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RESEARCH ARTICLE

Integrated View of Formaldehyde in Lung Toxicity: Molecular Mechanisms, Cellular Aberrations and Pathological Considerations

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Abstract

Formaldehyde (FA) is an indoor and occupational pollutant, with marked carcinogenic impact on the airways. The current investigation was initiated to determine the molecular, immunohistochemical and histopathological alterations in lung tissue of rats subjected to FA inhalation and to provide new reliable biomarkers of the hazardous insults of FA on the lung. Adult female *Wistar* rats were assigned into five groups; (1): negative control; (2): 10 ppm FA inhalation for 15 days; (3): 20 ppm FA inhalation for 15 days; (4): 10 ppm FA inhalation for 30 days and (5): 20 ppm FA inhalation for 30 days. After the experimental periods; rats were killed and lung tissues were harvested and prepared for molecular genetics, immunohistochemical and histopathological procedures. Molecular genetic results indicated significant overexpression in pulmonary Cyp2c6 gene in the group of rats submitted to FA inhalation (20 ppm) for 30 days. The expression level of pulmonary Bhmt gene showed significant upregulation in the groups of rats inhaled 10 and 20 ppm FA for 30 days. Pulmonary Mapk12, HLA-A and GsTpi gene expression levels revealed significant over expression in the groups of rats subjected to FA inhalation (10 ppm) for 30 days and (20 ppm) for 15 as well as 30 days. Meanwhile, the level of expression of pulmonary gene HLA-A recorded significant downregulation in the group of rats inhaled FA (10 ppm) for 15 days versus the control counterparts. In addition, immunohistochmical findings denoted positive immunoreactive responses for PCNA and cytochrome c in the lung tissue with different degrees according to the dose and duration of FA exposure. Histopathological description of lung tissue sections of rats underwent FA inhalation revealed various levels of histoarchitectural alterations depending on the dose and time interval of FA subjection. According to the before mentioned data, it seems that extreme FA burden on the lung leads to devastating effects on the molecular level (genotoxicity) and on the cellular level (proliferation and apoptosis). Also, morophological deformation depicted by histopathological investigation indicated the induction of conformational and structural alterations of lung tissue as a consequence of FA inhalation.

Introduction

Formaldehyde (FA, CH2O) is a one of the aldehyde group in which some chemical compounds like malondialdehyde, acetaldehyde, benzaldehyde, acrolein, vanillin and citral are found and it is an organic compound which has high reactive composition. FA is a familiar cosmetics indoor air pollutant present in clothes as it is widely employed in abundant industries[1,2] and it is also a byproduct of vehicle emissions and cigarette smoke[3]. Industrial applications of FA comes from fiber insulation and adhesives for wood-based materials as well as resins to biocides, textiles, timber covers, paper adhesives, leather, plastic, cosmetics and building materials[4]**.** It is a water soluble, colorless, reactive and flammable gas with a strong odor whose pure state is fetor and irritant. FA solid form (paraformaldehyde), in room temperature, could be turned into its gas form[5].

Liver is metabolized FA and transferred it into the blood stream as format and the FA is discharged through urine as format salts or *via* lungs through turning into CO2[6]. Inhalation of FA has toxic insults on the central nervous system[7]**,** respiratory system, reproductive system and bone marrow in addition to its well-known hepatotoxic effects[8]. Acute exposure to FA can cause eye, nose, throat, and skin irritation, brain changes as Alzheimer's disease[9]. FA produces inverse effects on the health on animals as well as humans from 2012; FA has been distributed as a carcinogen (Group 1) by the International Agency for Research on Cancer[10].

FA is an environmental air-polluting candidate, primarily; it gets in the body *via* respiration[11]. FA irritates the upper and lower respiratory tracts[12] and it is a potent trigger of inflammation in the lower respiratory tract[11]. It leads to impairment in the pulmonary function[13] and sensitized individuals asthmatics reactions[14].

FA might evoke airway ailments *via* many mechanisms such as, protein molecules binding mechanism, like albumin, to generate novel antigenic moieties[15]. This may in turn lead to the formation of special IgE antibodies which bounds mast cells, then the degranulation of most cells, as well as the liberation of inflammation mediators[16,17]. In addition, FA inhalation nonspecifically provokes airway mucosal inflammation[18,19]**.** Moreover, researches have demonstrated that inhaling animals of FA produce enhancement in malondialdehyde (MDA), reactive oxygen species (ROS), as well as DNA-protein cross-linking paralleled by levels of lungs reduced glutathione (GSH) as well as severe alterations in the antioxidant enzymes activity[20,11]. Acute FA poisoning has been found to cause suppression in the activity of CYP450 isoforms (CYP2C11, CYP3A2 and CYP2E1,) as well as enhancement in the rats CYP1A2 activity. In addition, animals subjected to FA inhalation (sub-acute, acute, chronic as well as sub-chronic) displayed, neoplastic as well as focal-nonneoplastic respiratory epithelium alterations of nasal mucous membrane[6]**.** Long-term exposure to FA has been shown tocause respiratory mucosa irritation, accompanied by marked airways carcinogenic effects[21].

The main targets of the present research were to assess the amalgamated genetic, immunohistochemical and histopathological insults committed in FA–induced lung toxicity and to address new validated biomarkers for the toxic impact of FA inhalation on the lung.

Material and Methods

Materials

Chemicals

- Formaldehyde (FA) was purchased from El Nasr Pharmaceutical Chemicals Co. (ADWIC), Cairo, Egypt. All other reagents and chemicals used in the present study were of analytical grade and high purity according to the International Standards.

Animals

Adult female albino rats *(Wistar* strain) of 3 month old weighing 130-150 g were procured from the Central Lab for Animal Care, of the National Research Centre, Giza, Egypt. They were

kept in a group of 4 in a cage, housed in animal facility in an environmentally conditioned room with respect to light, temperature, or air humidity and fed with standard laboratory food and water *ad libitum*. Before initiating the experiments, the rats were allowed to acclimatize for one week under the standard environmental conditions (12 h dark/ 12 h light cycle; temperature 20-22 °C) and relative humidity (40 %-60%). Animal care and experimental procedures were performed in accordance with Animal Welfare and Safety Legislation and approved by the National Research Centre Committee for Ethics in Medical Research No 08-129.

Experimental set-up

In this study, rats were randomly allocated into 5 groups, consisting of 8 rats each, as follow: (1): control group; (2): 10 ppm FA inhalation (6 h/day, 5 days/week) for 15 days[22]; (3): 20 ppm FA inhalation (6 h/day, 5 days/week) for 15 days[22]; (4): 10 ppm FA inhalation (6 h/day, 5 days/week) for 30 days[7] and (5): 20 ppm of FA inhalation (6 h/day, 5 days/week) for 30 days[7].

At the end of the experiment, diets were withheld from all the rats for 12 hours and the cervical dislocation was carried out following ethical human/animal euthanasia which was adopted with expertise. The abdominal cavity of each rat was opened up through a midline thoracic incision to expose the lung. The lung was excised and weighed using an electronic analytical and precision balance. Each lung was partitioned into two portions, the first portion was immediately frozen in liquid nitrogen and preserved in -80 °C for molecular genetics study and the other portion was fixed overnight in 10 % neutral buffered formalin (PH 7.4) for subsequent immunohistochemical and histopathological investigations.

