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Oxidative damage, inflammation, genotoxic effect, and global DNA methylation caused by inhalation of formaldehyde and the purpose of melatonin

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Abstract

Formaldehyde (FA) exposure has been proven to increase the risk of asthma and cancer. This study aimed to evaluate for 28 days the FA inhalation effects on oxidative stress, inflammation process, genotoxicity, and global DNA methylation in mice as well as to investigate the potential protective effects of melatonin. For that, analyses were performed on lung, liver and kidney tissues, blood, and bone marrow. Bronchoalveolar lavage was used to measure inflammatory parameters. Lipid peroxidation (TBARS), protein carbonyl (PCO), non-protein thiols (NPSH), catalase activity (CAT), comet assay, micronuclei (MN), and global methylation were determined. The exposure to 5-ppm FA resulted in oxidative damage to the lung, presenting a significant increase in TBARS and NO levels and a decrease in NPSH levels, besides an increase in inflammatory cells recruited for bronchoalveolar lavage. Likewise, in the liver tissue, the exposure to 5-ppm FA increased TBARS and PCO levels and decreased NPSH levels. In addition, FA significantly induced DNA damage, evidenced by the increase of % tail moment and MN frequency. The pretreatment of mice exposed to FA applying melatonin improved inflammatory and oxidative damage in lung and liver tissues and attenuated MN formation in bone marrow cells. The pulmonary histological study reinforced the results observed in biochemical parameters, demonstrating the potential beneficial role of melatonin. Therefore, our results demonstrated that FA exposure with repeated doses might induce oxidative damage, inflammatory, and genotoxic effects, and melatonin minimized the toxic effects caused by FA inhalation in mice.

Key words: pollutant, xenobiotic, oxidative damage, inflammation, toxicology

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Introduction

Formaldehyde (FA) is a contaminant air pollutant and a compound of particular concern due to its ubiquitous distribution as it is produced in many industries and has an extensive and versatile range of use. Thus, millions of people worldwide are exposed to this compound [1–3]. In medicine, FA is used in anatomy and pathology laboratories and embalming, for its sterilizing, preserving, and stabilizing properties [4]. In industry, FA is widely used in the production of several products such as resins, adhesives, binders for plywood, plastics, synthetic fibers, paints, and insulation foams, which are raw materials employed in furniture, upholstery, carpeting, drapery, and other household products, due to that many workers are occupationally exposed to FA [5–7].

International agencies such as the National Institute of Occupational Safety and Health, American Conference of Governmental Industrial Hygienists, and Occupational Safety and Health Administration suggest limits of 0.016, 0.3, and 0.75 ppm per 8 hours of work, respectively [8–10], while the World Health Organization recommends an internal FA limit of 0.08 ppm (0.1 mg/m^3) [11]. Nevertheless, toxicological studies demonstrated that airborne FA levels often exceed recommended exposure limits [12–14].

The primary effects of acute exposure to FA are irritation of the mucosa of the upper respiratory tract and the eyes, site of first contact [15]. However, it also affects metabolism in many different organs, since it is present in all cells of the human body [16]. Due to the fact that FA has high solubility and reactivity with nucleophilic groups of proteins and nucleic acids, it is able to form adducts and induce DNA damage [6, 17]. This characteristic explains the toxic and carcinogenic properties of formaldehyde [2, 18]. Based on genotoxic and carcinogenic effects, the International Agency for Research on Cancer classified this substance as a human carcinogen [19].

Damage to organ tissue from FA may be associated with oxidative stress and inflammation [20]. Recently, some studies demonstrated that FA inhalation increases the generation of reactive oxygen species (ROSs), and it causes a disruption of the physiological balance between oxidant and antioxidant enzymes in the lung tissue of rodents [7, 21]. ROSs are cytotoxic agents causing oxidative damage by attacking cell membrane and DNA and can contribute to a variety of diseases [22, 23]. Mechanisms of cellular defense can regulate oxidative stress through glutathione (GSH) and enzymes such as superoxide dismutase and catalase (CAT) [24].

Many studies have reported that antioxidant treatment can prevent oxidative stress in the tissues [7, 22, 25, 26]. Melatonin, an endogenous neurohormone produced by the pineal gland, acts on many physiological functions, including biological regulation of circadian rhythms, sleep, reproduction, and neuroimmunomodulation [27, 28] mainly through the activation of two G-protein-coupled plasma membrane receptors: MT1 and MT2 [27, 29]. It is small molecular size and its amphiphilic properties facilitate melatonin's penetration into subcellular compartments [30]. Melatonin protection actions have been previously reported against various xenobiotics [31–35], including the FA exposure [36–39]. Thus, melatonin and its metabolites play an important protective role in different pathophysiological conditions by attenuating oxidative stress and inflammation [32]. Melatonin neutralizes a host of toxic reactive molecules directly, stimulates the synthesis and the activation of antioxidant enzymes, and also inhibits pro-oxidative enzymes [28, 32, 40].

Therefore, the aim of this study was to evaluate for 28 days the FA inhalation effects on oxidative stress, inflammation process, and genotoxicity in mice as well to investigate the potential protective effects of melatonin administered intraperitoneally (i.p.) on reduction these parameters. For that, oxidative damage was assessed by levels of substances reactive to thiobarbituric acid (TBARS) and carbonylated proteins (PCO) beyond to antioxidant defenses such as levels of non-protein thiols (NPSH) and catalase activity (CAT) on lung, liver, and kidney tissues. Histological analysis of the lung was performed and bronchooalveolar lavage (BAL) was used to measure the levels of nitric oxide (NO) and inflammatory parameters. The comet assay, micronucleus test, and global DNA methylation were also assessed. To our knowledge, there are neither studies on the investigation of the melatonin influence (20 mg/kg; i.p.) to prevent oxidative damage induced by the FA inhalation exposure nor studies about the association between exposure to FA and global DNA methylation on mice.

Materials and Methods

Animals

Male adult Swiss mice (30–45 g) were kept in a temperature-controlled (22 ± 2°C) under a 12-h light/dark cycle with free access to water and food. The experiments were performed in accordance with national and international legislation (Guidelines of the Brazilian Council for Animal Experimentation—CONCEA and the US Public Health Service Policy on Human Care and Use of Laboratory Animals). The study was approved by the Institutional Animal Care and Use Committee of the Federal University of Santa Maria (process #4738290818).

