16)	1.5 ± 0.9 (16)	434 ± 126 (16)
***XRCC1* (rs25487)**				
Arg/Arg	0.6 ± 1.0 (34)	1.4 ± 1.5 (34)	1.2 ± 0.7 (34)	484 ± 411 (34)
Arg/Gln or Gln/Gln	0.7 ± 1.0 (59)	1.1 ± 1.2 (59)	1.5 ± 0.8 (59)	469 ± 250 (59)
***ATM* (rs600931)**				
CC	0.7 ± 1.1 (16)	1.1 ± 1.2 (16)	1.6 ± 0.8 (16)	448 ± 162 (16)
CT	0.6 ± 0.9 (41)	1.1 ± 1.4 (41)	1.4 ± 0.8 (41)	386 ± 124 (41)
TT	0.7 ± 1.1 (36)	1.3 ± 1.3 (36)	1.3 ± 0.8 (36)	587 ± 459 (36)*
***SOD2* (rs4880)**				
Val/Val	0.5 ± 0.8 (26)	1.1 ± 1.4 (26)	1.2 ± 0.6 (26)	421 ± 80 (26)
Val/Ala or Ala/Ala	0.7 ± 1.1 (67)	1.2 ± 1.3 (67)	1.4 ± 0.8 (67)	495 ± 368 (67)
Exposed				
***OGG1* (rs1052133)**				
Ser/Ser	0.8 ± 1.0 (53)	2.1 ± 2.8 (53)	1.3 ± 0.7 (53)	549 ± 474 (53)
Ser/Cys or Cys/Cys	1.0 ± 1.2 (47)	1.3 ± 1.7 (47)	1.5 ± 0.9 (47)	502 ± 353 (47)
***XRCC1* (rs1799782)**				
Arg/Arg	1.2 ± 1.5 (88)	2.1 ± 2.6 (88)	1.4 ± 0.8 (88)	539 ± 444 (88)
Arg/Trp or Trp/Trp	0.6 ± 1.0 (12)	0.9 ± 1.1 (12)	1.3 ± 0.8 (12)	440 ± 135 (12)
***XRCC1* (rs25487)**				
Arg/Arg	1.1 ± 1.6 (39)	2.0 ± 2.3 (39)	1.4 ± 0.8 (39)	471 ± 135 (39)
Arg/Gln or Gln/Gln	1.0 ± 1.3 (61)	1.9 ± 2.6 (61)	1.4 ± 0.8 (61)	563 ± 525 (61)

***ATM* (rs600931)**				
CC	1.7 ± 2.5 (14)	2.6 ± 3.0 (14)	1.4 ± 0.8 (14)	442 ± 103 (14)
CT	1.1 ± 1.2 (42)	2.2 ± 2.6 (42)	1.5 ± 0.9 (42)	546 ± 447 (42)
TT	0.9 ± 1.1 (44)	1.6 ± 2.1 (44)	1.3 ± 0.7 (44)	536 ± 458 (44)
***SOD2* (rs4880)**				
Val/Val	0.7 ± 0.9 (20)	1.5 ± 2.2 (20)	1.4 ± 0.7 (20)	436 ± 116 (20)
Val/Ala or Ala/Ala	1.2 ± 1.5 (80)	2.1 ± 2.5 (80)	1.4 ± 0.8 (80)	550 ± 464 (80)

n=number of individuals, MN=buccal micronucleus, NBUD=buccal nuclear bud, MDA=malondialdehyde, FRAP=ferric reducing ability of plasma, data are expressed as X ± SD. Genotype comparisons performed within the same group; $p>0.05$, except for *$p=0.0002$ in relation to CT at the same group.

Table 4- Effect of polymorphisms in different biomarkers evaluated by job occupation (only statistically significant results are included)

Biomarkers	Genotypes	Anesthesiologists (n)	Surgeons/technicians (n)
Buccal MN (‰)	***OGG1* (rs1052133)**		
	Ser/Ser	1.1 ± 1.4 (37)	0.6 ± 0.5 (16)
	Ser/Cys or Cys/Cys	1.1 ± 1.7 (37)	1.8 ± 1.2 (10)
	p value	0.9	**0.0004**
	***ATM* (rs600931)**		
	CC	2.2 ± 2.8 (10) [a]	0.5 ± 0.6 (4)
	CT	1.3 ± 1.3 (29) [b]	1.2 ± 1.0 (13)
	TT	0.9 ± 1.1 (35) [b]	1.0 ± 1.2 (9)
	p value	**0.01**	0.4
Buccal NBUD (‰)	***ATM* (rs600931)**		
	CC	2.5 ± 3.2 (10) [a]	3.0 ± 3.2 (4)
	CT	2.7 ± 2.8 (29) [a]	1.1 ± 1.7 (13)
	TT	1.3 ± 1.9 (35) [b]	2.4 ± 2.5 (9)
	p value	**0.03**	0.8
FRAP (µmol TE/l)	***XRCC1* (rs25487)**		
	Arg/Arg	438 ± 121 (25)	529 ± 144 (14)
	Arg/Gln or Gln/Gln	482.0 ± 273.8 (49)	893.3± 1014.5 (12)
	p value	0.3	**0.03**
	***ATM* (rs600931)**		
	CC	453 ± 120 (10)	415 ± 36 (4) [a]
	CT	504 ± 332 (29)	640 ± 641 (13) [b]