Methods

Inhalation Protocol

All rats had access to food and water in their home cages but not for the brief periods in the inhalation chambers. For each daily inhalation, the rats were transferred from the animal facility room to the laboratory, in their home cages, and positioned into an inhalation chamber. Inhalation of FA was carried out once a day for 6 hours to mimic the low and high doses inhalation in human. Inhalation of FA vapors was given in sealed 36-l cylindrical glass jars with acrylic lids similar to description in Bowen and Balster[23]. The lids were equipped with injection ports, a fan and a stainless steel mesh box holding filter paper. During FA inhalation, one dam was placed onto a grid floor 20 cm from the bottom and 30 cm from the filter paper in the lid of the chamber. The lid was replaced and a calculated amount of FA was injected onto filter paper from which the fan volatilized FA. At the end of the inhalation period, rats were removed immediately and returned to their home cages to wait the next inhalation period with the same procedure[24] which was repeated daily for the chosen periods of time (15 days and 30 days).

The dose of FA inhalation was calculated as follow:

$$\text{ppm} = \frac{\text{mg solute}}{10^6 \text{ mg water}} = \frac{\text{mg solute}}{\text{liter solution}}$$

$$\text{volume ml} = \frac{\text{weight of solute}}{\text{density of solution}}$$

Molecular Genetic Assay

Semi-quantitative RT-PCR

RT-PCR assay was conducted to determine the expression level of Cyp2c6, Bhmt, Mapk12, HLA-A and GSTP1 genes in the lung of rats underwent FA inhalation with the different doses and time intervals.

RNA Extraction

Total RNA was derived from 50 to 100 mg of lung tissue by the standard TRIzol extraction

method (Invitrogen, Paisley, UK) and recovered in 100 µl molecular biology grade water. In order to avoid any possible genomic DNA contamination, the total RNA specimens were pre-treated with DNA-free™ DNase and removal reagents kit (Ambion, Austin, TX, USA) following the guidance of the manufacturer's protocol.

Reverse Transcription

The complete Poly(A)+ RNA isolated from the lung samples was reverse transcribed into cDNA in a total volume of 20 µl using 1 µl oligo (dT) primer. The composition of the reaction mixture, termed as master mix (MM), consisted of 50 mM MgCl2, 10x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3), 10 mM of each dNTP (Amersham, Brunswick, Germany), and 50 µM of oligo $(dT)_{18}$ primer. The RT reaction was conducted at 25°C for 10 min, followed by 1 h at 42°C, and finished with denaturation step at 99°C for 5 min. Afterwards, the reaction tubes containing RT preparations were flash-cooled in an ice chamber until used for DNA amplification through polymerase chain reaction (PCR)[25].

Polymerase Chain Reaction (PCR)

The first strand cDNA from lung samples of rats in the different groups was used as templates for RT-PCR with a pair of specific primers. The sequence of specific primers and product sizes are listed in Table (1). ß-Actin was used as a housekeeping gene for normalizing mRNA levels of the target genes. The reaction mixture for RT-PCR consisted of 2.5 U of Taq Polymerase, 10 mM dNTP's, 50 mM MgCl2, 10x PCR buffer (50 mM KCl; 20 mM Tris-HCl; pH 8.3; Gibco BRL, Eggenstein, Germany), and autoclaved water. As shown in Table (1), programmable temperature cycling was performed with the following cycle profile: 94°C for 1 min and then 30-40 cycles each comprising denaturation for 30 sec at 94 °C, annealing for 45 sec at 54-55 °C, and extension for 45 sec at 72 °C. After the PCR cycles, the reaction tubes were maintained for 5 min at 72 °C and then at 4 °C. The PCR products were then loaded onto 2.0% agarose gel, with PCR products derived from ß-actin of the different lung samples[26].

Table 1: Primers sequence and reaction conditions in RT-PCR

Gene		Primers Sequences (5'- 3')	Annealing Temp (◦C)	Number of cycles	PCR product (bp)	References (Accession no.)
Cytochrome P450, subfamily IIc6 (Cyp2c6)	F	GTAAGCGCTCTACCACTGAG	55	40	142	Park et al.[27]
	R	TCTGTACTTCACCCAAAGGA				
Betaine-homocysteine methyltransferase (Bhmt)	F	AATGGAAGCAGTCAGAACAG	55	40	115	Park et al.[27]
	R	CAAGATCTTTTGAGCAGGAC				
Mitogen-activated protein kinase 12 (Mapk12)	F	AGTGTCCCCAAAACCTCTAT	55	40	183	Park et al.[27]
	R	TAAGCTTTCTGTCCAAGACC				
The major histocompatibility complex, class IA (HLA-A)	F	TCCGCTACTACAACCAGA	54	30	317	Lee et al.[28]
	R	CATTCAACTGCCAGGTCA				
Glutathione *S*-transferase pi (GSTPi)	F	CTGAACTCAGGTAGTCCAGC	55	30	329	Lee et al.[28]
	R	GGAGGTAGAAGTGCACAAAG				
ß-actin	F	ACCTTCAACACCCCAGCCATG-TACG	55	40	530	Park et al.[27]
	R	CTGATCCACATCTGCTGGAA-GGTGG				

Immunohistochemical Technique

The fixed lung tissues were prepared into paraffin wax blocks and serially sectioned. Three paraffin sections; 4 microns thick each was obtained from each block. One section was deparaffinized, rehydrated and immunostained using primary mouse monoclonal antibody against PCNA (Proliferating Cell Nuclear Antigen) Ab-1 (Clone PC10) Thermo Fisher Scientific; Fremont, CA 94538, USA; localized in the nucleus; to assess cellular proliferation. The second section from the same block was immunostained using mouse monoclonal antibody: anti-Cytochrome c, Clone: CTC03 (2B5), Thermo Scientific™ Lab Vision, Fremont, CA 94538, USA to assess apoptosis. UltraVision Plus Detection System Anti-Polyvalent, HRP/DAB from

Thermo Fisher Scientific; Fremont, CA 94538, USA was applied to detect both immunostains[29] using light microscope.

Histopathological Procedure

The third section of the same block was stained with haematoxylin and eosin for routine histopathological examination[30] under the light microscope.

Statistical Analysis

All data were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System[31] followed by Scheffé-test to assess significant differences between groups. The values are expressed as mean±SEM. The level of significance was set at $P < 0.05$.

Results

Molecular Genetics Results

Lung Cyp2c6, Bhmt, Mapk12, HLA-A and GSTPi genes expression levels of FA inhaled rats in the selected doses for different time intervals are summarized in Figs. (1-10). The results denotated that there is no significant difference ($P > 0.05$) in the Cyp2c6 gene expression level between rats inhaled FA (10 ppm and 20 ppm) for 15 days as well as (10 ppm) for 30 days when compared with that in the control counterparts. Meanwhile, the lung Cyp2c6 gene expression level of rats inhaled FA (20 ppm) for 30 days exhibited significant upregulation ($P<0.05$) comparative with that in rats of the control group (Figs. 1 and 2).