Chemicals

Formaldehyde (HCHO) was obtained from Merck (Darmstadt, Germany). Melatonin was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical quality and obtained from standard commercial suppliers. Melatonin was dissolved in saline solution (0.9%) containing 5% ethanol.

Experimental design and FA exposure

An inhalation chamber with 16 L (32 cm × 25 cm × 20 cm) coupled to a nebulizer (Inalamax NS®, Brazil) was used to generate an air stream from an aqueous FA solution. Ambient concentrations of FA within the chamber were determined using an Umex-100 passive sampler (SKC Inc., Eighty Four, USA), and FA analysis on the sampler was performed by gas chromatography in accordance with ISO 16000-4- 2004.

The animals were randomly divided into nine experimental groups with seven animals in each and then subdivided into two experiments. For the Experiment 1, the animals received inhalation of FA or their vehicle: Control Group (animals received FA vehicle); Group 0.5 ppm (animals received FA 0.5 ppm); Group 1 ppm (animals received FA 1.0 ppm); Group 5 ppm (animals received FA 5.0 ppm); and Group 10 ppm (animals received FA 10.0 ppm). For the Experiment 2, the animals received melatonin (20 mg/kg—i.p.) or their vehicle (i.p.) and 30 min after injection received inhalation of FA or their vehicle: Control Group (animals received melatonin vehicle + FA vehicle); Group 5 ppm (animals received melatonin vehicle + FA 5.0 ppm); Group Mel (animals received melatonin + FA 5.0 ppm); and Group 5 ppm + Mel: animals received melatonin + FA 5.0 ppm). Inhalations were performed 4 h per day, 5 times a week for 4 weeks, according to the OECD Test 412 [41] (Fig. 1). FA doses used in this study were

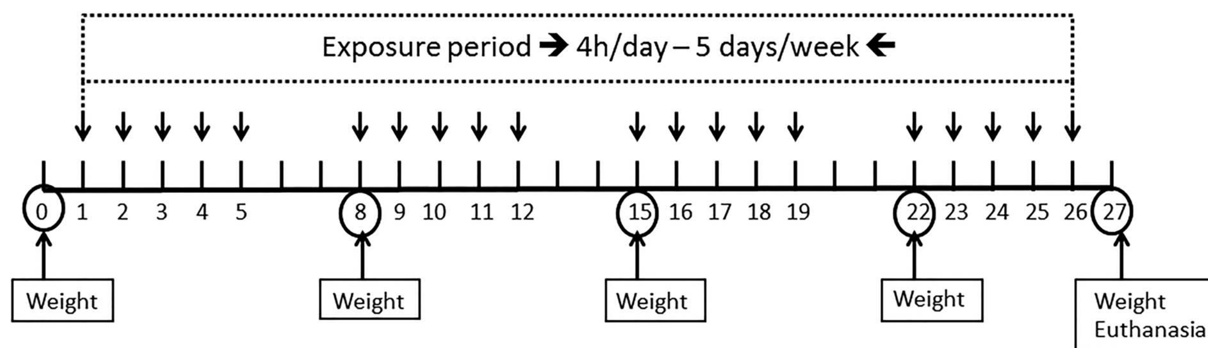

Figure 1: Experimental design.

chosen based on occupational exposure studies and reviews [42–46]. Regarding the melatonin dose, it was selected based on previous studies, indicating the nephroprotective activity observed in mice [32], in addition to the cardioprotective effect in rats [31]. After 24 h of the last FA exposure, the animals were euthanized with xylazine and ketamine (i.p.). Blood was collected directly from the heart ventricle. Subsequently, bronchoalveolar lavage (BAL) was collected and liver, kidneys, lungs, and femurs were removed. Tissue samples were homogenized in Tris/HCl 50 mM, pH 7.4 (1/10, w/v), and centrifuged at 2500 rpm for 10 min. The supernatants were separated and used for biochemical analysis. Total protein quantification was performed by the method described by Bradford [47] using bovine serum albumin as standard.

Collection and analysis of BAL

After euthanasia, the thorax of each animal was opened; the trachea was cannulated and perfused with 1 mL of PBS. The wash solution was collected, kept on ice until the end of the procedure to prevent cell lysis, and centrifuged at 2500 rpm for 10 min. The total cell number was determined in a Neubauer chamber using trypan blue staining. Giemsa stained slides were made for differential cell counting. The supernatant was used for the measurement of nitric oxide (NO) levels by the Griess method adapted to the Cobas Mira® automated system (Roche Diagnostics) according to Tatsch *et al.* [48] and the results expressed in μmol/L.

Biochemical marker

To assess liver toxicity, we determined the plasma activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) spectrophotometrically using Bioclin® kits according to manufacturer's specifications (Bioclin, Brazil).

Oxidative damage markers

Lipid peroxidation. Malondialdehyde (MDA) content, a measure of lipid peroxidation, was determined by testing the thiobarbituric acid reactive substances (TBARS) described by Buege and Aust [49]. To this end, 250 μL of tissue homogenates were incubated in a 100°C water bath with trichloroacetic acid (TCA) 10% and thiobarbituric acid (TBA) 0.6% for 15 min. They were then cooled on ice, added n-butanol, vortexed vigorously, and centrifuged at 3000 rpm for 10 min. The supernatant was used for absorbance reading in a spectrophotometer at 535 nm. Results were expressed in nmol TBARS/g tissue.

Carbonyl protein. Oxidative damage to proteins was evaluated by determining the carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH) according to the method described by Levine [50]. Briefly, proteins were precipitated by the addition of TCA 10%, redissolved in DNPH, and absorbance was read at 370 nm. Results were calculated using the extinction coefficient of 22 000 for aliphatic hydrazone. Results were expressed as nmol carbonyl protein/g tissue.

Non-enzymatic antioxidant defenses

Non-protein thiols. The levels of NPSH were assessed according to Ellman [51]. An aliquot of the homogenate was diluted with TCA 10% for protein precipitation and centrifuged. The deproteinized supernatant was incubated with potassium phosphate buffer (TFK 1 M pH 7.4), and 5,5 dithio-bis (2-nitrobenzoic acid) (DTNB) at room temperature. The developed yellow color was read immediately at 412 nm. Results were expressed as μM NPSH/mg protein.

