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RESEARCH ARTICLE

Ozone generated by air purifier in low concentrations: friend or foe?

Larissa Vivan Cestonaro[1] · Ana Maria Marcolan[2] · Luciana Grazziotin Rossato-Grando[1] · Ana Paula Anzolin[1] · Gabriela Goethel[3] · Angélica Vilani[1] · Solange Cristina Garcia[3] · Charise Dallazem Bertol[1]

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Abstract Ozone helps decontamination environments due to its oxidative power, however present toxicity when it is in high concentrations, by long periods of exposition. This study aimed to assess the safety of ozone generator air purifier at concentrations of 0.05 ppm in rats exposed to 3 and 24 h/day for 14 and 28 days. No significant differences are observed between groups in clinical signs, feed and water intake, relative body weight gain and relative weight of organs, macroscopy and microscopy of lungs, and oxidative plasma assay. In this exposure regime, ozone does not cause genotoxicity and no significant changes in pulmonary histology indicative of toxicity. Ozone generated in low concentrations, even in exposure regimes above the recommended is safe, both acute and sub-acute exposition.

Keywords Acute toxicity · Sub-acute toxicity · Rats · Ozone · Oxidative stress · Comet assay · Micronucleus

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✉ Charise Dallazem Bertol
charise@upf.br; charibertol@yahoo.com.br

[1] Curso de Farmácia, Instituto de Ciências Biológicas, Universidade de Passo Fundo, Campus I, Km 292, BR 285, Bairro São José, Passo Fundo, RS CEP 99052-900, Brazil

[2] Faculdade de Medicina, Universidade de Passo Fundo, Instituto de Patologia, Rua Teixeira Soares 885, 99010-081 Passo Fundo, RS, Brazil

[3] Laboratório de Toxicologia (LATOX), Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Avenida Ipiranga 2752, 90610-000 Porto Alegre, RS, Brazil

Introduction

Indoor pollution carries health risks (mucous membrane symptoms related to the eyes, nose, throat, dry skin, headache, and lethargy) due to pollutants such as microorganisms (Wallace 1996; Burge 2004; Toivola et al. 2004). The air purifiers ozone generators aim to improve air quality, reducing over 80% of fungi, bacteria, and viruses present in the environment (Thurston-Enriquez et al. 2005; Hudson et al. 2007; Lin et al. 2007; Murray et al. 2008; Bertol et al. 2012; Petry et al. 2014).

On the other hand, high ozone levels (0.08–3 ppm) in the environment are considered toxic and it has been associated with various adverse health effects, including increases the formation of reactive oxygen species (ROS), reduction in lung function induced airway inflammation through increases the infiltration of neutrophils and macrophages in both healthy individuals, and those who already have some kind of respiratory disease, exacerbation of respiratory illnesses, and increased rates of hospital admissions (Crapo 1986; Mehlman and Borek 1987; Mustafa 1990; Devlin et al. 1991; Aris et al. 1993; Peden et al. 1995; Devlin et al. 1997; Krishna et al. 1998; Bhalla and Gupta 2000; Long et al. 2001; Alexis et al. 2004; Viebahn-Hänsler et al. 2012).

According to current legislation, the exposure to ozone is limited at 0.1 ppm by 8 h and does not exceed 5×10^{-8} (OSHA 1992; IEC 2002; Brasil 2016). Here, we aim to evaluate the effects of continuous exposure to ozone generated by air purifiers following manufacturer guidelines and exceeding daily recommended exposure in order to answer: acute and sub-acute exposure to ozone generated by air purifier in low concentrations (0.05 ppm) is safe?

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Materials and methods

Animal model

Wistar male rats (5–6-week old) were supplied by CECAL (Centro de Criação de Animais de Laboratório/FIOCRUZ, Rio de Janeiro—RJ). Animals were accommodated individually in plastic cages of 40 × 32 × 17 cm, at 18–22 °C, 12/12 h light/dark cycle, with access to water and diet (commercial feed) ad libitum for 4 weeks.

Rats were divided into three groups (12 animals per group): control not exposed to ozone, rats exposed 3 h/day to ozone as recommended by manufacturers, and rats exposed without interruption to ozone for 24 h. Animals were exposed to these regimen for 14 (acute study) and 28 days (sub-acute study). The 12 plastic cages (containing one animal/cage) were allocated in three rooms with 1.5 × 1.5 m^2 each, one room for each group. This number of animals (n = 6 for acute and sub-acute treatment) was used considering the three Rs related to animal experimentation (replacement, refinement and reduction), reducing the number, but without impairing the results and statistical analysis. The male rats were chosen to avoid the hormonal influence.