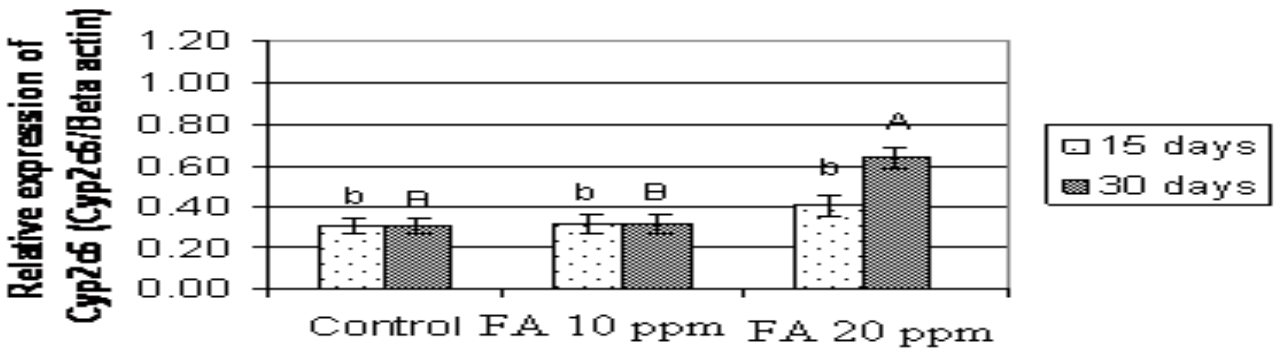


Fig. 1: Expression level of Cyp2c6 gene in lung tissue of rats underwent FA inhalation determined by semi-quantitative RT-PCR. Within each column, means superscripts with different letters are significantly different ($P \leq 0.05$

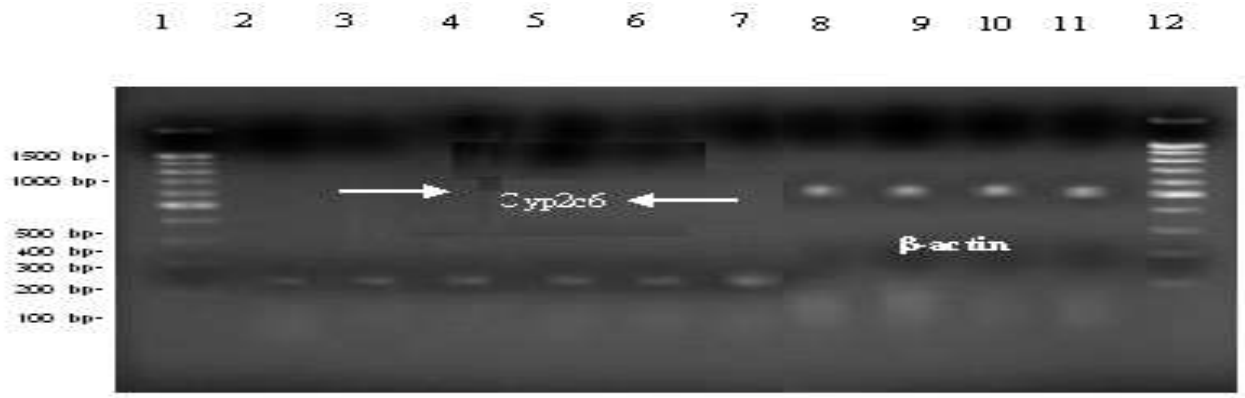


Fig. 2: Semi-quantitative RT-PCR analysis of Cyp2c6 gene in lung tissue of rats underwent FA inhalation. The RNA recovery rate was estimated as the ratio between the intensity of Cyp2c6 gene and the β-actin gene. Lanes 1 and 12 represent DNA ladder. Lanes 2 and 5 represent control group. Lanes 3 and 6 represent group inhaled 10 ppm of FA. Lanes 4 and 7 represent group inhaled 20 ppm of FA. Lanes 8 to 12 represent mRNA of the β -actin gene.

The findings of Bhmt gene expression level in the lung tissue of rats submitted to FA inhalation revealed that there is no significant difference between rats inhaled FA (10 ppm and 20 ppm) for 15 days and rats in the control group. However, the lung Bhmt gene expression level of rats inhaled FA (10 ppm and 20 ppm) for 30 days showed significant up-regulation versus that in rats in the control group (Figs. 3 and 4).

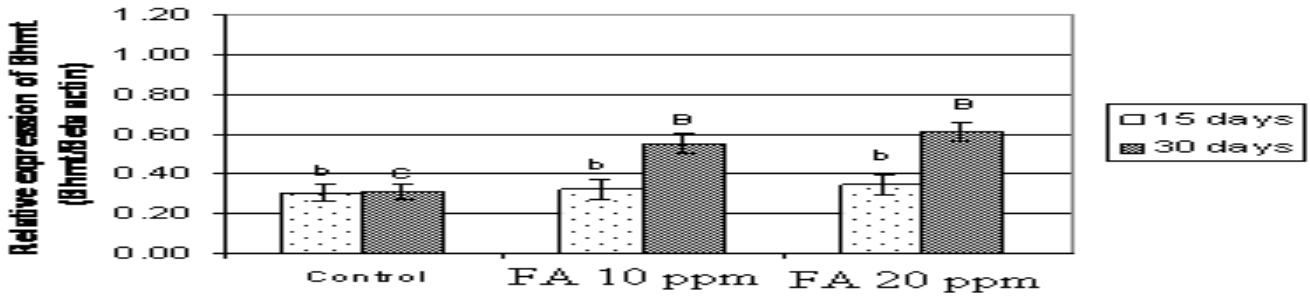


Fig. 3: Expression level of Bhmt gene in lung tissue of rats underwent FA inhalation determined by semi-quantitative RT-PCR. Within each column, means superscripts with different letters are significantly different (P ≤ 0.05).

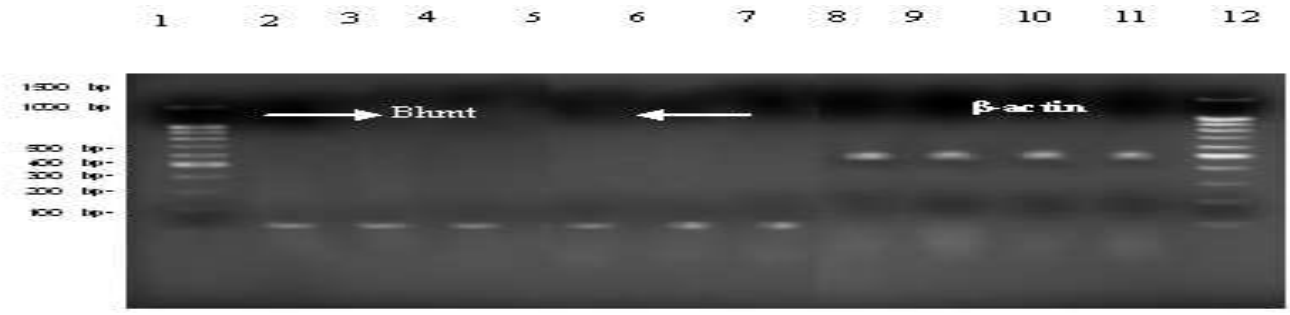


Fig. 4: Semi-quantitative RT-PCR analysis of Bhmt gene in lung tissue of rats underwent FA inhalation. The RNA recovery rate was estimated as the ratio between the intensity of Bhmt gene and the β -actin gene. Lanes 1 and 12 represent DNA ladder. Lanes 2 and 5 represent control group. Lanes 3 and 6 represent group inhaled FA (10 ppm). Lanes 4 and 7 represent group inhaled FA (20 ppm). Lanes 8 to 12 represent mRNA of the β -actin gene.

The data illustrated in Figs (5 and 6) showed that the lung Mapk12 gene expression level of rats inhaled 10 ppm FA for 15 days exhibits no significant difference in respect with that in rats in the control group. Meanwhile, the lung Mapk12 gene expression level of rats inhaled 10 ppm FA for 30 days showed significant up-regulation in comparison with that in rat in the control group. In addition, significant up- regulation in lung Mapk12 gene expression level of rats inhaled 20 ppm FA for 15 days and 30 days when compared to that in rats in the control group.