TT	441 ± 144 (35)	906 ± 919 (9) [c]
p value	0.8	**0.03**

n=number of individuals, MN=buccal micronucleus, NBUD=buccal nuclear bud, FRAP=ferric reducing ability of plasma, data are expressed as X ± SD. Distinct letters differ significantly among analyzed genotypes of the same group.

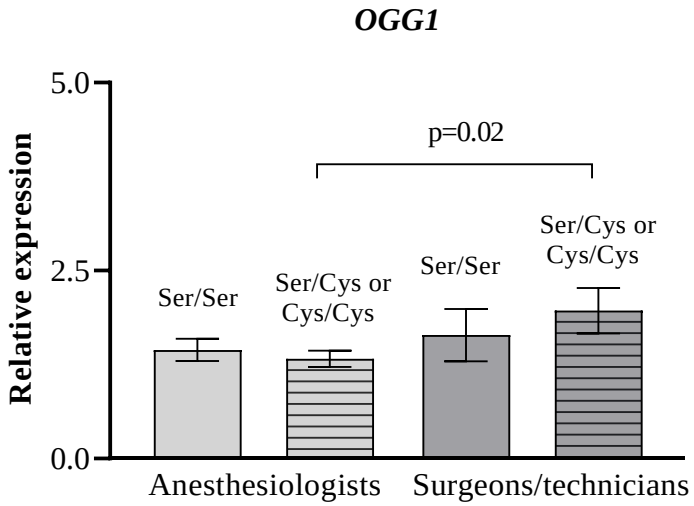

Figure 4 *OGG1* expression according to rs1052133 polymorphism genotypes by job occupations (anesthesiologists and surgeons/technicians). Data are expressed as X ± SEM.

4 DISCUSSION

A possible hypothesis of interaction between halogenated inhalational anesthetics and DNA was proposed by Jaloszyński et al. [1999] through the N7 alkylation of purines and/or the generation of ROS. Additionally, damage to macromolecules resulting from the imbalance between ROS formation and antioxidant capacity can lead to oxidative stress and, consequently, contribute to genotoxicity/mutagenicity [Jones, 2008].

It is known that individuals have different responses to anesthetics and differ in their ability to repair genetic damage. Drawing a parallel with a clinical study, patients anesthetized with isoflurane exhibited lower *OGG1* expression on the first postoperative day than before or during anesthesia [Braz et al., 2011]. A recent study conducted by our research group observed up-regulation of *OGG1* and *NRF2* (a gene involved in the antioxidant response), during a short period of occupational exposure to WAGs in medical residents [Aun, 2021]. Differently, in line with our findings, Souza et al. [2021] did not observe changes in *OGG1* expression in physicians exposed to a mean of 15 years exposure to WAGs compared to an unexposed group. In addition, no changes between groups were reported in the aforementioned study regarding oxidized DNA damage, contributing to the lack of differences concerning gene expression [Souza et al., 2021]. The fact that we did not observe significant changes in the expression of *OGG1* and *SOD2* considering a chronic exposure to WAG does not rule out the possibility that other genes are differentially expressed, but more studies are needed to evaluate gene networks involved in repair mechanisms and oxidative stress facing WAG occupational exposure, especially when most modern anesthetics are applied.

In order to observe whether professionals showed differences in exposure time and in the assessed biomarkers, we categorized the exposed group into anesthesiologists and surgeons/technicians. Interestingly, among exposed professionals, anesthesiologists had longer duration of WAG exposure (h/week and years) and consequent oxidative stress, resulted from

higher levels of MDA and lower FRAP and *SOD2* expression compared to surgeons/technicians.

To the best of our knowledge, our study is the first to report that oxidative stress, including *SOD2* expression, is affected by job occupation/duration of exposure to WAGs. To effectively eliminate ROS, through interaction with superoxide anions and conversion into hydrogen peroxide and oxygen, superoxide dismutase (SOD) plays important role in maintaining cellular homeostasis [Miao and Clair, 2009]. Due to the importance of this gene concerning oxidative stress, the longer exposure (time) to WAGs resulted in greater production of ROS (high levels of MDA), which certainly contributed to our observed results. In fact, young physicians exposed to isoflurane and to a lesser degree to sevoflurane and N_2O exhibited changes in antioxidant enzymes during medical residency [Costa Paes et al., 2014]. Interestingly, in a small longitudinal study conducted in medical residents, who have a large workload, oxidative stress was reported (decreased SOD levels with increased: oxidized DNA damage, MDA and glutathione peroxidase), indicating a possible modulation of antioxidant capacity pathways on exposure to WAG [Aun, 2021]. Therefore, we suggest that long exposure to high anesthetic pollution may depress the antioxidant defense system.