Ferric Antioxidant Power Test. Ferric Antioxidant Power Test (FRAP) was determined according to the Benzie and Strain method [52]. The assay measures the ability of antioxidants to reduce the $[Fe\ (TPTZ)_2]^{3+}$ complex to the blue $[Fe\ (TPTZ)_2]^{2+}$ complex. Plasma was added to FRAP reagent (TPTZ 10 mM and $FeCl_3$ 20 mM in acetate buffer 300 mM, pH 3.6), and absorbance was measured at 593 nm. Ferrous sulfate ($FeSO_4$) was used as a standard. The antioxidant capacity of the samples was expressed in μMol Fe^{2+}/ml of plasma and was calculated by interpolating the absorbance values on the calibration curve.

Enzyme antioxidant defense

Catalase activity. CAT enzyme activity was determined according to Aebi [53]. Briefly, the reduction in the absorbance of a reaction mixture containing 30 mM hydrogen peroxide (H_2O_2) was measured at 240 nm for 90 s with a spectrophotometer. Results were expressed as CAT enzyme activity (IU/mg protein).

Genotoxicity

Comet assay. The alkaline comet assay is used for the detection of DNA strand breaks in cells or nuclei isolated from various

tissues that have been exposed to potentially genotoxic materials. Five microliters of heparinized whole blood were added to 95 µL of low melting agarose (0.75%). The mixture was spread on a slide coated with a normal melting (1.5%) agarose layer, covered with a coverslip, and stored at 4°C to solidify. After 2 h, the coverslip was removed and the slides were placed in a lysis solution (NaCl 2.5 M, EDTA 100 mM, Tris–HCl 10 mM, distilled water, DMSO 10%, and Triton X-100 1%) overnight. Subsequently, slides were incubated in an alkaline buffer (NaOH 300 mM and EDTA 1 mM, pH 13) and acclimatized for 20 min. The DNA was electrophoresed for 20 min at 25 V and 300 mA, and the buffer was neutralized with 0.4 M Tris (pH 7.5) for 15 min in the dark, dry, fixed in ethanol 96% for 5 min. After drying, the nucleoids were stained with ethidium bromide and examined at ×500 magnification under a fluorescence microscope (Olympus IX-71, Japan). Random images of 100 cells (50 cells from each of the two replicate slides) were analyzed from each animal. The damage score is based on tail moment and tail DNA amount and is considered a sensitive measure of DNA damage [54]. Cell quantification was performed using mean tail moments and DNA tail using free CometScore™ software.

Micronucleus test. Both femurs of each mouse were 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. Centrifugations and cell suspension were spread on slides and stained with MayGrunwald stain for 7 min, washed with water, and stained with Giemsa stain (diluted 1/10 with distilled water) for 1 min. The slides were washed with water and dried at room temperature. Polychromatic erythrocytes (immature erythrocytes) were stained light blue and normochromatic erythrocytes (mature erythrocytes without ribosome) were stained red-tiled. After staining, the slides were analyzed by light microscopy. The presence or absence of micronuclei (MN) was observed through the formation of small points near the cell nucleus. One thousand cells were counted for each sample in duplicates. Results were expressed as MN frequency per 1000 cells [54].

Global methylation

The DNA extraction from liver tissue was based on a method established by Lahiri and Numberger [55]. After the DNA extraction, the amount of DNA was quantified by a NanoDrop spectrophotometer and diluted to 2 µg of DNA in each sample. The samples treatment was based on established methods [56, 57]. After the treatment, the samples were centrifuged at 14 000 rpm for 5 min and 70 µL of the supernatant were injected in high-pressure liquid chromatography with diode array detection according to an established method by Barbosa *et al.* [5]. The relative content of 5-methyl-deoxy-cytidine (5mdC) was expressed as a percentage in respect to the total amount of deoxycytidine (dC) and was calculated according as Global DNA methylation (%) = [5mdC/(dC + 5mdC)] × 100.

Histopathological evaluations

After collecting the blood, the mice were euthanized under anesthesia and complete macroscopic evaluation of all body cavities was conducted. For histological analysis, the lungs were removed and immersed in buffered formalin fixative solution.

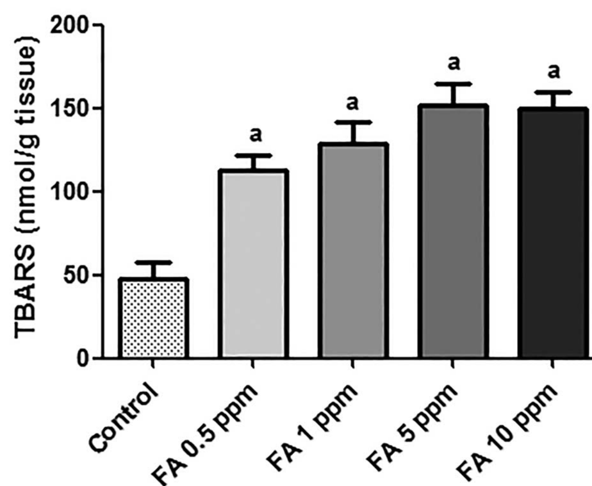

Figure 2: Level of lipid peroxidation in the liver of mice exposed to FA in different concentrations ($n = 7$). Data were expressed as mean ± SEM. [a]Significant difference in relation to the control group ($P < 0.0001$).

Subsequently, 5-µm-thick sections were prepared from paraffin-embedded slices and then stained with Masson-Goldner [58]. Stained slides were photographed using Zeiss AX10 microscopy (Carl Zeiss AG, Oberkochen, Germany).

Statistical analysis

Statistical analyzes were performed using the GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). Results were compared using one-way analysis of variance, followed by *post hoc* tests for multiple comparisons (Newman–Keuls test) and expressed as mean ± standard error of the mean (SEM). Differences between groups were considered significant when *P*-values were less than 0.05 ($P < 0.05$).

Results

Effect of FA and melatonin in the animals' weight

There were no significant changes in animals' weight in any group (data not shown).

Determination of FA concentration

Inhalation exposures with different FA concentrations were performed (0.5, 1, 5, and 10 ppm). Based on this experiment, it was observed that TBARS levels in the liver of mice exposed to the mentioned concentrations happens in a dose-dependent way (Fig. 2). It was observed significant differences among the groups exposed to FA. This way, as the highest FA concentrations (5 and 10 ppm), there was no difference in relation to hepatic TBARS levels, so we chose the 5 ppm concentration to continue the FA exposure experiments with melatonin.