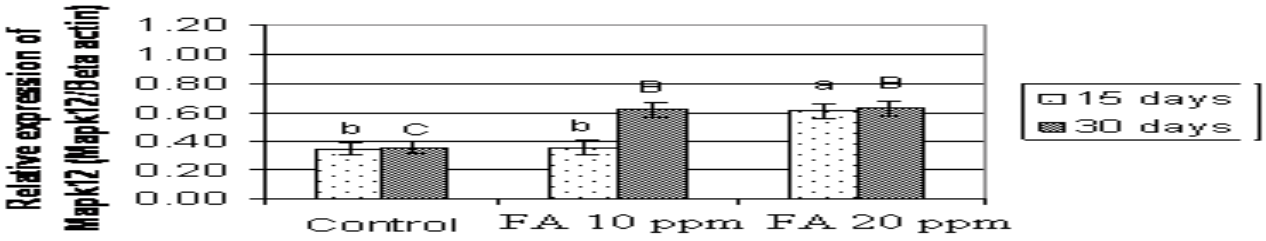


Fig. 5: Expression level of Mapk12 gene in lung tissue of rats underwent FA inhalation determined by semi-quantitative RT-PCR. Within each column, means superscripts with different letters are significantly different (P < 0.05)

Fig. 6: Semi-quantitative RT-PCR analysis of Mapk12 gene in lung tissue of rats underwent FA inhalation. The RNA recovery rate was estimated as the ratio between the intensity of Mapk12 gene and the β -actin gene. Lanes 1 and 12 represent DNA ladder. Lanes 2 and 5 represent control group. Lanes 3 and 6 represent group inhaled FA (10 ppm). Lanes 4 and 7 represent group inhaled FA (20 ppm). Lanes 8 to 12 represent mRNA of the β -actin gene.

Regarding the lung HLA-A gene expression level, the data revealed that there is no significant difference between rats underwent FA (10 ppm) for 15 days and rats in the control group. While, significant up-regulation in the lung HLA-A gene expression level of rats underwent FA inhalation (10 ppm) for 30 days as well as those underwent FA inhalation (20 ppm) for 15 days and 30 days in comparison with that in rats in the control group (Figs. 7 and 8).

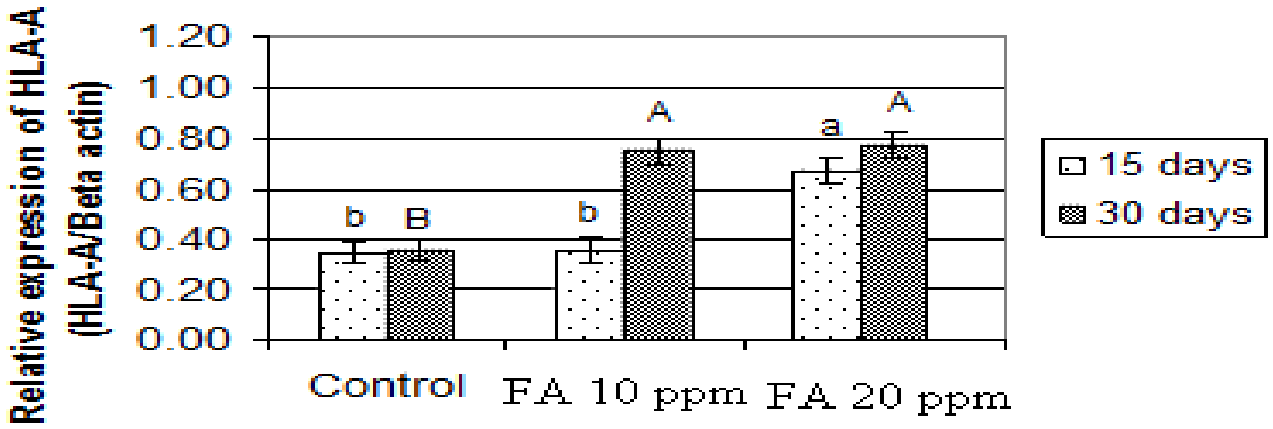


Fig. 7: Expression level of HLA-A gene in lung tissue of rats underwent FA inhalation determined by semi-quantitative RT-PCR. Within each column, means superscripts with different letters are significantly different (P < 0.05)

Fig. 8: Semi-quantitative RT-PCR analysis of HLA-A gene in lung tissues of rats underwent FA inhalation. The RNA recovery rate was estimated as the ratio between the intensity of HLA-A gene and the β -actin gene. Lanes 1 and 12 represent DNA ladder. Lanes 2 and 5 represent control group. Lanes 3 and 6 represent group inhaled FA (10 ppm). Lanes 4 and 7 represent group inhaled FA (20 ppm). Lanes 8 to 12 represent mRNA of the β -actin gene.

Regarding the lung GSTPi gene expression level, the data revealed no significant difference in rats exposed to FA inhalation at dose 10 ppm for 15 days and rats in the control group. However, significant up-regulation in the lung GSTPi gene expression level of rats inhaled FA at dose 10 ppm for 30 days as well as those exposed to FA inhalation at dose 20ppm for 15 and 30 days relative to that in rats of the control group (Figs 9 and 10).

Immunohistochemical Reactions

Lung tissue section from control rat immunostained with the proliferation marker antibody (PCNA) showed negative immunoreactivity for PCNA (Fig. 11a). Sections of lung tissue from rats inhaled 10 as well as 20 ppm FA for 15 and 30 days immunostained with the proliferation marker antibody for PCNA revealed positive immunoreactivity manifested through the brown color with numerous nuclei (Fig. 11 b-e). Section of lung tissue from rat inhaled 10 ppm FA for 15 days revealed moderate nuclear positivity for the proliferation marker PCNA. Section of lung tissue of rat subjected to 20 ppm FA for 15 days represented numerous proliferating epithelial cells lining bronchioles and alveoli. Lung tissue section from rats underwent FA (10

ppm) for 30 days showed proliferating cells within bronchiolar epithelium and lining the alveoli. Lung tissue section from rat submitted FA (20 ppm) for 30 days revealed highly proliferating epithelium lining bronchioles and alveoli. Of note, FA inhalation for 30 days revealed higher proliferation compared to 15 days. Lung tissue section from control rat immunostained with the antibody of the apoptotic marker (cytochrome c) showed negative immunoreactivity for cytochrome c (Fig. 12a).

Section of lung tissue of rats inhaled 10 as well as 20 ppm FA for 15 and 30 days immunostained within the antibody of apoptotic marker for cytochrome c demonstrated a positive immunoreactivity with the numerous cells cytoplasm revealing apoptosis stimulation (Fig. 12 b-e). Section of lung tissue from rat inhaled 10 ppm FA for 15 days showed positive mild cytoplasmic immunostaining of the apoptotic marker (cytochrome c). Lung tissue section from rats subjected to FA (20 ppm) for 15 days revealed moderate positivity for cytochrome c within cytoplasm of epithelium lining bronchioles and alveoli. Section of lung tissue from rat underwent 10 ppm FA for 30 days represented positive moderate cytoplasmic immunostaining for cytochrome c. Section of lung tissue from rat inhaled 20 ppm FA for 30 days illustrated a moderate positively immunoreactive cytochrome c within cytoplasm of epithelium lining bronchioles and alveoli. Noteworthy, FA inhalation for 30 days revealed weaker apoptosis compared to 15 days.

