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Oxidative effects on lung inflammatory response in rats exposed to different concentrations of formaldehyde☆

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ABSTRACT

The formaldehyde (FA) is a crosslinking agent that reacts with cellular macromolecules such as proteins, nucleic acids and molecules with low molecular weight such as amino acids, and it has been linked to inflammatory processes and oxidative stress. This study aimed to analyze the oxidative effects on pulmonary inflammatory response in Fischer rats exposed to different concentrations of FA. Twenty-eight Fischer rats were divided into 4 groups (N = 7). The control group (CG) was exposed to ambient air and three groups were exposed to different concentrations of FA: 1% (FA1%), 5% (FA5%) and 10% (FA10%). In the Bronchoalveolar Lavage Fluid (BALF), the exposure to a concentration of 10% promoted the increase of inflammatory cells compared to CG. There was also an increase of macrophages and lymphocytes in FA10% and lymphocytes in FA5% compared to CG. The activity of NADPH oxidase in the blood had been higher in FA5% and FA10% compared to CG. The activity of superoxide dismutase enzyme (SOD) had an increase in FA5% and the activity of the catalase enzyme (CAT) showed an increase in FA1% compared to CG. As for the glutathione system, there was an increase in total glutathione (tGSH), reduced glutathione (GSH) and oxidized glutathione (GSSG) in FA5% compared to CG. The reduced/oxidized glutathione ratio (GSH/GSSG) had a decrease in FA5% compared to CG. There was an increase in lipid peroxidation compared to all groups and the protein carbonyl formation in FA10% compared to CG. We also observed an increase in CCL2 and CCL5 chemokines in the treatment groups compared to CG and in serum there was an increase in CCL2, CCL3 and CCL5 compared to CG. Our results point out to the potential of formaldehyde in promoting airway injury by increasing the inflammatory process as well as by the redox imbalance.

1. Introduction

The increased morbidity and mortality of the respiratory inflammatory diseases such as asthma, emphysema and bronchitis have been correlated with the exposure of individuals to the environmental pollutants including particulate matter and chemical substances such as formaldehyde (FA) (Lambert et al., 2003; Fujimaki et al., 2004; Green-McKenzie and Hudes, 2005; Ezratty et al., 2007). Pollutants may cause injury to the airway epithelium, inducing changes in the local immune response, favoring the induction and maintenance of inflammation as in asthma, and even causing lung cancer and cardiovascular disease (Dales and Raizenne, 2004; Güleç et al., 2006; Sul et al., 2007). In particular health professionals as well as all individuals that leads with

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methodologies applied in anatomy, pathology and histology field are in potential risk to the damage actions of the FA due to their labor activities (Wantke et al., 2000).

FA is one of the most studied chemicals nowadays (NAS, 2007) and occurs as a natural product in most living systems and the environment and it has been used in aqueous solution as a disinfectant and preservative and more recently in various industrial applications, including the production of adhesives, timber covers, plastic, textiles, leather, chemical manufacturing (Bosetti et al., 2008), building materials and cosmetics (Li et al., 2007). It is also found in emissions from cars due to the use of methanol as an alternative to fossil fuels because its enzymatic biotransformation generates FA, the kitchen gas burning (Lino dos Santos Franco et al., 2009) and tobacco smoke (Li et al., 2007). FA causes local irritation; skin sensitization after acute and sub-acute exposure in animal experiments and in humans (Swenberg et al., 1980); irritation to the eyes, nose, throat and airway; mild neuropsychological disorders (Ezratty et al., 2007) and is a potent trigger of inflammation of the lower airways (Lima et al., 2015; Bardana and Montanaro, 1991), in particular mediated by eosinophils, neutrophils and phagocytic cells that amplify this inflammatory process through inflammatory cytokines and chemokines (Persoz et al., 2010, 2012).

FA is a well-known crosslinking agent that reacts with cellular macromolecules, such as proteins, nucleic acids and amino acids with cytotoxic, haematotoxic, immunotoxic and genotoxic effects (Cheng et al., 2003; Liteplo and Meek, 2003; Metz et al., 2004). It is also able to induce DNA damage, growth inhibition and DNA repair delay after UV irradiation in various types of human cells (Grafstrom et al., 1983, 1984; Cosma and Marchok, 1988; Emri et al., 2004). In 2006, the International Agency for Research on Cancer (IARC) classified inhaled FA as a chemical able to cause nasopharyngeal cancer in humans. Recently, the IARC highlights that FA can cause leukaemia in humans, expanding the last classification from 2006 (IARC, 2006, 2012).