Effect of FA and melatonin in the BAL

The results showed that 5-ppm FA induced an increase in the number of cells in BAL, but this increase was not significant in relation to other groups ($P > 0.05$). In addition, it was observed that the percentage of lymphocytes and monocytes did not

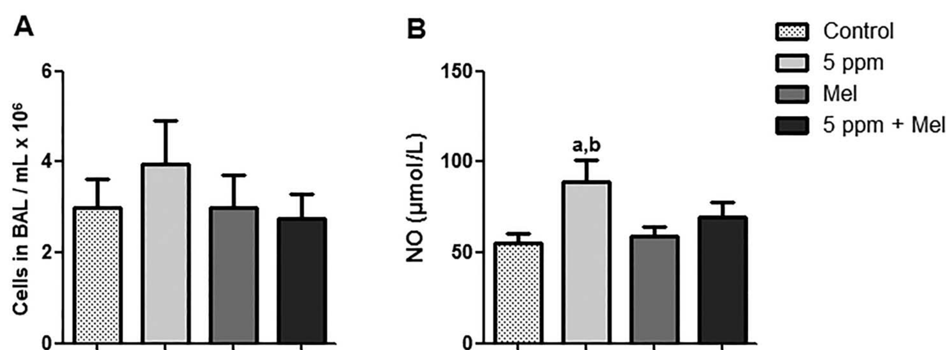

Figure 3: Effect of FA and melatonin in BAL. (A) Inflammatory cells in BAL of mice. (B) NOx levels in BAL of mice. Values expressed as mean ± SEM. [a]Significant difference in relation to the control group ($P < 0.05$); [b]Significant difference in relation to Mel group ($P < 0.05$).

significantly differ among groups (data not shown). Besides, it was possible to observe that the 5 ppm + Mel group had a decreased number of cells in BAL; however, this decrease did not show any significant difference in relation to other groups ($P > 0.05$) (Fig. 3A). It was quantified the NO levels in the BAL supernatant. Figure 3B shows that the exposition to 5-ppm FA (89.33 ± 11.79 µmol/L) increased NO levels in comparison to the control group (55.60 ± 5.20 µmol/L) and to Mel group (59.00 ± 5.51 µmol/L) ($P < 0.05$). Melatonin reduced NO levels in the 5 ppm + Mel group (69.60 ± 8.23 µmol/L) when compared with the 5-ppm group; therefore, this reduction had no significant difference ($P > 0.05$).

Effect of FA and melatonin in lung tissue

According to data shown in Fig. 4A, TBARS levels in the lung were significantly higher in the group exposed to 5 ppm FA (161.6 ± 14.21 nmol/g tissue) in relation to the control group (108.5 ± 9.85 nmol/g tissue) ($P < 0.05$). The 5 ppm + Mel group showed a decrease in TBARS levels (123.7 ± 11.32 nmol/g tissue); however, this effect did not present significant difference in comparison to 5 ppm FA group ($P > 0.05$).

Regarding NPSH levels in lung tissue, the exposition to 5-ppm FA decreased this marker (4.44 ± 0.2 µM/mg ptn) when compared with the control group (8.34 ± 0.74 µM/mg ptn) ($P < 0.01$). In the 5 ppm + Mel group, although no significant, NPSH levels presented an increase (5.75 ± 0.71 µM/mg ptn) when compared with 5-ppm FA group; however, it did not reestablish the control group levels (Fig. 4B). Besides this, histological analysis (Fig. 4C–F) showed damage to the lung tissue observed in the thickening of alveolar walls and consequent decrease in alveolar lumen plus an increase in the infiltration of inflammatory cells in the 5-ppm group. In the 5 ppm + Mel group, these damages were reduced.

Effect of FA and melatonin in plasma of mice

It was observed that AST activity, determined in plasma, increased in the 5-ppm group; however, there was no significant difference among groups ($P > 0.05$). In addition, animals in the 5 ppm + Mel group had similar AST levels to the control group and Mel group (Fig. 5A). The ALT activity did not show significant difference among groups ($P > 0.05$) (Fig. 5B).

Figure 5C shows the plasma antioxidant capacity measured through the FRAP reducing power. It was observed that, although the exposure to 5-ppm FA induced a decrease in FRAP levels and the pretreatment with melatonin increased this measure, no significant difference was observed. Besides, the group treated only with melatonin presented higher antioxidant power when compared with other groups.

Effect of FA and melatonin in hepatic and renal tissues

Table 1 shows the oxidative stress markers in hepatic and renal tissues. Regarding the liver, it was observed that 5-ppm FA exposition promoted a significant increase in TBARS ($P < 0.0001$) and PCO ($P < 0.05$) levels when compared with the control group. In addition, melatonin decreased TBARS ($P < 0.05$) and PCO ($P < 0.01$) levels in the 5 ppm + Mel group with the last parameter being almost restored to the levels presented in the control group. Regarding TBARS and PCO levels in the kidney, it was observed a small decrease in the 5-ppm FA group in relation to the control group and an increase in the 5 ppm + Mel group in relation to the 5-ppm FA group (Table 1).

Considering the NPSH levels, the exposition to 5-ppm FA caused a decrease in NPSH levels in the liver and kidney when compared with the control group. Such difference was only significant in the liver. The pretreatment with melatonin failed to reverse the NPSH in both tissues (Table 1). No significant change was observed among the treated groups in relation to CAT enzyme activity; however, the exposure to 5-ppm FA decreased the activity of this enzyme in both liver and kidney and the pretreatment with melatonin increased its activity in the kidney (Table 1).

Effect of FA and melatonin in genotoxicity

In comet assay, the DNA strand break in the cells was expressed in the tail moment. According to Fig. 6A, the results showed that the exposition to 5-ppm FA significantly increased the magnitude of the DNA strand break when compared with the control group ($P < 0.01$). However, melatonin was unable to protect this DNA damage. Observing the MN frequency in bone marrow cells, a significant increase was seen in the 5-ppm FA group when compared with the control group ($P < 0.05$). Melatonin was able to prevent MN formation in bone marrow cells of the animals exposed to 5-ppm FA, but this reduction in MN frequency was not significant (Fig. 6B).