The experiments followed the ethical guidelines for animal experimentation according to the Brazilian Society of Science in Laboratory Animals (SBCAL 2016) and Arouca Law 11.794, of October 8, 2008 (Brasil 2008). The study was approved by Ethics Committee on Animal Use (CEUA) in research at the University of Passo Fundo obtaining the no. 032/2012.

Ozone

Ozone was generated by Brizzamar® air purifier (Sentinela Eletrônicos/Ronda Alta/RS) at concentrations of 0.05 ppm. The amount of ozone released was monitored by EcoSensorModelOS-4 sensor (Ozone Switch™).

Perform evaluations

The consumption of water, feed, and body mass gain were monitored every 2 days. After 14 and 28 days, the animals were anesthetized with xylazine/ketamine (10 mg/kg, 100 mg/kg) intraperitoneally. Blood (about 8 mL) was withdrawn by intracardiac puncture in syringe containing heparin. Animals were euthanized by diaphragm rupture. Plasma was centrifuged (1500 rpm/15 min) and stored at −20 °C.

Organs (lung, liver, spleen, kidneys, and heart) were washed with phosphate buffer (pH 7.4), dried, and weighed. The organs weights were normalized according to the mass of the animal. The lungs were analyzed macroscopically and microscopically.

Microscopic and macroscopic analysis of the lungs

The lungs were observed macroscopically, placed in bottles containing 10% buffered formalin for histological analysis. HE (hematoxylin-eosin) and Giemsa dyes were used. Degree of tissue disorganization were observed (microscopy at ×50 magnification) and were scored 0 (no disorganization areas), 1 (with < 1/3 of disorganization areas), 2 (between 1/3 and 2/3 of disorganization areas), and 3 (> 2/3 of disorganization areas). The bronchioles, alveolar septa (presence of thickening, congestion, infiltration of macrophages and neutrophils), alveoli (presence of red blood cells, macrophages and neutrophils), and blood vessels (congestion, neutrophils infiltrates and neutrophils paving) were observed using HE dye. Giemsa dye was used to observe mast cells. Neutrophil and mast cell count were performed in ten fields of the right and left lungs of each rat at ×400 magnification (Olympus Microscope CH20).

Oxidative stress

Oxidative stress markers at the plasma were evaluated: lipid peroxidation, non proteic thiols, and nitric oxide. Results were normalized with protein content (Lowry et al. 1951). Lipoperoxidation was evaluated by measuring the amount of thiobarbituric acid reactive species (TBARS) (Ohkawa et al. 1979). The thiol group is an indirect assay for the determination of reduced glutathione. The amount of non proteic thiols was determined by Ellmann method (Ellman 1959). Measurement of nitric oxide was determined by reaction with Griess reagent (Bracht and Ishii-Iwamoto 2003).

Comet assay

Comet assay was performed according to the slightly modified protocol (Da Silva et al. 2000). Mutagenic agent MMS (methoxy methyl sulfonate) (70 μM) was injected intraperitoneally 6 h before the euthanasia in one rat of the control group and was used as positive control (Sasaki et al. 2007). Five microliters of whole blood (with heparin) was added to 95 μL of agarose low melting (0.75%). The mixture was poured into a frosted microscope slide coated containing a layer of normal-melting-point agarose (1.5%), covered with a cover slip, and stored at 4 °C. After 1 h, the cover slip was removed and the slides were disposed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, distilled water, 10% DMSO, and 1% Triton X-100) remained overnight. Subsequently, the slides were incubated in alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 13) and acclimatized for 20 min. The DNA was submitted to electrophoresis for 20 min at 25 V (0.90 V/cm) and 300 mA, and the buffer was neutralized with 0.4 M Tris (pH 7.5) for 15 min, in the dark, dried, fixed in 96% ethanol for 5 min. After drying, the