Exposure to chemical and physical agents as well as cellular aerobic metabolism and inflammation are sources of reactive oxygen species (ROS), including singlet oxygen, hydrogen peroxide, superoxide anion and hydroxyl radical (Lima et al., 2015). These reactive species are important mediators of cell damage, play an important role in the redox imbalance and may contribute to a variety of disorders (Halliwell, 1997). Polyunsaturated fatty acids associated with the membrane are likely to react with ROS, resulting in lipid peroxidation (Campos et al., 2013). This process can interfere with the fluidity of the membrane and cellular compartments, which can lead to cell lysis (Datta and Namasivayam, 2003). In this context, our study aimed to analyze the oxidative effects on pulmonary inflammatory response in Fischer rats exposed to different concentrations of FA.

2. Methods

2.1. Experimental design

Eight-week-old male Fischer rats were housed under controlled conditions in standard laboratory cages (Laboratory of Experimental Nutrition, Department of Food, School of Nutrition, Federal University of Ouro Preto – UFOP) and given free access to water and food. All *in vivo* experimental protocols were approved by the ethics committee (#2011/01) from UFOP. Rats (N = 28) were evenly divided into 4 groups: control group exposed to ambient air (CG), group exposed to 1% formaldehyde (FA1%), group exposed to 5% formaldehyde (FA5%) and group exposed to 10% formaldehyde (FA10%) using an inhalation chamber with 30 L (25 cm × 30 cm × 40 cm) coupled to an ultrasonic nebulizer (Unique Group, Indaiatuba, São Paulo, Brazil). The exposures of 20-min were performed during three times a day for five consecutive days, totaling 60 min/day (Lima et al., 2015; Maiellaro et al., 2014; Oliveira et al., 2015).

2.2. Assessment and analysis of Bronchoalveolar Lavage Fluid (BALF)

Immediately after euthanasia, the chest of each animal was opened to collect BALF. The left lung was clamped, and the right lung and the trachea were cannulated and perfused with 3 ml of saline solution (3 × 1.000 μl). The samples were kept on ice until the end of the procedure to avoid cell lysis. Total mononuclear and polymorphonuclear cell numbers were previously stained with trypan blue and determined in a Neubauer chamber. A differential cell count was performed on cytospin preparations (Shandon, Waltham, MA, USA) stained with fast panoptic coloration kit (Laborclin, Pinhais, Paraná) (Bezerra et al., 2011).

2.3. Assessment of hematological paramenters

For the complete blood count, the blood was diluted with saline (1:2) and erythrocyte hematological parameters, hematocrit and hemoglobin were evaluated using electronic counting device (ABX diagnostics, micro 60) of the Pilot Clinical Analysis Laboratory (LAPAC-UFOP).

2.4. Processing and homogenizing tissue

After BALF collection, the right ventricle of each rat was perfused with saline to remove blood from the lungs. The right lung was clamped so that just the left lung could be perfused with 4% buffered formalin (pH 7.2) at a pressure of 25 cm H_2O for 2 min via the trachea. The left lung was removed and immersed in a fixative solution for 48 h. The material was then processed as follows: tap water bath for 30 min, 2 baths in 70% and 90% alcohol each for 1 h, 2 baths in 100% ethanol for 1 h each, and embedding in paraffin. Serial 5-μm sagittal sections stained with hematoxylin and eosin were obtained from the left lung for histological analyses. After the removal of the left lung for histology, the right lung was immediately removed and stored in crushed ice in labeled tubes. The lungs were subsequently homogenized in 1 ml potassium phosphate buffer, pH 7.5 and centrifuged at 1500 × *g* for 10 min. The supernatant was collected, and the final volume of all samples was adjusted to 1.5 ml with phosphate buffer. The samples were stored in a freezer (−80 °C) for biochemical analyses.

2.5. Immunoassays for inflammatory markers

Plasma and lung tissue homogenates were used for evaluation of the inflammatory chemokines CCL2, CCL3 and CCL5. The immunoassays were performed in 96-well plates which were added 100 of monoclonal antibody to the protein (or peptide) of interest, diluted in PBS containing 0.1% bovine serum albumin - BSA (SIGMA). After incubation for 12 h at room temperature, the unabsorbed antibodies were discarded and the plates blocked with 300 uL/well of a PBS solution containing 1% BSA for 1 h at 37 °C. The plasma samples/or the supernatant were applied in a volume of 100 μl to each well. The reading of the marking intensity was performed on ELISA reader using a wavelength of 490 nm (Martins et al., 2013). All the chemokine ELISA kits were purchased from Peprotech (Ribeirão Preto, Brazil) and particularities in this methodology performed according to the manufacturer.