Global DNA methylation

Regarding the global DNA methylation (Fig. 6C) was observed that the global DNA methylation levels increased in the 5-ppm group. However, there was no significant difference among

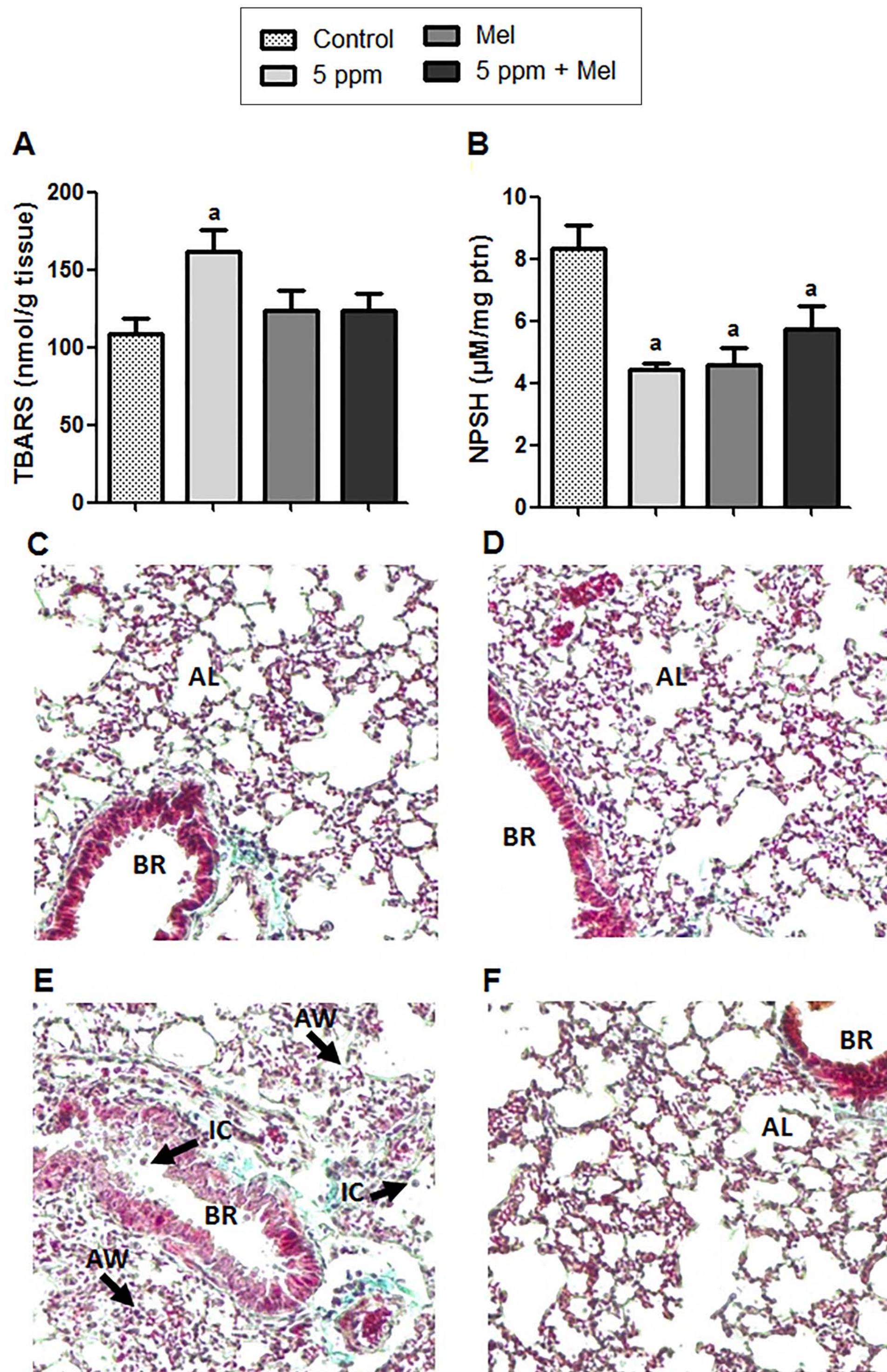

Figure 4: Effect of FA and melatonin in lung tissue. (A) TBARS levels. (B) Levels of non-protein thiols (NPSH). (C–F) Histology of control groups, Mel, 5 ppm and 5 ppm + Mel, respectively; 10x. Data expressed as mean ± SEM. [a]Significant difference in comparison to the control group ($P < 0.05$); BR represents the bronchioles; IC represents inflammatory cells; AW represents the thickening of alveolar wall; AL represents the alveolar lumen.

groups ($P > 0.05$). The global DNA methylation levels were 2.56, 3.12, 2.26, and 2.61% for control, 5 ppm FA, Mel, and 5 ppm + Mel, respectively. In the 5 ppm + Mel group, the global DNA methylation showed a decreased when compared with 5-ppm FA group; however, this reduction was not significant ($P > 0.05$).

Discussion

It is known that FA is a substance that causes toxicity not only in the upper respiratory tract [59] but also in several organs as lung [60], liver [25], and kidney [61]. The concentration of 5-ppm FA was chosen based on experiment 1, which showed that

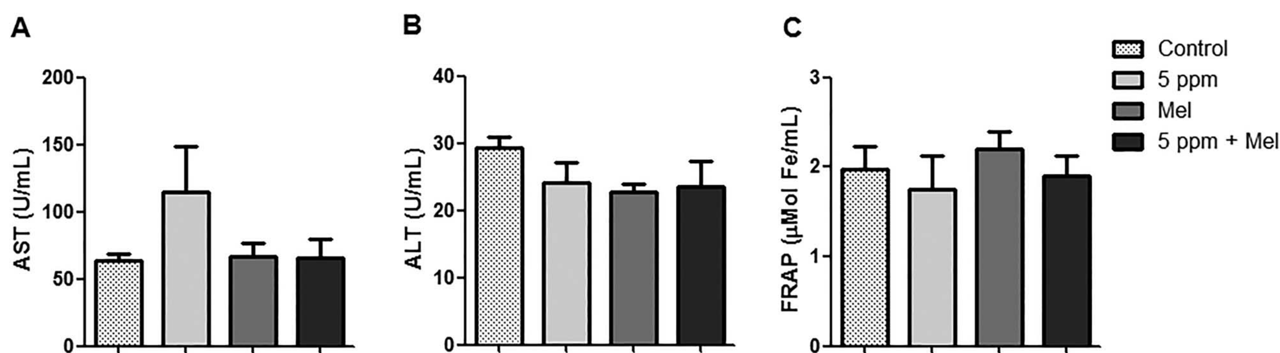

Figure 5: Effect of FA and melatonin in plasma of mice. (A) AST activity. (B) ALT activity. (C) FRAP. Data were expressed as mean ± SEM. There were no significant differences among groups.