In relation to genetic instability, MN assessed in buccal cells has already been investigated in healthcare subjects occupationally exposed to WAG [Chandrasekhar et al., 2006; Souza et al., 2016; Çakmak et al., 2019; Braz et al., 2020b]. However, we did not detect difference for MN and NBUD among the exposed professionals. Interestingly, and supporting our findings, a study conducted in medical residents comparing those from anesthesiology field (exposed for 60 h/week) and surgery field (exposed for 18 h/week) reported a significant increase in buccal MN in anesthesiology residents when compared to unexposed residents (control); the results from surgical residents were between those highly exposed and the nonexposed, without a significant difference between them [Braz et al., 2018]. Therefore,

although we did not detect a statistical significance between the two job occupations, in a previous study of our research group, we have reported increased buccal MN and NBUD frequencies, involved in chromosomal damage and gene amplification, respectively, in professionals exposed to WAGs compared to a control group [Silva et al. unpublished results; submitted].

The recognition, catalysis and removal of oxidative DNA damage occurs through the base excision repair (BER) pathway, whose initiator element consists of 8-oxoguanine DNA glycosylase 1 (OGG1) transcribed by the gene located in important region in several polymorphisms' studies involving genetic instability and cancer [Goode et al., 2002; Mitra et al., 2011; Peng et al., 2014]. The switch from serine to cytosine at position 326 of *OGG1* (rs1052133) was associated with a significant reduction in the enzymatic activity and influence on genomic stability when compared to the wild-type homozygote [Yamane et al., 2004; Hill and Evans, 2006]. As a result, in occupational exposure studies, the alternative allele (-/Cys) was associated with increased DNA damage in exposed workers [Kahl et al., 2018a; Rohr et al., 2011; Miglani et al., 2021]. In relation to *SOD2* polymorphism (rs4880), the switch from valine to alanine at the 16th amino acid of the beginning of the signal sequence, affects the conformation and consequent transport of SOD2 to the mitochondria [Duarte et al., 2016]. Therefore, taking into account the enzymatic functionality, the amino acid valine is associated with a 30% to 40% reduction in SOD2 [Sutton et al., 2003]. Making a comparison, Kahl et al. [2018b] showed an association between *SOD2* Val/Val genotype and increased frequency of MN and NBUD and decreased antioxidant activity in professionals occupationally exposed to pesticides and nicotine.

Genetic susceptibility of these two SNPs in professionals exposed to WAGs has not yet been investigated. Therefore, our findings show a lack of influence of *OGG1* Ser326Cys and *SOD2* Val16Ala for the analyzed biomarkers; however, influence of amino acid Cys on *OGG1*

expression was found in control subjects. In addition, interestingly, we observed a significant increase in MN frequency in surgeons/technicians carrying -/Cys when compared to professionals who were wild-type homozygous (Ser/Ser), and that surgeons/technicians carrying -/Cys had higher *OGG1* expression than anesthesiologists with the same genotype. Even with less exposure than anesthesiologists, surgeons/technicians seem to be more susceptible to genetic instability when carrying -/Cys genotype. As a response, repair-related gene expression pathways may increase to try to maintain DNA integrity. Thus, we cannot discard that "less exposure" can be accompanied by adaptive changes to ensure the balance of redox status and genetic stability. However, we recognize the relative small sample size of surgeons/technicians in our study, requiring further investigations on associations of *OGG1* polymorphisms and their influence on transcripts, considering factors, such as job occupation and exposure time, among others (sex and age).

The current study also contributed to show lack of modulation of *SOD2* polymorphism in *SOD2* expression. In this polymorphism, the amino acid switch is known to affect the conformation of the SOD2 protein [Shimoda-Matsubayashi et al., 1996; Ascencio-Montiel et al., 2013]. It was hypothesized that in addition to the alteration in the amino acid sequence in the coding region, this SNP could affect gene expression due to structural alteration of mRNA [Johnson et al., 2008]. However, in a clinical study, Alachkar et al. [2017] found no correlation between transcript levels and *SOD2* Val16Ala genotypes in patients with acute lymphoblastic leukemia (ALL). For the first time in occupational exposure to WAGs, our results also did not show changes in *SOD2* expression influenced by those genotypes.