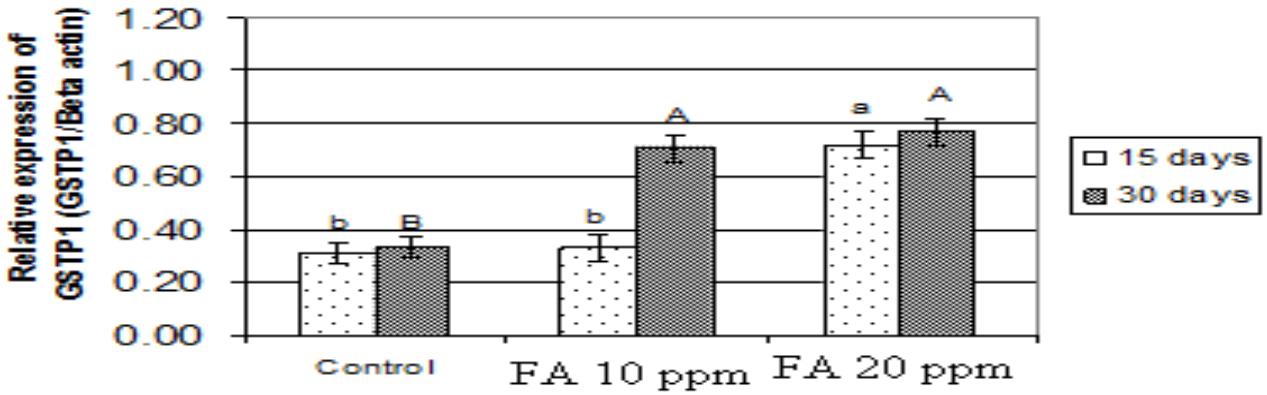


Fig. 9: Expression level of GSTPi gene in lung tissue of rats underwent FA inhalation determined by semi-quantitative RT-PCR. Within each column, means superscripts with different letters are significantly different (P < 0.05)

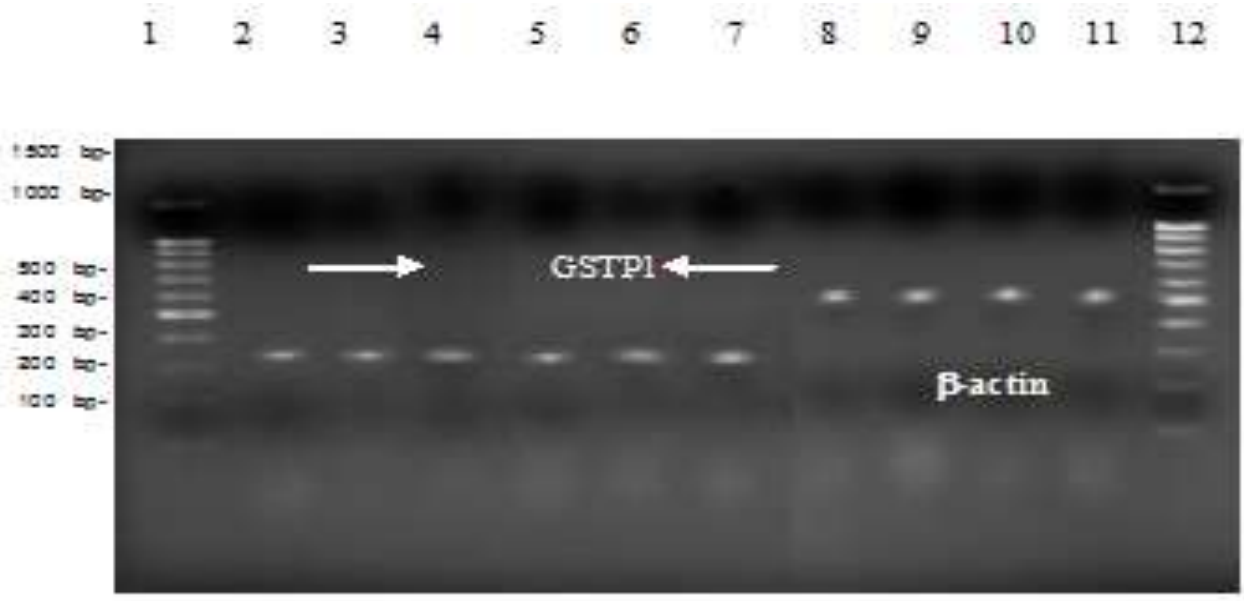


Fig. 10: Semi-quantitative RT-PCR analysis of GSTPi gene in lung tissue of rats underwent FA inhalation. The RNA recovery rate was estimated as the ratio between the intensity of GSTPi gene and the β -actin gene. Lanes 1 and 12 represent DNA ladder. Lanes 2 and 5 represent control group. Lanes 3 and 6 represent group inhaled FA (10 ppm). Lanes 4 and 7 represent group inhaled FA (20 ppm). Lanes 8 to 12 represent mRNA of the β -actin gene

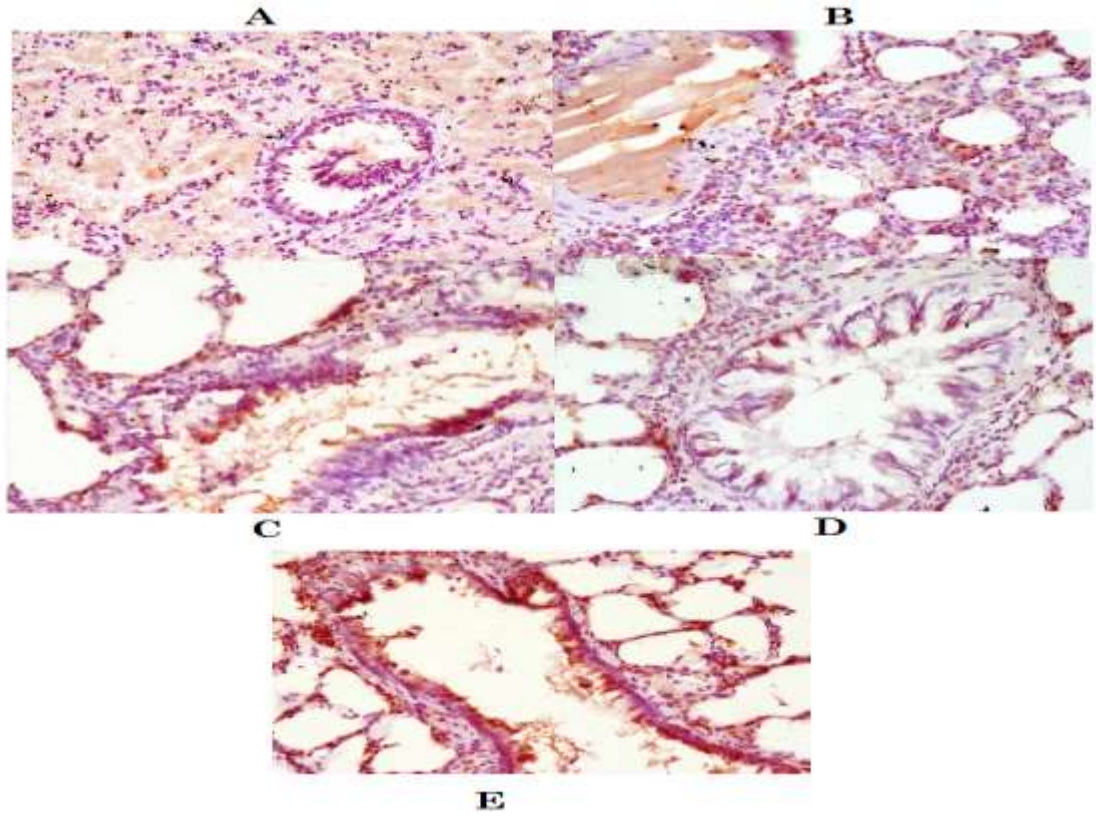


Fig. 11: (A) Lung tissue section from control rat showing negative immunoreactivity for PCNA (immunoperoxidase, PCNA X200)

(B) Lung tissue section from rat inhaled 10 ppm of FA for 15 days showing moderate nuclear positivity for the proliferation marker PCNA (immunoperoxidase, PCNA X400)

(C) Lung tissue section from rat inhaled 20 ppm of FA for 15 days showing numerous proliferating epithelial cells lining bronchioles and alveoli (immunoperoxidase- PCNA X400)