Table 1: Effect of FA and melatonin in oxidative stress parameters in mice

aa	Groups	TBARs nmol/g tissue	PCO nmol/g tissue	NPSH μM/mg protein	CAT μmol/mg protein
Liver	Control	40.84 ± 7.80	1.89 ± 0.15	30.46 ± 4.53	25.68 ± 3.85
	5 ppm	152.1 ± 13.00[a,b]	3.25 ± 0.31[a,b]	15.28 ± 3.25[a]	22.24 ± 1.09
	Mel	64.26 ± 11.56	1.86 ± 0.52	19.21 ± 2.93[a]	21.48 ± 2.15
	5 ppm + Mel	112.5 ± 12.34[a,b,c]	1.97 ± 0.23[c]	12.97 ± 2.35[a]	21.17 ± 2.05
Kidney	Control	191.0 ± 10.30	3.71 ± 0.37	16.90 ± 1.56	25.17 ± 2.22
	5 ppm	182.2 ± 12.32	2.11 ± 0.41[a]	11.41 ± 2.76	22.39 ± 2.31
	Mel	176.6 ± 13.29	3.11 ± 0.43	16.86 ± 1.37	23.20 ± 2.98
	5 ppm + Mel	190.5 ± 12.33	1.75 ± 0.49[a]	9.26 ± 0.29[a,b]	24.44 ± 1.71

Data are represented as mean ± SEM.
[a]Significant difference in relation to the control group.
[b]Significant difference in relation Mel group.
[c]Significant difference in relation 5 ppm group; differences were considered significant when $P < 0.05$.

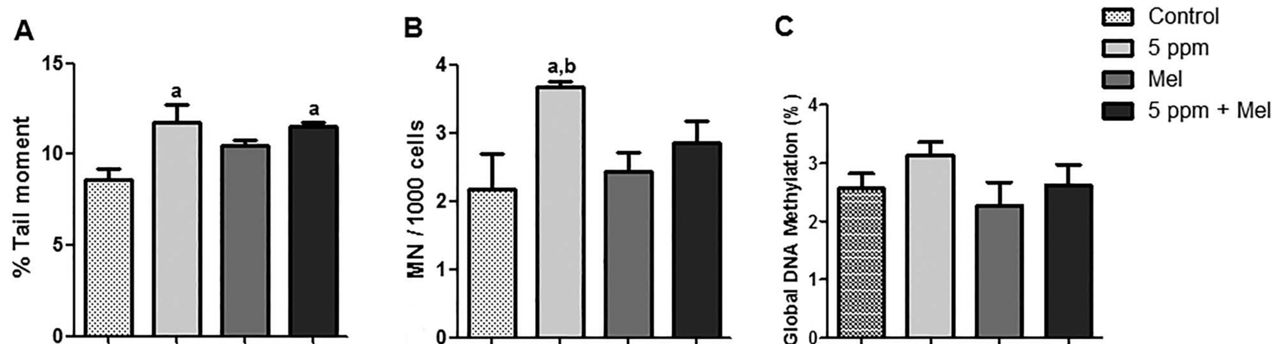

Figure 6: DNA damage. (A) Comet assay. (B) Micronucleus. (C) Global DNA methylation. Data were expressed as mean ± SEM. [a]Significant difference in relation to the control group ($P < 0.05$); [b]Significant difference in relation to Mel group ($P < 0.05$).

TBARS levels in the liver of mice exposed to different FA concentrations happens in a dose-dependent way (Fig. 2). Besides, 5 and 10 ppm did not differ in the observed effect, justifying the use of 5 ppm concentration to perform the experiments with melatonin. Regarding the melatonin dose applied in this study, it was based on recent studies where nephroprotective activity was observed in mice [32], in addition to the cardioprotective effect in rats [31]. To our knowledge, there are no studies on melatonin influence (20 mg/kg; i.p.) to prevent oxidative damage induced by the FA inhalation exposure associated with epigenetic studies on mice.

In this study, it was evaluated the FA effects on different parameters as oxidative stress, inflammation, and genotoxicity, as well as the melatonin activity to prevent the damage caused by the inhalation exposure to 5-ppm FA. The main results showed that the inhalation exposure to 5-ppm FA caused an increase in NO levels and inflammatory cells recruited for BAL. Besides, the pretreatment with melatonin reduced both parameters. Oxidative stress in the lung was evident and characterized by an increase in TBARS levels and a decrease in NPSH levels in animals exposed to 5-ppm FA. It was observed that the pretreatment with melatonin before inhaling FA decreased TBARS levels and increased NPSH levels. Regarding DNA damage, it was observed that melatonin decreased MN formation in bone marrow cells in animals exposed to FA. Thus, it was demonstrated that FA triggered oxidative damage in lungs and liver of mice and such damage may be prevented with melatonin administration. Studies show that FA inhalation exposure induces airway inflammation [24, 62, 63]. In this context, it was evaluated the presence of inflammatory cells in the lung parenchyma of mice submitted

to FA exposure and to the treatment with melatonin analyzing the total and differential counts of inflammatory cells in BAL.

Results showed a higher number of cells recruited for BAL in mice exposed to FA; however, there was no significant difference. In agreement with the results found in this study, Fujimaki *et al.* [64] investigated the effects of FA exposure in low concentrations (40, 80, and 2000 ppb for 12 weeks) on the inflammatory response in mice. It was demonstrated that FA exposure did not cause an increase in inflammatory cells in BAL. Another study, carried out by Maiellaro *et al.* [65], also found no difference in the number of cells recruited for BAL among pregnant rats exposed to 0.75-ppm FA for 21 days. In contrast to what was observed in BAL cells, other authors demonstrated an increase in inflammatory cells in lungs of rats exposed to FA associated with an increase in macrophages, lymphocytes, and neutrophils [24, 60]. In both studies, the used FA concentrations were higher (1, 5, and 10% for 5 days) when compared with those used in this study. In addition, the increase in the number of BAL leukocytes from rats exposed to FA can occur in a time-dependent way as shown by the study of Lino dos Santos Franco *et al.* [66]. This way, the first line of defense against harmful agents is the mobilization of leukocytes, which migrate to the inflammatory site through the endothelial cell barrier. Thus, mice exposed to FA 5 ppm to inhalation exposure for 28 days showed a modest inflammatory response.