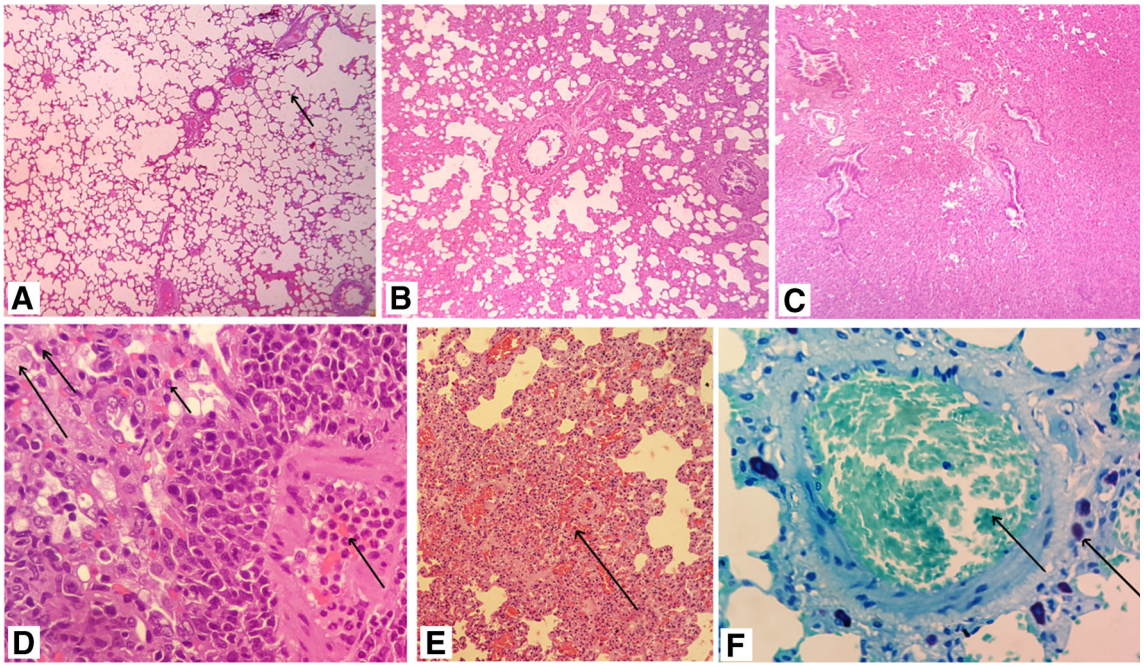

Fig. 1 Lung microscopy, represented (**a**) normal areas (arrow indicate thin alveolar septum) (R10, sub-acute toxicity, ozone 24 h/day), (**b**) focal areas of inflammation (R8, sub-acute toxicity, ozone 3 h/day), (**c**) focal areas of intense inflammation (R11, sub-acute toxicity, control group), (**d**) arrows indicate macrophages, xanthomatous macrophages, neutrophils, blood vessel with neutrophils inflammation (R8, sub-acute toxicity, ozone 3 h/day), (**e**) areas of thickening, septum congestion, and bleeding (R11, sub-acute toxicity, control group), (**f**) mast cells (arrows indicate the mast cells in purple and red blood cells in green) (R8 sub-acute toxicity, control group). Photos **A**, **B**, **C**, **E**, ×100 magnification, dye HE. Photos **D** (dye HE) and **F** (dye Giemsa), ×400 magnification

nucleoids were stained with ethidium bromide and examined at ×500 magnification in a fluorescence microscope (Olympus IX-71, Japan). Images of 100 randomly cells (50 cells from each of two replicated slides) were analyzed from each rat. Damage score is based on the tail moment, and the amount of DNA in the tail and is considered a sensitive measure of DNA damage (Hellman et al. 1995). The quantification of the cells was carried using average tail moments and percentage tail DNA using CometScoreTM free software.

Micronucleus assay

The femur from each rat was dissected, the epiphyses were cut, and the bone marrow was removed with a syringe containing a needle. The marrow was diluted in fetal bovine serum; centrifuged and cell suspension were spread on slides and stained with MayGrunwald dye for 7 min, washed with water, and stained with Giemsa dye (diluted 1/10 with distilled water) for 1 min. Slides were washed with water and dried at room temperature. Polychromatic erythrocytes (immature erythrocyte-PCE) were stained light blue and normochromatic erythrocytes (mature erythrocytes without ribosome—NCE) were stained red-tile. After staining, the slides were analyzed by optical microscopy. The presence or not of micronucleus assay (MN) was observed, through the formation of small dots next to the cell nucleus (Choy 2001). One thousand cells were counted for each sample, in duplicates. Results were expressed as MN frequency per 1000 cells.