(D) Lung tissue section from rats inhaled 10 ppm of FA for 30 days showing proliferating cells within bronchiolar epithelium and lining the alveoli (immunoperoxidase- PCNA X400)

(E) Lung tissue section from rat inhaled 20 ppm of FA for 30 days showing highly proliferating epithelium lining bronchioles and alveoli (immunoperoxidase, PCNA X400)

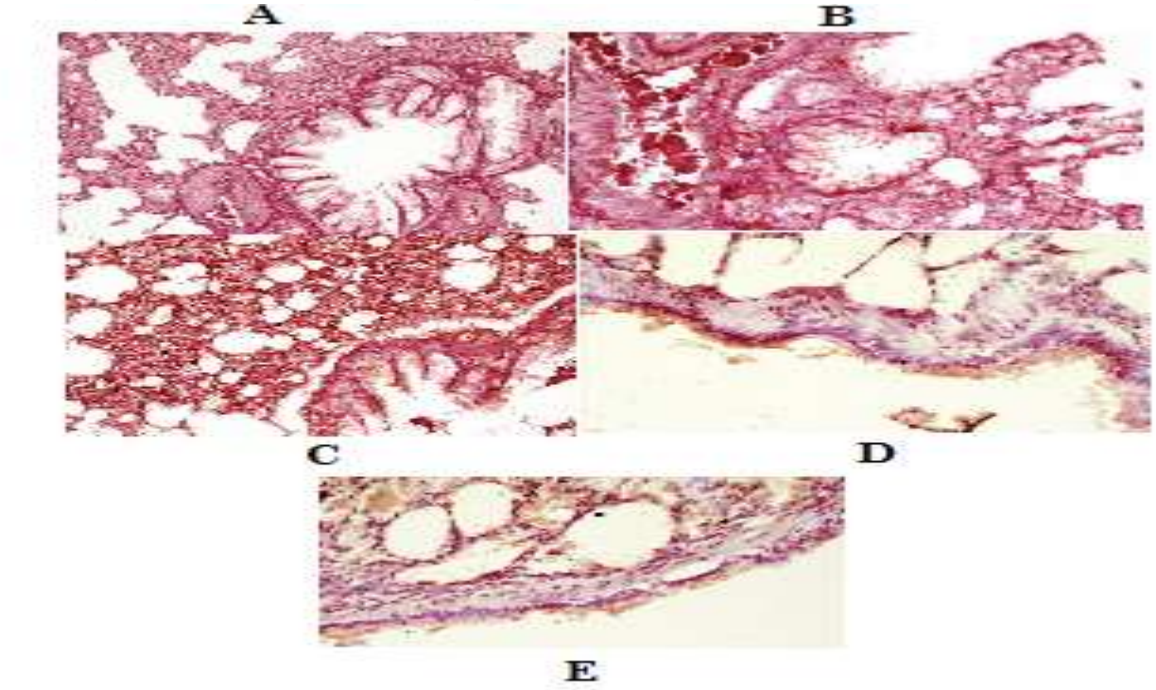


Fig. 12: (A) Lung tissue section from control rat showing negative immunoreactivity for cytochrome c (immunoperoxidase-cytochrome c, X200).

(B) Lung tissue section from rat inhaled 10 ppm of FA for 15 days showing mild positive cytoplasmic mmunostaining for the apoptotic marker cytochrome c (immunoperoxidase-cytochrome c, X200).

(C) Lung tissue section from rat inhaled 20 ppm of FA for 15 days showing moderate positivity for cytochrome c within cytoplasm of epithelium lining bronchioles and alveoli (immunoperoxidase-cytochrome c, X200).

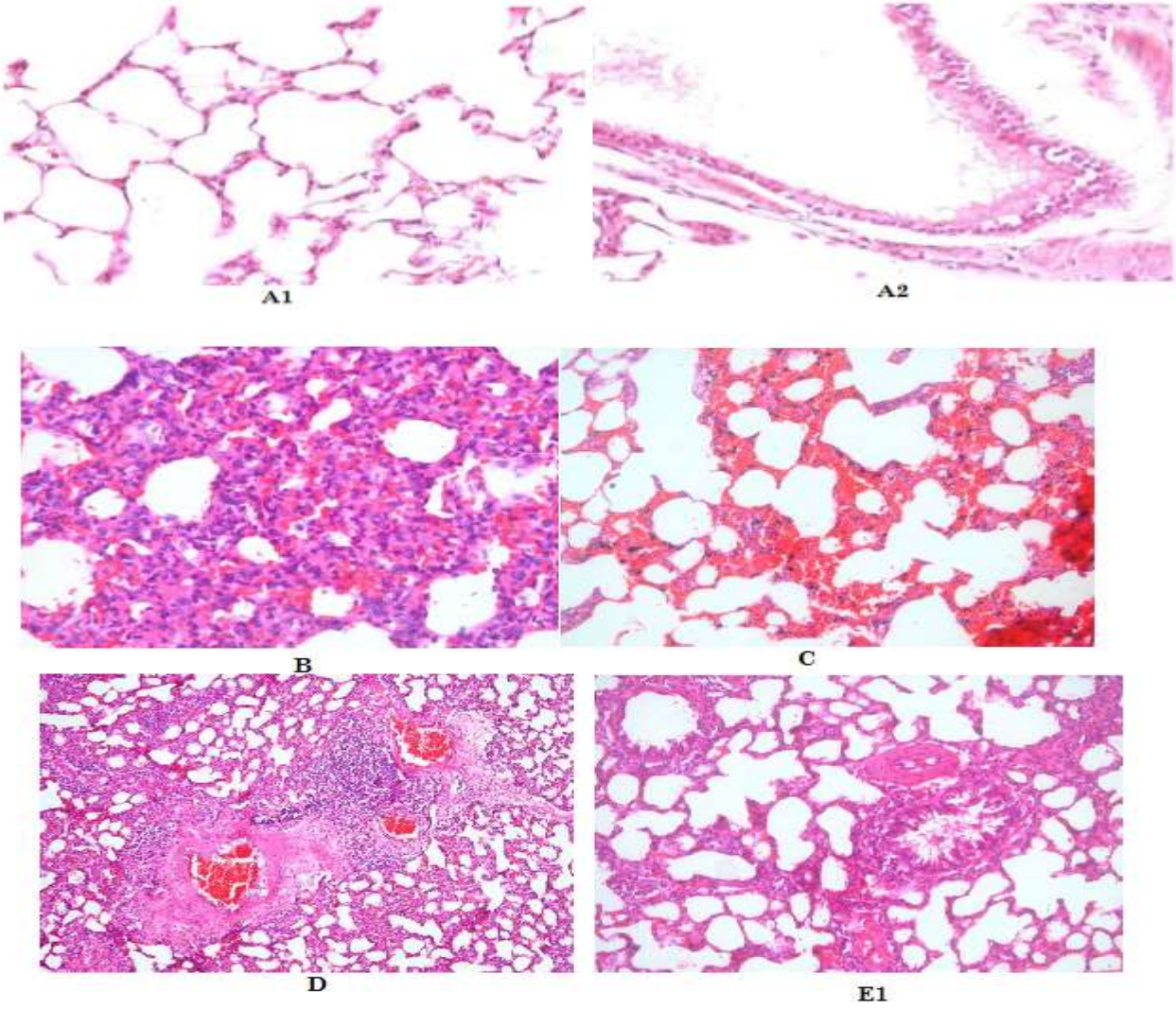
(D) Lung tissue section from rat inhaled 10 ppm of FA for 30 days showing moderate positive cytoplasmic immunostaining for cytochrome c (immunoperoxidase-cytochrome c, X400).