It is known that NO is a modulator of the leukocyte-endothelial cell interaction, controlling leukocyte adhesion to the endothelium during the inflammatory process [67]. In addition, NO can be eliminated by ROS to form the peroxynitrite anion, which can cause oxidative damage and it also play a role in airway inflammation [23, 24, 68]. Our results showed an increase in NO levels in BAL due to the exposure to FA. Our findings according to Lino dos Santos Franco *et al.* [67] who observed an exacerbated release of nitrites by BAL cells in rats exposed to 1% FA for 3 days. Likewise, mice exposed for 90 days to a mixture of volatile organic compounds had increased NO levels in the lung, as observed by Wang *et al.* [23]. Based on these reported studies, FA has the potential to cause irritation and injury to the lung parenchyma, triggering an inflammatory process in a non-specific manner.

This study showed that FA increased the cellular inflow in the lung, what was evidenced by the increase in NO levels and the leukocytes amount in BAL. Additionally, it was observed in the differential count that the leukocytes recruited in the lung were composed of mononuclear cells. Regarding lung histology, it was observed an increase in the infiltration of inflammatory cells and a thickening of the airway walls with visible remodeling of the airways probably due to the oxidative damage induced by FA exposure. According to our findings, Murta *et al.* [60] reported an increase in the inflammatory response plus an increase in the area of the alveolar lumen and a decrease in density of the volume of alveolar septa. These same results were supported by Liu *et al.* [69] that found the same effects cause by FA inhaling exposure.

Oxidative stress occurs in the cell when the production of oxidants is greater than the ability to remove them by endogenous antioxidants. The produced ROS react with several biological macromolecules, including membrane lipids, proteins, and nucleic acids, changing their functions [70–72]. Oxidative stress is considered one of the potential mechanisms behind FA-induced systemic toxicity [25, 60, 73–75]. In this sense, to evaluate the effect of melatonin on the damage caused by FA exposure, some important biomarkers of oxidative stress in the pulmonary, hepatic, and renal tissues were evaluated.

In the present study, mice exposed to 5-ppm FA presented elevated levels of TBARS in lung and liver, while elevated levels of PCO were found in the liver. TBARS is an indicator of lipid peroxidation, a result from the reaction of ROS with phospholipids present in biological membranes, and PCO formation, on the other hand, is the reaction of ROS with proteins [76, 77], indicating that lipid and protein damage were induced in these tissues. The results are in line with previous studies where high levels of lipid peroxidation and PCO were observed in different rodent tissues [22, 60, 75, 78, 79].

When oxidative stress occurs, cells try to neutralize oxidative effects and restore redox balance, activating or silencing genes that encode defensive enzymes, transcription factors, and structural proteins [70]. The antioxidant defense system operates through enzymatic and non-enzymatic components [22]. Considering this, CAT activity was evaluated together with non-enzymatic antioxidants such as NPSH levels in tissues and FRAP in plasma. There were no significant changes in CAT activity in the liver and kidney exposed to FA. According to our findings, Ramos *et al.* [79] did not observe significant changes in CAT in the renal tissue of *Fisher* rats exposed to FA at 1, 5, and 10% for 5 days. Similarly, Lino dos Santos Franco *et al.* [24] showed no difference in CAT activity in the lung of *Wistar* rats after 3 days of exposure to 1% FA.

Regarding NPSH levels, results showed that FA exposure triggered a decrease in this marker in the liver, kidney, and lung. This result is corroborated by Brandão *et al.* [80] that found an NPSH levels decrease in testicles of mice exposed to cadmium chloride. GSH, the main NPSH in tissues, acts as a free radical scavenger, helps in the regeneration of other antioxidants [80], and plays a vital role as a coenzyme in the detoxification of many chemicals, including FA [71, 81]. This way, FA exposure may have depleted GSH in cells. This was observed by a decreasing in NPSH levels in lung and liver, interrupting the balance between oxidants and antioxidants and causing oxidative stress, which was observed by the increase in TBARS and PCO in these tissues.

FA is a strong mutagen that induces damage to oxidative bases and breaks in the DNA strand and DNA-protein crosslinks [5, 82]. In this study, genetic damage was assessed using comet assay and MN test. Results showed an increase in the tail moment and MN frequency with the exposure to FA. Liu *et al.* [83] observed that the exposure to FA 1 at 10 mg/m^3 for 20 weeks increased MN frequency in bone marrow of mice, but the difference was not significant. In an *in vitro* study with rat bone marrow mesenchymal stem cells, comet assay showed that FA-induced DNA strand breakage increased in a dose-dependent way [84].

Epigenetic changes [DNA methylation, histone modifications, and microRNA (miRNA) expression] can regulate gene expression without changes DNA sequence [85, 86]. DNA methylation is the most studied epigenetic modification in mammals [87, 88] and includes hypomethylation, which can lead to gene overexpression and hypermethylation, which can cause silencing of gene expression [88–90]. It is known that exposure to toxic agents is associated with DNA methylation changes, which may be increased, as demonstrated in the study by Qiu *et al.* [91] in rats exposed to vinyl chloride and in the study by Li *et al.* [88] with mice exposed to particulate matter 2.5. Regarding FA, epigenetic studies have shown that FA can lead to changes in the expression and activity of DNA methyltransferases (DNMTs), which are enzymes that are responsible for maintaining methylation status in the genome and that catalyze the transfer of a methyl fraction of S-adenosyl-Lmetionin (SAM) for the cytosine of a CpG dinucleotide [92]. Liu *et al.* [92] showed that long-term exposure to

FA reduced DNA methylation in 16HBE cells. On the other hand, Barbosa *et al.* [5] observed an increase in DNA methylation in individuals occupationally exposed to FA. However, studies on the influence of FA on global DNA methylation in mice are still scarce, and to our knowledge, our study is the first that evaluates this biomarker. Regarding the epigenetic results, we did not find a significant difference among the groups, but our study showed a trend toward an increase in global DNA methylation levels in the mice exposure to FA. In this line, more research is required to extend this study to better understand the mechanisms of epigenetic changes induced by the FA.