2.6. Oxidative stress biomarkers in lung homogenates and antioxidant defense

As an index of lipid peroxidation, we used the formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction as previously described by Draper et al. (1993). Briefly, the TBARS level was estimated in accordance with the method described by Buege and Aust (1978). Carbonyl protein levels were determined according to the method described by Levine et al. (1994). Lung homogenates were used to determine CAT activity. This method was based on the enzymatic decomposition of H_2O_2 observed spectrophotometrically at 240 nm for 5 min. The results were expressed as activity per milligram of protein. One unit of CAT was equivalent to the hydrolysis of 1 μmol of H_2O_2 per minute (Aebi, 1984). The SOD enzyme activity was estimated by the inhibition of auto-oxidation of adrenaline measured spectrophotometrically at 480 nm (Bannister and Calabrese, 1987). Glutathione is present in the cells mainly in its reduced form (GSH) representing around 90% and the remainder appears in the form of oxidized glutathione (GSSG). This assay was adapted from commercial kit Sigma # CS0260, and used a kinetic method for measuring the total levels of glutathione (GSH + GSSG) in biological samples by reducing the 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in TNB. The total protein content was determined according to the method described by Lowry et al. (1951), using bovine serum albumin as a standard.

2.7. Reactive oxygen species production by neutrophils

2.7.1. Isolation of neutrophils

Blood was obtained by cardiac puncture and collected in heparinized tubes. Neutrophils were isolated using two gradients of different density, Monopaque (d = 1.08) and Leucopaque (d = 1.12), in accordance with the procedures described by Bicalho et al. (1981) with minor adjustments. The cell viability of each sample was higher than 95%, as determined by the trypan blue exclusion test.

2.7.2. Chemiluminescence assay

To measure ROS production, chemiluminescence assays were carried out as described by Martins Chaves et al. (2000). For each assay, 1×10^6 neutrophils were incubated in Hank's solution (pH 7.4) with 500 μL of luminol (10^{-4} M). Photon emission was determined at each minute for 30 min using a luminometer (Lumat^3 LB 9507; Berthold Technologies, Bad Wildbad, Germany). The values were expressed as relative units of light/min (RLU/min).

2.8. Morphometric and stereological analyses

All morphometric analyses were performed at the Multiuser Laboratory of the Research Center for Biological Sciences of the Federal University of Ouro Preto. To determine the alveolar lumen area (Aa), twenty random images obtained from the histological slides of the lungs were digitized with Leica Application Suite software in association with a Leica DM5000b optical microscope and CM300 digital micro-camera. All images were scanned with a 40× objective. With the aid of Image J software, we used a representative image of a 40× magnification with a 100-μm ruler to calibrate a ruler in pixels derived from the program so that 434 pixels equaled 100 μm. Five alveolar areas of each slide prepared from each animal were measured (Ávila DeL et al., 2013; Campos et al., 2014). The analysis of the volume density of the alveolar septa (Vv) was performed on a test system consisting of 16 points and a known test area (Starcher, 2000) in which the forbidden line was considered a boundary to avoid an overestimation of the number of structures. The test system was connected to a monitor attached to a microscope. The number of points (PP) that touched the alveolar septa was assessed according to the total number of test points (PT) in the system using the equation $Vv = P_p/P_t$. To obtain uniform and proportional lung samples, we analyzed 18 random fields in a cycloid test system attached to the monitor screen. The reference volume was estimated by point-counting using the test point (PT) system. A total area of 1.94 mm^2 was analyzed to determine the volume densities of alveolar septa (Vv) in slides stained with hematoxylin and eosin (Nagato et al., 2012; Mandarim-de-Lacerda, 2003).

2.9. Statistical analysis

The data with normal distribution were assessed through univariate analysis of variance (one-way ANOVA) followed by the Tukey pos-test. Data were expressed as mean ± standard error of mean. We used the Kruskal–Wallis test followed by Dunn's post-test for discrete data and expressed them as median, minimum and maximum values. In both cases, the difference was considered significant when the p value was <0.05. All analyses were performed with GraphPad Prism version 5.00 (GraphPad Software; San Diego, CA, USA) for Windows 7.