(E) Lung tissue section from rat inhaled 20 ppm of FA for 30 days showing moderate positive immunoreactivity for cytochrome c within cytoplasm of epithelium lining bronchioles and alveoli (immunoperoxidase-cytochrome c, X400)

Histopathological Description

Lungs tissue section from rat in the control group revealed ordinary alveolar spaces with thin walled interalveolar septae (Fig. 13 a(1)). Also, the bronchiolar lining formed of single layered, regular, cubiodal, mucus secreting, ciliated epithelium with bland basophilic nuclei (Fig 13 a (2)). Several adverse histopathologic alterations have been noticed in lung tissue sections extracted from rats subjected to 10 ppm as well as 20 ppm FA inhalation for 15 and 30 days. The intensity of histoarchitectural changes in 20 ppm FA inhalation showed moderate increase compared to 10 ppm FA inhalation. Again, the intensity of structural distortions showed marked increase in the 30 days of inhalation compared to 15 days of inhalation.

Section of lung tissue from rat inhaled 10 ppm FA for 15 days revealed mononuclear cell aggregates within thickened interavelveolar septae (Fig. 13 b). Lungs tissue section from rat underwent FA inhalation (20 ppm) for 15 days indicated marked congestion with broken interalveolar septae and widened alveolar spaces (Fig 13c). Section of lung tissue from rat inhaled 10 ppm FA for 30 days represented moderate congestion and lymphoid aggregates in between pulmonary alveoli (Fig 13 d). Section of lung tissue from rat inhaled 20 ppm FA for 30 days denoted partial loss of ciliated epithelium lining bronchiole (Fig. 13e(1)). Moreover, dense lymphoid aggregates with lymphoid hyperplasia within pulmonary tissue and sloughing of lining epithelium of bronchiole have been seen (Fig. 13e(2)). Furthermore, hyperplastic epithelial lining of bronchiole with few scattered atypical nuclei and sloughing within the lumen have been noticed (Fig 13 e(3)



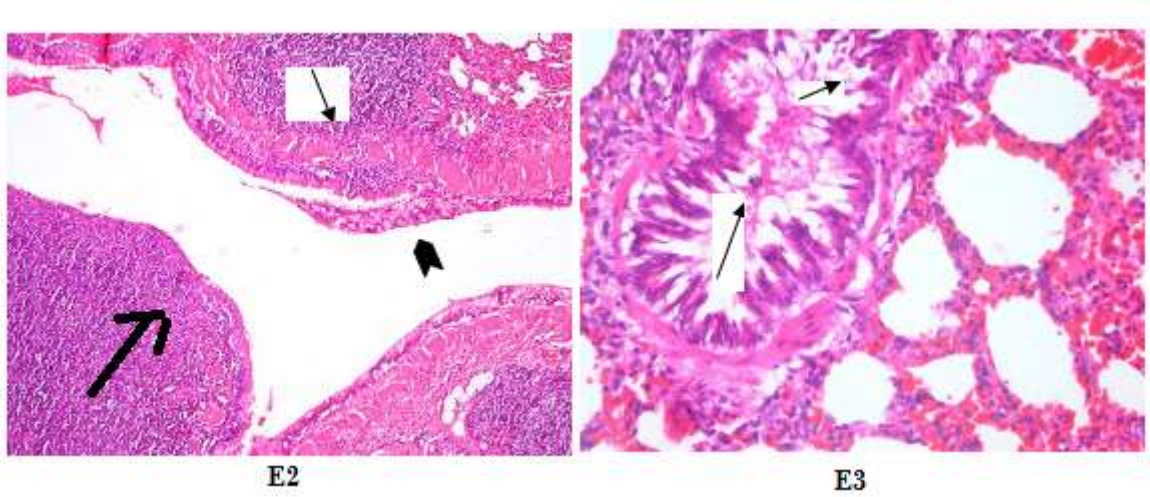


Fig. 13: (A1) Lung tissue section from control rat showing ordinary alveolar spaces with thin walled interalveolar septae (H&E X400).
(A2) Lung tissue section from control rat showing bronchiolar lining formed of single layered, regular, cuboidal, mucus secreting, ciliated epithelium with bland basophilic nuclei (H&E X400).
(B) Lung tissue section from rat inhaled 10 ppm of FA for 15 days showing mononuclear cell aggregates within thickened interalveolar septae (H&E X400).
(C) Lung tissue section from rat inhaled 20 ppm of FA for 15 days showing marked congestion with broken interalveolar septae and widened alveolar spaces (double headed arrow) (H&E X200).
(D) Lung tissue section from rat inhaled 10 ppm of FA for 30 days showing moderate congestion and lymphoid aggregates in between pulmonary alveoli (H& E X100).
(E1) Lung tissue section from rat inhaled 20 ppm of FA for 30 days showing partial loss of ciliated epithelium lining a bronchiole (arrow) (H&E X200).
(E2) Lung tissue section from rat inhaled 20 ppm of FA for 30 days showing dense lymphoid aggregates (arrow) with lymphoid hyperplasia within pulmonary tissue and sloughing of lining epithelium of bronchiole (arrow head) (H&E X200).
(E3) Lung tissue section from rat inhaled 20 ppm of FA for 30 days showing hyperplastic epithelial lining of bronchiole with few scattered atypical nuclei (arrow) and sloughing within lumen (H&E X400).

Discussion

This study was tailored to justify the molecular mechanisms, immunohistochemical reactions and histopathologic aberration of the lung of rats inhaled FA chronically as well as the study aimed to explore new relevant biomarkers for the devastating insults of FA inhalation on the lung. DNA–protein cross-link (DPC) Formation, which is determined the level of DNA damage, has been previously found to be increased at FA concentration (10 μM as well as 2 mM, (dose-dependently)) in V79 Chinese hamster cells *in vitro* assays and aorta endothelial cells of rat[32,33]. Also, FA-induced modification in apoptosis, metabolism, immunity, as well as signaling transduction involved genes[34,35,36].

Present data denoted significant up-regulation in the lung tissue gene expression levels of Cyp2c6, Bhmt, Mapk12, HLA-A as well as GSTPi of rats inhaled FA. Cyp2c6 over expression in the lung of rats exposed to FA may represent the accumulation of oxidative stress[37]. Formaldehyde has been found to increase cellular reactive oxygen species as well as cell death promotion[38,39]. Moreover, various phase I as well as phase II oxidative stress and xenobiotic metabolism regulating genes, the glutathione as well as cytochrome P450 family, respectively, were modified in FA inhaled rats comparing with control[34]. The enzyme system of cytochrome P450 (CYP) is included in humans metabolizing drugs; it is always expressed in the intestine as well as liver[37]. The CYPs activity and expression may be stimulated or suppressed *via* exogenous as well as endogenous materials[40]; reactive oxygen species found *in vitro* as well as *in vivo* have been documented in many events, resulting in CYP alteration during inflammation[41]. A family of mammalian heme-thiolate enzymes called cytochromes P450 (P450) is included in the endogenous as well as exogenous lipophilic compounds oxidative metabolism. Poor coupling of the P450 catalytic cycle results in continuous manufacturing of ROS, that affects cellular functions as well as signaling pathways. P450 production of ROS is mainly managed *via* gene transcription regulation and *via* alteration in the reactions between monooxygenase protein constituents which affects its coupling, activity as well as stability.

Malfunction of these mechanisms may yield an overflow of ROS production that may lead to oxidative stress as well as lipid peroxidation[42].