The *ATM* gene encodes mutated ataxia-telangiectasia kinase (ATM), which has role in apoptotic and repair responses, as well as in proteins involved in cell cycle checkpoints and regulatory pathways of the antioxidant response [Ditch and Paull, 2012; Cirotti et al., 2021]. It is suggested that polymorphisms located in the non-coding region of *ATM* may influence the

maturation process of mRNA and interfere with protein formation [Wang et al., 2009]. However, little is known about the influence of SNP rs600931 on ATM functional modification. Regarding genetic instability, Wang et al. [2009] associated the homozygous wild-type *ATM* genotype (rs600931) with lower susceptibility to MN formation in lymphocytes from workers exposed to polycyclic aromatic hydrocarbons. To the best of our knowledge, no study has explored polymorphisms in *ATM* on buccal genetic instability and oxidative stress biomarkers regarding occupational exposure.

Different from Wang et al. [2009], our results showed increased MN frequency for the *ATM* CC genotype together with increased NBUD frequency for C allele in anesthesiologists. Therefore, in this study, the C allele was associated with greater genetic instability in anesthesiologists exposed to WAGs. Higher antioxidant capacity was observed in *ATM* TT controls when compared to *ATM* CT controls. In the exposed group, slightly higher means of antioxidant capacity were observed for the T allele compared to the wild-type homozygote, but these differences were not significant. Among the exposed group, surgeons/technicians presented higher FRAP values when they were carrying at least one T allele, similar to observed in the control group. Contrarily, anesthesiologists showed no differences in FRAP values for *ATM* polymorphism, although the mean for the TT genotype was slightly lower. In fact, this profession category exhibited reduced antioxidant capacity, increased lipoperoxidation and longer exposure time to WAGs when compared to surgeons/technicians, with no genotype influence on antioxidant status. Considering that *ATM* plays important role in keeping balance in redox state and is critical to lifespan [Miura and Endo, 2010], we suggest that the *ATM* TT (rs900931) genotype is associated with higher antioxidant defense in some of the participants included in the study, which is linked to ability to deal with ROS, reducing DNA damage. However, further studies with a larger sample size and assessment of damage/repair pathways and oxidative stress are necessary to prove this hypothesis.

XRCC1 encodes a protein with several domains capable of interacting with repair components of single-strand DNA breaks formed after exposure to ionization and alkylating agents, being a gene responsible for the participation of the BER-type repair mechanism [Abbotts and Wilson, 2016]. It is believed that the glutamine variant of rs25487 (-/Gln) may alter the interaction between the enzymatic components of the repair pathways, leading to susceptibility to genotoxicity [Ginsberg et al., 2011]. In relation to this gene, surgeons/technicians genotyped with -/Gln exhibited increased antioxidant capacity (FRAP) compared to those carrying Arg/Arg. No influence of *XRCC1* Arg399Gln was observed in antioxidant capacity in children with ALL [Dincer et al., 2015]. On the other hand, higher antioxidant activity in individuals with Gln allele compared to those carrying Arg allele has been reported [Orhan et al., 2016], supporting our data concerning increased FRAP with -/Gln. Whether the increase in antioxidant capacity in the variant genotype is associated with an attempt to compensate the oxidative damage process in a shorter exposure time to WAGs warrants further investigation.

Therefore, our findings suggest that occupationally exposed professionals do not exhibit changes in *OGG1* and *SOD2* expressions compared to the control group. Among exposed professionals, anesthesiologists had a longer duration of exposure to WAGs, resulting in greater oxidative stress and decreased *SOD2* expression compared to surgeons/technicians. Additionally, decreased expression in *OGG1* -/Cys anesthesiologists was observed compared to surgeons/technicians of the same genotype. *ATM* TT genotype was associated with better antioxidant capacity in the control group and in surgeons/technicians. On the other hand, this polymorphism was associated with increased MN in CC genotype and increased NBUD in CC/CT in anesthesiologists. In surgeons/technicians, *OGG1* -/Cys was associated with increased MN frequency and *XRCC1* -/Gln with increased antioxidant capacity. Thus, our study contributes to knowledge regarding modulation of genetic susceptibility, gene expression, and

DNA damage and oxidative stress endpoints, including the differences of job occupation, in response to occupational exposure to WAGs.

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Anexo

Aprovação do Projeto pelo Comitê de Ética em Pesquisa

UNESP -FACULDADE DE MEDICINA DE BOTUCATU

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Papel de polimorfismos, instabilidade genética, resposta oxidativa, expressão e metilação gênicas na exposição ocupacional aos anestésicos

Pesquisador: MARIANE APARECIDA PEREIRA SILVA

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 2

CAAE: 30055120.7.0000.5411

Instituição Proponente: Departamento de Anestesiologia

Número do Parecer: 4.063.468

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

Assinado por:
SILVANA ANDREA MOLINA LIMA
(Coordenador(a))

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