3. Results

To evaluate the presence of inflammatory cells in the lung parenchyma of rats exposed to formaldehyde in different concentrations, we performed total and differential counts of inflammatory cells in the BALF. Our results showed that the concentrations of FA1% and FA5% did not alter the amount of pulmonary inflammatory cells after exposure (Fig. 1). Furthermore, the exposure to the FA10% promoted an increase of inflammatory cells compared to CG while the exposure to FA5% and FA10% induced changes in inflammatory cell profiles. There was an increase in the percentage of macrophages in FA10% compared to CG as well as an increase in the percentage of lymphocytes in FA5% and FA10%, when compared to CG (Fig. 1).

The morphometric analysis of the lung parenchyma demonstrated that exposure to FA1% did not promote significant changes in pulmonary histologic structure (Figs. 3 and 2). The exposure to FA5% reduced the Vv of the alveolar septa and increased the Aa in the lung parenchyma compared to the CG, respectively. There was a decrease of the Aa in FA10% when compared to FA1% and FA5% (Figs. 3 and 2).

We determined the chemokine production in the lung parenchyma and plasma in those animals exposed to formaldehyde at different concentrations. The exposure to FA1% induced an increase of CCL5 in the lung parenchyma. Likewise, it was observed increased in plasma also associated with increased of plasma CCL3. The exposure to FA5% also increased the levels of CCL5 in the pulmonary parenchyma and plasma CCL3. Finally, the exposure to FA10% led to an increase of CCL2 and CCL5 in the lung parenchyma and plasma CCL2 in animals from this group (Tables 1 and 2).

In addition, the exposure to FA5% and FA10% increased the NADPH oxidase activity compared to the CG (Table 3). An increase of CAT activity in FA1% was also observed as well as an increase of SOD activity in FA5%, ever in comparison to CG. Besides, an increase in tGSH, GSH and GSSG and the alteration in the ratio of GSH/GSSG in FA5% was observed when compared to the CG (Table 3). The imbalance between oxidants and antioxidants in the lung parenchyma was evaluated by measuring redox imbalance biomarkers such as TBARS and carbonyl protein, which indicate damage to cell membranes and oxidative modification of proteins. Finally, an increase in lipid peroxidation in FA10% was observed when compared

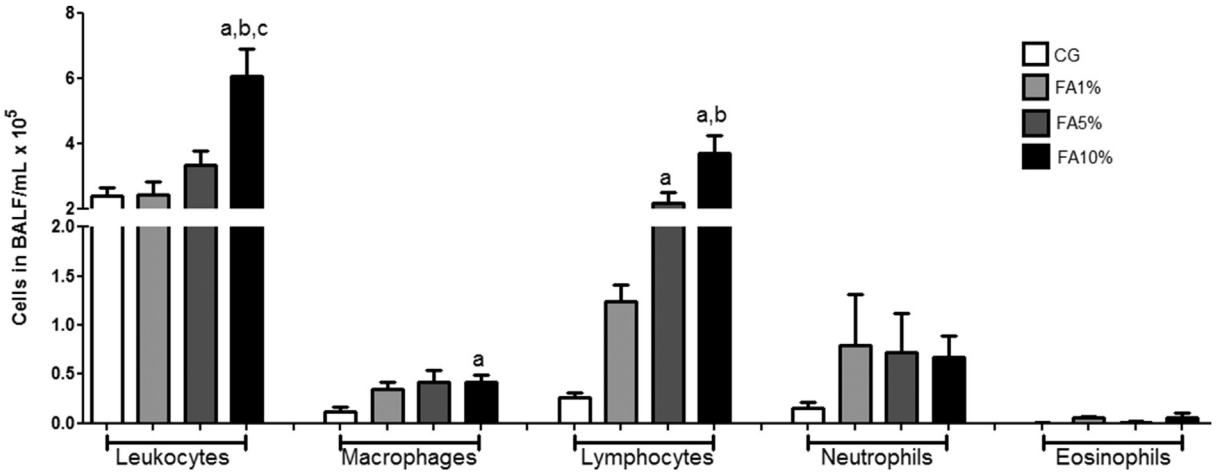

Fig. 1. Effects of exposure to formaldehyde on cellular influx in Bronchoalveolar Lavage Fluid from the CG, FA1%, FA5% and FA10% groups. The letter (a) represents a significant difference compared to CG. Data were expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Bonferroni post-test ($p < 0.05$).