The cytosolic enzyme, Bhmt, catalyzes the conversion of homocysteine and betaine into methionine as well as dimethylglycine, respectively[43]. The semi quantitative RT-PCR analysis results in the current study revealed up-regulation of Bhmt gene in lung of rat inhaled FA. It has been reported that organic solvents as ethanol inhalation revealed a remarkable inhibition on methionine synthase (MS) in the 1st week of inhalation and a dramatic elevation in the activity of Bhmt at the 2nd of inhalation. These results fixed the chance that the stimulation of Bhmt activity clarified a mechanism by which the liver adapted to the prevention of S-adenosylmethionine (SAM) as well as methionine production resulting from methionine synthase (MS) inhbition[43]. This mechanism may be the underlying mechanism by which Bhmt is overexpressed upon inhalation of FA for a long period of time.

The stimulation of the MAP kinase family members is a main mechanism for the extracellular signals transduction. Mitogen-activated protein kinase 12 (MAPK12 = p38 kinase = stress-activated protein kinase) has been found to be stimulated *via* bacterial endotoxin inflammatory cytokines or cellular stresses. Therefore, it is occasionally known as the stress-activated protein kinase (SAPK)[44]. The induction of the pulmonary Mapk12 expression as a result of FA inhalation as confirmed by the RT-PCR analysis in the current study could be ascribed to an increase in the extracellular-regulated kinases 1 and 2 (ERK 1/2) and JNK activity. McCarroll et al.[44] study has been proved this explanation by their findings which indicated the increased phosphorylation of ERK 1/2 and JNK downstream substrates.

The major histocompatibility (MHC) class I antigen presentation pathway possess a major role in the infected cells immune system modification. Molecules of MHC class I are expressed on the all nucleated cells cell surface and present peptide fragments comes from the intracellular proteins. Antigen processing and presentation by MHC class I molecules is critical for immune surveillance. Molecules of MHC class I are expressed at the virtually all somatic nucleated cells cell surface are composed of a polymorphic heavy chain non-covalently accompanied ß2-microglobulin, as well as an antigenic peptide comes from proteins degraded in the cytosol and translocated into the endoplasmic reticulum[45]. Major histocompatibility (MHC) class I molecules surface levels on antigen presenting cells are *vital* to produce an appropriate immune response. There is a great number of evidences that up- or down-regulation of MHC class I expression could results in immune modulations[46]. Aberrant MHC class I levels elevations could attribute to the occurrence of various pathological conditions as hypomyelination[47] and an- kylosing enthesopathy[48]. MHC class I molecules expression is regulated mostly at the level of transcription[49], and cis -acting regulatory sequences that are transcription factors binding sites as retinoid X receptor, CIITA as well as NF- U B that are found to be important in the MHC class I genes expression[50]. The expression of MHC class I may be up-regulated *via* cytokines such as IFN-Q, IFN- K as well as TNF-K[51].

Formaldehyde inhalation could results an imbalance in between Th1 as well as Th2 cytokines secreted *via* human bronchial epithelial cells (16HBE cells), as well as elevation of TNF-α and IL-8 expression[52]. Also, it has been demonstrated that FA inhalation aggravates the cytotoxic and proinflammatory effects[53]. The introduction of cellular Reactive oxygen species and promotion cell death after FA inhalation lead to motivation of cellular ROS and cell death promotion[38,39], intracellular signaling axis to increase the production of enzymes of detoxification as Glutathione S-transferases (GSTs). Oxidative stress as well as the ROS generation are important inflammation component and associated the polymorphism of GSTPi gene with bronchial asthma as well as airway hypersensitivities[54,28].

Inhalation of FA in our study caused marked increase in cell proliferation marker as evidenced by nuclear immunoreactivity for antibody to PCNA in lung tissue. This agrees with Szende and Tyihák[55] who observed that specific FA concentration can cause cell proliferation stimulation. Tyihák et al.[56] found that the influential effect of FA inhalation on cell proliferation as well as apoptosis; *via* enhancement or inhibition; depended on the used FA concentration. Aizenshtadt

et al.[57] contributed the stimulated proliferative effect to epidermal growth factor cell receptor activation by FA because of its interaction with FA.

A normal biologic process, apoptosis, is being modified on toxicants inhalation[58]. Enhancement of lungs apoptotic cell death induced by 10 and 20 ppm FA inhalation for 15 days; was documented in the current study by positive immunoreactivity for cytochrome c. These results are in agreement with Sandikci et al.[59] study who noticed apoptosis stimulation in young as well as adult rats after FA inhalation. The study of Lim et al.[60] reasoned the apoptosis stimulating effect of FA inhalation to produce the oxidative stress, *via* suppression of an endogenous antioxidant, peroxiredoxin 2 proteins, through p38 mitogen stimulated protein kinase (p38MAPK). Interestingly, the present investigation showed apoptosis, after 15 days inhalation of FA, was marked as well as more intense compared to inhalation for 30 days. This could be attributed to the longer inhalation duration (30 days) allowed more intense as well as stronger cells proliferation that takes place, as revealed by the PCNA, the proliferation marker. Nuclear proliferation, hyperchromasia as well as focal atypia were outgrown and prominent, giving no chance for apoptosis. Thus, long inhalation duration may enhance nuclear atypical changes accumulation as well as alterations, that are eventually and detrimental hazardous. Finally, this could result the atypia precipitation as well as its progression to premalignant as well as even malignant lesions due to the long durations of FA high dose exposure[5].

Lower respiratory tract affection by rat's inhalation of FA was the concern of the current study; as inhalation of 10 as well as 20 ppm FA for 15 and 30 days leads to histopathological modifications. Also, inhalation of FA induced histoarchitectural alterations were duration as well as dose dependent. These findings come in line with that of **Kamata et al[61]** who revealed that formaldehyde induces modifications in dose dependent manner. Loss of epithelium lining the alveoli and destruction of alveolar septae with widening of alveolar spaces, noticed in the histopathological investigation in our study indicate the blunting of glandular secretions of the surfactant down to the pharynx with consequent reduction of surface tension within the alveoli. This will interfere with respiration as it hinders alveolar collapse. The next step is affection of pulmonary function through reducing alveolar ventilation by lessening the flow of air from terminal bronchioles to alveoli[62]. Also, researchers have found that inhalation of FA is accompanied with increased lungs reactive nitrogen species (RNS) / ROS levels as well as antioxidant enzymes activity alterations[11]. Afterward the oxidative damage induced by protein biotransformation as well as lipid peroxidation represented *via* individuals inhaled FA; there was inflammatory cells recruitment[63]. In rats, at the concentrations of FA > 6 ppm, DNA-protein cross link (DPC)s was increased significantly accompanied with histopathological alterations of hyperplasia as well as the nasal lateral meatus squamous metaplasia[64] Furthermore, inhaled FA causes hypoxia which decreases tissue oxygen[65]. Lungs hypoxia may induce inflammation as well as oxidative stress that could lead to pulmonary oedema, bronchial vasoconstriction, pulmonary hypertension and vascular remodeling[66].

Conclusively, the present *in vivo* study involving inhalation of FA in two doses (10 ppm and 20 ppm) at different time intervals (15 days and 30 days)in rat clearly demonstrated that FA inhalation has significant detrimental insults on the lung. The adverse impacts of FA on the lung tissue is contributed to (1) genotoxicity by potentiating over expression of genes responsible for apoptosis, inflammation and oxidative stress and (2) stimulation of lung cell proliferation and apoptosis as evidenced by immunohistochemical investigation. The aberration in these key events in response to FA inhalation leads to subsequent distortion in the structural organization of the lung as confirmed by histopathological features. Therefore, the selected biomarkers under this study might be considered as reliable indicators for the mechanistic aspects in favor of FA-induced lung toxicity.

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