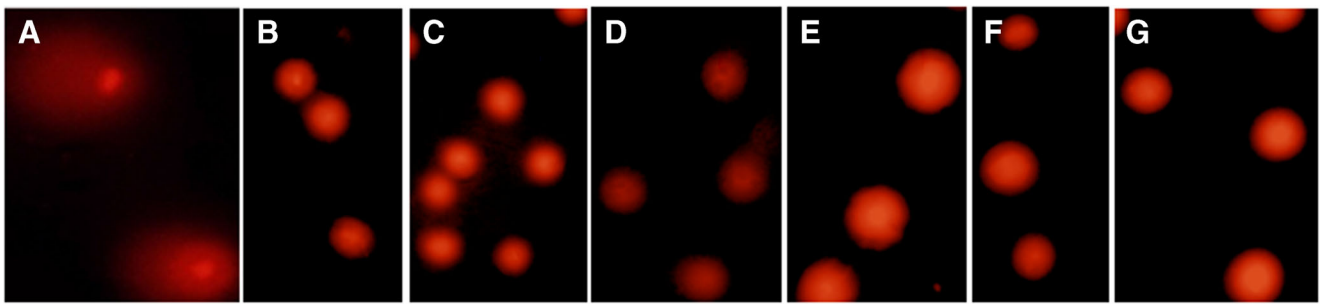

Fig. 2 Photos of blood cells subjected to the comet assay after 14 days of study (×400 magnification). **a** Positive control—R6 control group. **b** R2 cells of control group. **c** R2 cells of ozone 3 h/ day. **d** R2 cells of ozone 24 h/day; and after 28 days of study. **e** R7 cells of control group. **f** R7 cells of ozone 3 h/ day. **g** R7 cells of ozone 24 h/day

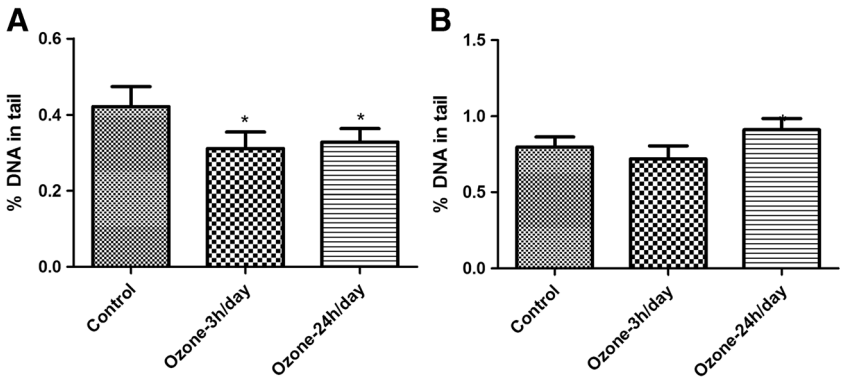

Fig. 3 Percent DNA in tail. **a** 14 days. **b** 28 days of study

Statistical analysis

Histological results were submitted to descriptive statistical analysis. The pattern of distribution of the data was analyzed using the Kolmogorov-Smirnov test. The results were statistically analyzed by comparing the means by analysis of variance (ANOVA) (one-way to parametric data), followed by Tukey's test with minimum significance level of $p < 0.05$. The data for feed consumption, water, and relative weight were analyzed by two-way ANOVA (repeated measures) followed by Tukey's test ($p < 0.05$). Data were expressed as mean ± standard deviation.

Results and discussion

Main results of this work highlight that ozone generated by ozone purifier in low concentrations is safe, both using the scheme recommended by the manufacturer (3 h/day) as well as above recommended, in an acute and sub-acute regimen.

No significant differences are observed between groups in clinical signs, feed, and water intake, relative body weight gain and relative weight of organs (data not shown). No macroscopic and microscopic changes indicative of pulmonary toxicity are observed on lungs of rats after 14 or 28 days treatment in comparison to control animals.

Microscopic analysis found some inflammatory cells only in focal areas in all groups (Fig. 1) with low number of neutrophils. In the alveolar septa observed thickening due to recruitment of inflammatory cells and epithelial cells (type 1 and 2 pneumocytes lining the alveoli, Robbins and Cotrans 2010), congestion in the capillaries around the areas of consolidation and infiltrates of macrophages and neutrophils and foam cells (Fig. 1d). The presences of red blood cells without phagocytosis, in the alveoli, indicates recent hemorrhage due intracardiac puncture (Fig. 1e) (Leslie and Wick 2011). Excess of red blood cells into blood vessels indicate congestion. The bronchioles showed without inflammatory cells (Fig. 1a). The slides showed similar findings in all groups studied.