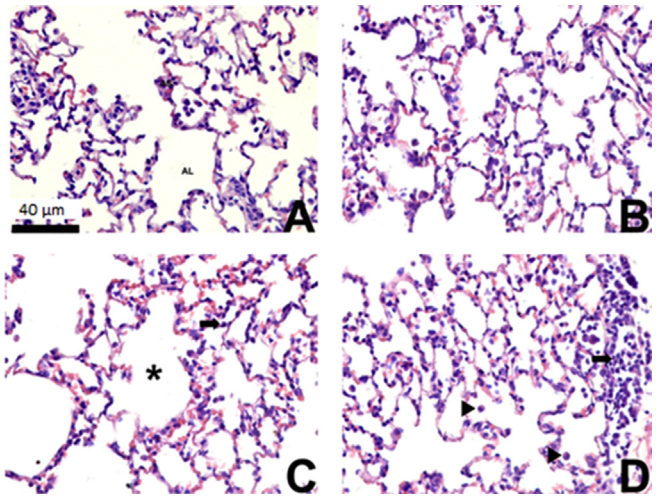

Fig. 2. Photomicrographs of lung sections stained with hematoxylin and eosin. There is a reduction in volume density (Vv) in the septum in FA5% (C) compared to CG (A), FA1% (B) and FA10% (D). AL: alveolar light; (*) increased alveolar lumen area; arrows: lymphocytes; arrowheads: macrophages. Bar = 40 microns.

Table 1
Levels of CCL2, CCL3 and CCL5 of the pulmonary parenchyma in lung samples from the CG, FA1%, FA5% and FA10% groups.

	CG	FA1%	FA5%	FA10%
CCL2 (µg/mL)	16.9 ± 0.8	18.2 ± 0.3	18.4 ± 0.5	23.0 ± 2.4[a]
CCL3 (µg/mL)	245.7 ± 29.9	255.0 ± 39.7	283.5 ± 39.6	400.0 ± 89.4
CCL5 (µg/mL)	238.6 ± 53.7	487.0 ± 52.0[a]	489.2 ± 64.0[a]	491.0 ± 54.0[a]

The letter (a) represents a significant difference compared to CG. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA ollowed by Bonferroni post-test ($p < 0.05$).

Table 2
Levels of CCL2, CCL3 and CCL5 in plasma from the CG, FA1%, FA5% and FA10% groups.

	CG	FA1%	FA5%	FA10%
CCL2 (µg/mL)	75.2 ± 2.4	84.0 ± 0.6	84.0 ± 0.5	90.0 ± 3.4[a]
CCL3 (µg/mL)	68.0 ± 8.5	187.0 ± 27.0[a]	155.0 ± 24.0[a]	142.0 ± 11.0
CCL5 (µg/mL)	316.0 ± 39.5	498.0 ± 52.0[a]	331.0 ± 43.0	335.0 ± 38.0

The letter (a) represents a significant difference compared to CG. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Bonferroni post-test ($p < 0.05$).

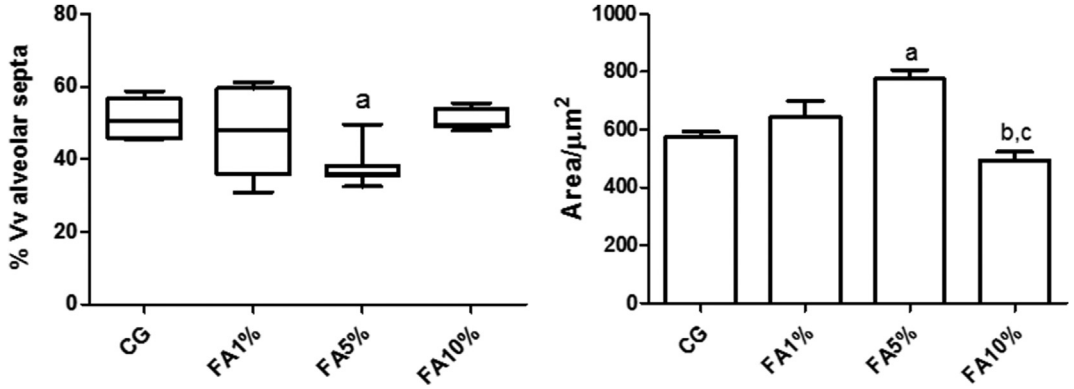

Fig. 3. Effects of exposure to formaldehyde on volume density of the alveolar septa and alveolar lumen area. The letter (a) represents a significant difference compared to CG. Letters (b,c) represent significant differences compared to FA1% and FA5%. Data were expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Bonferroni post-test and Kruskal–Wallis test followed by the Dunn's post-test ($p < 0.05$).

to all groups and the protein carbonyl formation in FA10% compared to CG (Table 3).