Studies have reported that the use of antioxidants prevented the lipid and protein damage resulting from FA. Gurel *et al.* [22] observed that the treatment with vitamin E showed an inhibitory effect of lipid peroxidation and protein oxidation on the frontal cortex and hippocampus of rats that received FA 10 mg/kg (i.p.) for 10 days. Zararsiz *et al.* [26] used omega 3 along with FA 10 mg/kg (i.p.) for 14 days and found a reduction in lipid oxidation in the renal tissue of *Wistar* rats. These findings are in agreement with the results of this study, which showed a reduction in lipid peroxidation levels and protein oxidation by applying melatonin.

Melatonin is especially effective as an antioxidant because it uses a wide variety of means to reduce oxidative stress [93], since it has several characteristics of an ideal antioxidant [94]. Due to its small molecular size and amphiphilic properties, its penetration into subcellular compartments is facilitated [30, 94, 95], which makes it present in adequate amounts in cells. Thus, melatonin eliminates several toxic reagents, including the hydroxyl radical (OH) and the peroxynitrite anion ($ONOO^-$) (two main initiators of the peroxidation of fatty acids), and also takes advantage of its metabolites resulting from its reaction with ROS, in which also they are highly efficient radical scavengers [30, 93–95]. These effects are because the metabolism of melatonin, in addition to being carried out by enzymatic processes (CP450), is also performed through its interaction with ROS and reactive nitrogen species [96, 97]. In addition, melatonin has the ability to stimulate the activity of antioxidant enzymes including glutathione peroxidase and glutathione reductase, in addition to increasing glutathione synthesis [28].

In this context, it is possible to suggest that melatonin inhibited lipid peroxidation in the lung and liver as well as the formation of carbonyl proteins in the liver. This antioxidant effect of melatonin can be explained by its property of directing eliminating reactive oxygen and nitrogen species [28, 29] through a cascade reaction where the metabolites produced [2-OH-melatonin, 3-OH-melatonin-cyclic, 4-OH-melatonin, N1-acetyl-N 2-formyl-5-methoxyquinuramine (AFMK) and N1-acetyl-5-methoxyquinuramine (AMK)] neutralize reactive species [30, 93, 96]. Besides, due to its small molecular size and amphiphilic properties that facilitate its penetration in subcellular compartments, this substance can be incorporated into a superficial spot in the lipid layers of the membrane near the polar heads of these molecules, consequently protecting lipid molecules from being attacked by reactive species [30].

It is known that high levels of ROS are among the leading causes of DNA damage, in such a way that this oxidative damage to DNA can compromise genomic integrity. Thus, due to its antioxidant capacity, several mechanisms may be behind the protection of melatonin against damage to DNA, including the direct elimination of ROS and indirectly by stimulating antioxidant enzymes or modulating the repair pathways [98, 99]. Its amphiphilic property allows it to quickly cross morphophysiological barriers and provide local protection to DNA against locally generated ROS [93, 98, 100]. Pérez-González *et al.* (2018) found that melatonin metabolites can repair radical cations centered on guanine by transferring electrons to oxidized sites and radicals centered on C, the sugar portion of 2′-deoxyguanosine (2dG) by hydrogen atom transfer. In addition, the 6-hydroxymelatonin and 4-hydroxymelatonin metabolites must also repair OH adducts in the imidazole ring. Thus, these study's results strongly suggest that the role of melatonin in preventing DNA damage can be mediated by its ability, combined with that of its metabolites, to directly repair oxidized sites in DNA through different chemical routes.

Regarding epigenetic changes, our results showed that pretreatment with melatonin tended to decrease overall DNA methylation, although without significant difference. A study by Ozen *et al.* [37] found that melatonin decreased FA-induced apoptosis in rat testicles. In addition to being an antioxidant, melatonin is probably an epigenetic regulator, as it and its metabolites can regulate DNMTs, as well as apoptosis, either by masking target sequences or by blocking the enzyme's active site [101]. This fact is because melatonin reaches intracellular organelles, including the nucleus, where it accumulates and interacts with specific nuclear binding sites [30, 71, 94, 102]. Thus, it can be suggested that melatonin can protect against changes in DNA methylation induced by FA.

In contrast, pretreatment with melatonin did not significantly change NPSH levels or CAT activity in the tissues. However, in the lung, there was a slight but insignificant increase in NPSH. This lack of effect of melatonin in increasing the activity of antioxidant enzymes may be the result of a reduced ROS rate due to the potent elimination of direct free radicals by the melatonin itself. Thus, we suggest that the main mechanism by which melatonin shows antioxidant activity, under the conditions of this study, was due to its cascade mechanism in which the interaction with free radicals leads to the formation of secondary and tertiary metabolites. These metabolites are capable of neutralizing toxic derivatives of oxygen and nitrogen, also playing an important role in maintaining the integrity of the genome.

Regarding the limitations of this study, it is highlighted that variable tissue responses may reflect differences in FA levels. Different findings among studies may happen due to the use of different species of rats or mice, the doses applied, time intervals, and test conditions. In addition, differently from this study, most of the works mentioned have evaluated FA effects in a short period of exposure and sometimes with higher doses than the ones necessary to induce detectable toxic effects. Another point to consider was the use of a full body inhalation chamber, which makes the airflow and the transport of inhaled FA throughout the respiratory tract different for each animal. Therefore, further studies on chronic and controlled exposure are needed to determine FA toxic effects, the protective effects of melatonin, and related parameters.

Conclusion

The findings of this study showed that the exposure to 5-ppm FA in mice resulted in oxidative damage to the lung and liver tissues. Also, an increase in NO levels, infiltration of inflammatory cells, and a thickening of the airway walls were observed. Besides, FA significantly induced DNA damage. Moreover, pretreatment with melatonin reduced oxidative damage and inflammatory in lung and liver tissues and prevented DNA damage in mice. Therefore, our results demonstrated that FA exposure with repeated doses might induce oxidative damage, inflammatory and genotoxic effects, and melatonin seems to minimize some of these toxic effects caused by FA inhalation in mice.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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