Ozone significantly reduces the number of mast cells in the lung, probably due to its ability to reduce hypersensitivity reactions involved in allergic process. Previously, controlled exposure to ozone (0.4 ppm for 2 h) increased neutrophilia and mCD14 expression in airway macrophages and monocytes (Alexis et al. 2004). Conversely, rats subchronically exposed to ozone (0.12 and 0.25 ppm 12 h/day for 6 weeks; 0.06 ppm for 5 days, then to 0.12 to 0.25 ppm and returned to 0.12 ppm for 9 h in a 3 or 13 weeks) presented increased alveolar epithelium volume and cellular injury (Chang et al. 1991). The differences in these results and ours are related to the higher exposure pattern performed by Alexis and colleagues and Chang and colleagues.

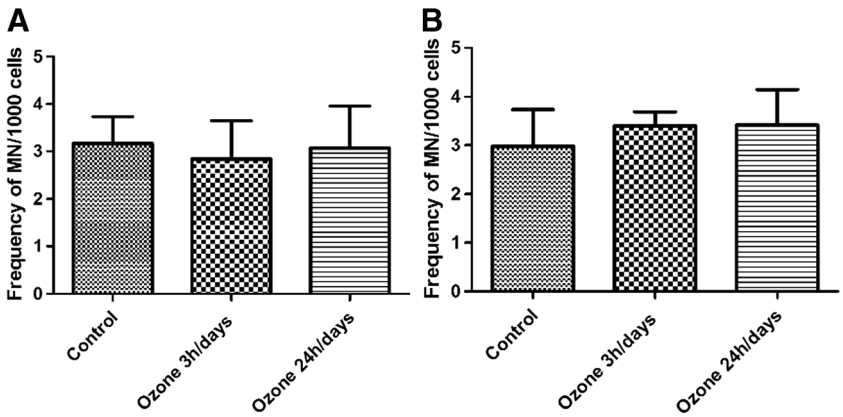

Fig. 4 Polychromatic erythrocytes (PCE)/erythrocyte normochromic (NCE) ratio per thousand cells per animal. **a** 14 day and **b** 28 day of study. The mean frequency of the PCE/NCE ratio was analyzed by the one-way ANOVA, followed by Tukey's test.

No significant changes in oxidative stress biomarkers are observed in none exposure regimen tested.

Ozone exposures even in regimens higher than indicated by the manufacturer are not genotoxic in the tested conditions. Interestingly, in the acute assay, residual DNA damage in ozonated groups are lower than that observed in control group. In sub-acute groups, the 24-h/day-ozonated animals present higher damage in relation to other groups, but although statistically significant, this damage is not considered genotoxic since DNA tail is lower than 1% (Figs. 2 and 3). MN assay has used to evaluate DNA damage at the chromosomal level (Collins 2004). Statistical analysis comparing the frequency of MN between the groups showed that, in both, they did not present a mutagenic effect when compared to the control, as can be observed in Fig. 4. Studies evaluating the bone marrow MN after exposure to ozone are not commonly found, bringing important data and easy-to-perform techniques to assess mutagenicity in rodents.

In summary, our results demonstrate that ozone generated by air purifiers are safe when following manufacturer instructions and even at higher exposure periods in acute and subacute regimen. Ozone was removed from the list of indoor pollutants published by WHO (WHO 2010). However, some authors considered ozone as an indicator of environmental pollution (Bell et al. 2004; Ruidavets et al. 2005; Tager et al. 2005; Anenberg et al. 2010; Silverman and Ito 2010; Goudarzia et al. 2013; Silva et al. 2013; Bell et al. 2014; Chen et al. 2015). In fact, ozone concentration increases due to the formation of smog as well as many other reactive oxygen or nitrogen species are increased, and these other species are directly related to damage caused in the respiratory function, leading to synergistic effect (WHO 1979; Lee et al. 1983).

Ozone generated by air purifiers in concentration about 0.05 ppm is safe, either in the use of 3 h/day as 24 h/ day in the acute and sub-acute regimens.

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