Table 3
Analysis of NADPH oxidase activity in rat blood and activities of SOD, CAT, Glutathione Fractions, TBARS content and Protein carbonyl in lung samples from the CG, FA1%, FA5% and FA10% groups.

	CG	FA1%	FA5%	FA10%
NADPH oxidase (RLU/min)	215.4 ± 21.3	144.0 ± 32.7	351.0 ± 45.0[a]	482.5 ± 24.0[a]
SOD (U/mg ptn)	15.8 ± 4.4	32.6 ± 4.5	63.8 ± 9.8[a]	20.8 ± 4.9
CAT (U/mg ptn)	8.9 ± 0.08	14.6 ± 2.4[a]	10.5 ± 0.3	6.8 ± 1.0
GSHt (nmol/ml)	5.6 ± 1.3	7.9 ± 1.8	13.3 ± 1.8[a]	5.4 ± 1.0
GSSG (nmol/ml)	2.0 ± 0.5	2.7 ± 0.8	5.8 ± 0.8[a]	2.0 ± 0.4
GSH (nmol/ml)	3.5 ± 0.8	5.2 ± 1.3	7.5 ± 0.9[a]	3.4 ± 0.6
GSH/GSSG (nmol/ml)	1.8 ± 0.2	1.5 ± 0.1	1.3 ± 0.1[a]	1.7 ± 0.2
TBARS (nmol/mg ptn)	0.3 ± 0.05	0.17 ± 0.01	0.17 ± 0.008	0.54 ± 0.04[a,b,c]
Protein carbonyl (nmol/mg ptn)	98.5 ± 9.5	99.0 ± 7.3	85.6 ± 7.8	133.7 ± 9.3[a,c]

The letters represent significant differences between groups. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Bonferroni post-test ($p < 0.05$).

For the determination of systemic inflammatory parameters, evaluations were performed in the peripheral blood of animals exposed to different concentrations of formaldehyde. Animals exposed to FA1% do not elevate the total number of leukocytes neither caused any change in the individual subtypes of these inflammatory cells (Table 4). The exposure to FA5% promoted increase in total blood leukocytes associated with a high number of lymphocytes. Likewise, it was observed an increase of total leukocytes in rats exposure to FA10%, but without differences in the subtypes of leukocytes (Table 4). Furthermore, only in FA10% it was observed a reduction in the average volume of platelets.

4. Discussion

In this study, we characterized the influx of inflammatory cells into the pulmonary parenchyma of rats exposed to different concentrations of formaldehyde during 5 days, the damage caused by the oxidation of cellular membranes and proteins, the profile of the activity of oxidant enzymes as NADPH oxidase and antioxidant enzymes as SOD, CAT and glutathione system as well as the pulmonary and systemic inflammatory biomarkers and the assessment of the volume density of alveolar septa (Vv) and the alveolar lumen area (Aa).

The exposure of FA10% caused the increase of inflammatory cells in the lung associated with the increase of macrophages and lymphocytes while the exposure of FA5% only caused increase in the lymphocytes population. Other study showed a significant increase in the number of leukocytes from BALF of rats exposed to FA in a time-dependent manner (Lino-dos-Santos-Franco et al., 2011). Jung et al. (2007) also observed an increase in the number of total cells from BALF of mice exposed to FA while Wang et al. (2014) observed an increase in the number of inflammatory cells from BALF in mice exposed to a mixture of volatile organic compounds (formaldehyde, xylene, toluene, benzene). Those researches corroborate with our study since we showed an increase of inflammatory cells from BALF indicating the occurrence of the inflammatory response triggered by the exposure to FA. According to our data and other studies the formaldehyde potentially causes irritation and/or lesions in the pulmonary parenchyma triggering an inflammatory process in a nonspecific manner. Moreover, it can be noted that the FA1% was ineffective to promote changes in cell populations in the pulmonary parenchyma in this experimental model.

Studies by Valença and Porto (2008) and Campos et al. (2014), showed an increase in the alveolar lumen area in mice exposed to cigarette smoke. In our study, even using a different harmful stimulus, we also observed an increase of the alveolar lumen area and a decrease of the septum volume density in the group of rats exposed to FA 5%, but not to FA10%. Our data suggest that the tissue damage might occur with FA in a dependent-concentration condition. The decrease of the volume density of alveolar septa (Vv) and the increase of the alveolar lumen area (Aa), showed in the exposure to FA 5%, might be related to the production of matrix metalloproteinases (MMPs) by inflammatory cells. Lymphocytes, elevated in this FA5% group, could be potential sources of MMP that act on the remodeling and degradation process of matrix extracellular.

The inflammatory events are usually mediated by the expression and production of cytokines and chemokines which are essential to activation and recruitment of inflammatory cells into the injured tissue. The cellular expression and raising in the chemokine levels as CCL2, CCL3 or CCL5 in organic fluids have been related to the influx of monocytes/macrophages and lymphocytes in acute events (Cocchi et al., 1995; Conti and DiGioacchino, 2001). Our data showed an elevation of CCL2 and CCL5 in the lung as well as an increasing of CCL2, CCL3 and CCL5 in the plasma, followed by a detection of a high number of macrophages and lymphocytes in the lungs and lymphocytes in the blood of animals exposed to FA. In the FA5% exposure, a high production of CCL5 was related to the highest influx of lymphocytes in this tissue. Similarly, the increase of CCL2 and CCL5 levels in the pulmonary microenvironment inforce, in part, the large influx of these macrophages and lymphocytes.

Table 4
Leukocytes and platelets in the blood from the CG, FA1%, FA5% and FA10% groups.

	CG	FA1%	FA5%	FA10%
Total leukocytes ($\times 10^3/mm^3$)	3.9 ± 0.4	5.0 ± 0.8	7.0 ± 0.7[a]	6.9 ± 0.9[a]
Neutrophils ($\times 10^3/mm^3$)	1.4 ± 0.6	1.0 ± 0.3	1.04 ± 0.2	1.6 ± 0.8
Lymphocytes ($\times 10^3/mm^3$)	2.4 ± 0.2	3.6 ± 0.6	5.3 ± 0.4[a]	4.0 ± 0.5
Monocytes ($\times 10^3/mm^3$)	0.04 ± 0.02	1.0 ± 0.04	0.04 ± 0.04	0.02 ± 0.02
Eosinophils ($\times 10^3/mm^3$)	0.0 ± 0.0	0.4 ± 0.2	0.6 ± 0.2	0.6 ± 0.2
Basophils ($\times 10^3/mm^3$)	0.4 ± 0.4	0.3 ± 0.2	0.6 ± 0.6	1.2 ± 0.7
Mean platelet volume ($\times 10^6/mm^3$)	6.5 ± 0.1	6.0 ± 0.1	6.0 ± 0.2	6.0 ± 0.1[a]

The letter (a) represents a significant difference compared to CG. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Bonferroni post-test ($p < 0.05$).

In the study of pulmonary parenchyma, changes were observed in the plasma in the animals exposed to FA5% and FA10%. Sul et al. (2007) noticed that functionally related genes in the development process, coagulation, gene expression, cellular homeostasis and oncogenesis of rats were suppressed after exposure to FA. The reduction in mean platelet volume, one of the plasma change described in the 10% of the FA exposure, can be directly related to the FA action on the genes responsible to the coding of proteins related to coagulation.

In an environment of pathological tissue exposed to FA, the activity of NADPH oxidase generates superoxide anion and it is directly related to oxidative stress. Cells with high activity of NADPH oxidase, as macrophages and neutrophils, can contribute to the oxidative damage in the lungs (Júnior et al., 2005). Vaid and Katiyar (2014) observed an increase of oxidative stress mediated by NADPH oxidase in cells exposed to cigarette smoke. Lino-dos-Santos-Franco et al. (2011) concluded that the NADPH oxidase, NOS (nitric oxide synthase) and COX (cyclooxygenase) are, at least in part, responsible to the increase of ROS after the exposure to FA. In this study, we identified an increase in the NADPH oxidase activity as well as its dependence on different concentrations to FA.

FA is a substance that has been related to the development of lung inflammatory process (Lino-dos-Santos-Franco, 2011), once macrophages and neutrophils present in the lungs contribute to the imbalance between oxidants and antioxidants (Lin and Thomas, 2010). Oxidants are sub products inherent of activated leukocytes and promote the inflammatory response and the tissue damage. Furthermore, the direct increase of the oxidative condition produced by the release of oxygen species from inflammatory neutrophils and macrophages has relevance to the imbalance between oxidants and antioxidants as well as the establishment of oxidative stress (Lanzetti et al., 2008). The SOD is the main enzymatic defense in the lungs against the hazardous effect of O^{-}_{2} by converting O^{-}_{2} into H_2O_2, a substrate to CAT and GPx (Campos et al., 2013). In our present study an increase of inflammatory cells in the pulmonary parenchyma was associated with all groups exposed to FA and an increase of SOD activity was detected in the group exposed to FA5% while the CAT activity was increased in the group exposed to FA1%. Gurel et al. (2005), administered FA intraperitoneally in rats and observed that the activity of SOD and CAT in the frontal cortex and hippocampus was significant lower in the FA group than the control group. The activities of both enzymes were mantained in a similar level of the control group in animals that received FA and vitamin E. Gulec et al. (2006) have found decreases in activity of SOD and CAT in the liver of rats that received intraperitoneal injections of FA (10 mg/kg) during 10 days with consequent increase in the lipid peroxidation and proteins oxidation. Those results compared to our data suggest that the exposure to FA, prolonged or at high concentrations, suppresses the protected mechanism driving the immune response to lung damage. However, an acute exposure in lower concentrations of FA5% is not enough to cause tissue damage.

The GPx catalyzes the hydrogen peroxide (H_2O_2) and organic peroxides to their corresponding alcohols by converting GSH into GSSG. The GR is an enzyme that catalyzes the GSSG metabolism in two molecules of GSH. The reduction of the GR activity in cells induces the decrease of glutathione level in the cells, a process related to several diseases such as AIDS, Parkinson's Disease and Diabetes (Erat and Ciftci, 2006). FA leads to the decrease of the GR expression causing the loss of the conversion capacity of reactive substances in nontoxic compounds resulting in lipid peroxidation and protein oxidation (Adibhatla et al., 2003; Adibhatla and Hatcher, 2008). In this study we observed the increase in the GSSG and GSH formation and the decrease of GSH/GSSG ratio when exposed to FA at 5%, therefore, this is an indication of a higher activity of the antioxidant defenses in this group.

Previous studies suggest that the exposure to FA in experimental models promote the antioxidant system depression due to the increase of lipid peroxidation and the free radicals formation (Datta and Namasivayam, 2003; Farooqui et al., 1986). Usually, the lipid peroxidation is considered a hazardous process able to lead to structural changes of proteins and lipids such as biomembranes and lipoproteins and it is associated with poor cell function (Kühn and Borchert, 2002). In this study we showed that both the increase in formation of protein carbonyl and the lipid peroxidation occur in a dose-dependent form and it was noticed in the group exposed to FA10%. Sul et al. (2007) verified that malondialdehyde (MDA) levels, an indicator of lipid peroxidation, were not different from the control in the lung of rats treated with 5 ppm FA. However, the MDA levels were significantly higher when the rats were exposed to 10 ppm FA. Teng et al. (2001) verified that adding FA to the culture of hepatocytes led to a ROS generation and lipid peroxidation in time-dose dependent form. Tang et al. (2003) showed that the MDA levels were increased when testicular germ cells of rats were exposed to high levels of FA. Furthermore, Sul et al. (2007) observed that the presence of carbonyl protein associates with similar patterns of lipid peroxidation, where the carbonyl content in the lungs of rats treated with 5 ppm formaldehyde was not different from their control but was significantly increased in the lungs of rats exposed to 10 ppm formaldehyde, showing a dose-dependent relationship damage. Gurel et al. (2005) observed an increase of protein carbonyl formation in the frontal cortex and the hippocampus of rats receiving FA (10 mg/kg) intraperitoneally for 10 days and when the animals were treated with vitamin E, the protein carbonyl formation was reduced suggesting that this change is due to oxidative stress caused by FA.

The exposure to FA alters the organism function increasing the inflammatory stimuli. Chemokines are released leading to the increase of inflammatory cells into the injury site and, possibly modifying the antioxidant response (Fujimaki et al., 2004). The increase of SOD activity and the GSH/GSSG ratio reduction in the group exposed to FA5% suggest a reaction of the organism; an attempt to protect itself from damage due to the exposure to FA. It can be confirmed by the levels of lipid peroxidation and protein carbonyl that were not different from the control group. Regarding the group exposed to FA10%, there was an increase in inflammatory cells in the lung parenchyma followed by increased levels of lipid peroxidation and protein oxidation, without an increase in the activity of antioxidant enzymes. This study revealed that several biochemical and histological changes are produced in the pulmonary parenchyma and blood in rats in response to the exposure to different concentrations of FA. These effects are, at least in part, mediated by the production of ROS and the reduction of the activity of antioxidant enzymes as well as by chemokine production. Our results suggest that the formaldehyde has a potential in promoting airway injury both by increasing the inflammatory process as the redox imbalance, which occurs in adose-dependent manner.

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