

Light and Scanning Microscopic Study of the Effect of Car Fuel (Gasoline) Inhalation on Guinea Pig Respiratory System at Station

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Abstract: Exposures to gasoline vapor among workers had hazards impact on pulmonary function and structure. This study was designed to evaluate the effect of outdoor exposure to gasoline vapor on trachea and lung of guinea pigs. Twenty five animals were divided into control (A) and experimental (B). The experimental group was subjected to gasoline inhalation at station for 30 (B1) and 90 (B2) days. Those exposed for 30 days were subdivided into two subgroups; one received ascorbic acid during exposure (B1a) and the other did not receive it (B1b). Trachea, using light and scanning microscope visualized shortening and even loss of cilia as well as focal epithelial desquamation resulted from exposure to gasoline vapor. Significant decrease in tracheal goblet cells occurred due to gasoline inhalation (90 days). Both mucosa and submucosa showed cellular infiltration. Exposure of lung to gasoline vapor (30 days) resulted in focal inflammatory cell infiltrate, intra-alveolar hemorrhage and focal emphysematous changes. Alveolar septum thickening and accumulation of intra-alveolar exudate was observed in 90 days exposed animals. Bronchi showed elongation of the mucosal fold, sloughing of some epithelial cells and accumulation of secretions. Inflammatory cell infiltration appeared in the wall of both bronchi and bronchioles. Animals received vitamin C during exposure to gasoline vapor had less histopathological changes in both trachea and lung. Gasoline vapor had adverse effect on histological structure of lungs and trachea, which could be partially ameliorated by vitamin C supplement.

Key words: Gasoline, inhalation, scanning, microscopy, trachea, lung

INTRODUCTION

Vehicular pollution is one of the most significant contributors to urban air pollution in recent time. Inhalation is one of the most important routes of human exposure to various automobile exhausts and result in exacerbation of illness from respiratory diseases (Zanobetti *et al.*, 2000). There is growing evidence that environmental pollutants could have some impact on the immunologic function that is involved in the occurrence of asthma and chronic obstructive pulmonary disease (Lee *et al.*, 2004).

Gasoline (car fuel) is a volatile mixture of toluene, xylene, benzene and over 100 hydrocarbons. It is produced by simple distilling from petroleum oil (Hill, 1982). Today's tendency is to remove lead from gasoline, which was earlier responsible for intoxication due to lead poisoning (Takamiya, 2003). Methyl Tert Butyl Ether (MTBE) is an oxygen containing compound used nowadays as a component of unleaded gasoline

(Shim *et al.*, 2004; Zhang *et al.*, 2006). Previous studies proved that exposure to inhaling MTBE as an additive to gasoline might result in tachycardia, tachypnea, hepatocellular hypertrophy, hepatocellular carcinoma, Leydig cell tumors in the testis and bleeding tendency (Lington *et al.*, 1997; De Peyster *et al.*, 2003; Lin *et al.*, 2005). Benzene toxicity and benzene induced tumor development in the lung should be taken into consideration for risk assessment in humans. Inhalation is the most common route through which humans are exposed to benzene, where the lung is one of target organs for benzene toxicity (Yoon *et al.*, 2004).

The general pathological response to an inhaled toxicant is said to be epithelial cell injury and the triggering of acute inflammatory immune processes. The epithelial cells of the airway and alveoli encounter particles after inhalation, which result in a number of respiratory diseases (Sureshkumar *et al.*, 2005). Gu *et al.* (2003) found that supplementation of vitamin C and E ameliorates laryngeal tracheal eosinophilia induced by

toluene diisocyanate exposure. So, the present study aimed to investigate the effect of car fuel on histological structure of guinea pigs trachea and lung and the effectiveness of vitamin C in protection against these changes.

MATERIALS AND METHODS

Twenty five Dunkin Hartley males guinea pigs weighing between (400-600 g) were purchased from animal's house in King Fahd Medical Research Center (KFMRC). Guinea pig has been used because they are similar to humans with respect to respiratory system and vitamin C metabolism (Panda *et al.*, 2000; Moller *et al.*, 2003). Animals were housed in a polycarbonate cage with stainless steel wire lids and kept one week for conditioning under constant temperature, humidity and 12:12 h day/night cycles with free access to water and a balanced diet. All experiments were undertaken with the consent of the animal ethics in accordance with the guidelines set out by the Canadian Council on Animal Care. The animals were randomized into two main groups.

The control group (A, n = 10), were subjected for 24 h day⁻¹ to fresh air outside the KFMRC (exposed to dust, temperature etc.). The experimental group (B, n = 15) was subjected to inhalation of gasoline by putting their cages near the station tanks for 30 (B1) and 90 (B2) days, respectively. So they were exposed to open, intermittent vapor generation. Those exposed for 30 days were subdivided into two subgroups; one group received vitamin C supplementation (B1a, n = 5) during exposure and the other group (B1b, n = 5) did not receive it. Vitamin C (10 mg/kg/day) was supplied by feeding tube according to Kang (1998).

At the date of sacrifice, animals were anesthetized by ketamine and setun. The chest was opened and perfusion was carried via the heart with normal saline followed by 10% neutral buffered formalin. The trachea and lungs were immediately removed. Lungs of each animal were weighted, samples from each lobe passing by the hilum were refixed in the same fixative then processed for 3-4 µm for paraffin sections, stained with Hematoxyalin and Eosin (H and E) and Perl's for hemosiderin. Mid tracheal sectors were processed by the same procedure for light microscopy. Some samples were fixed in gluteraldehyde (3%) in phosphate buffer for scanning electron microscopy.

For counting goblet cells in tracheal epithelium, An area about 1 cm above the tracheal carina was chosen because there was less variation in the number of goblet cells here than in other sites in the trachea. Counting was carried in paraffin sections stained with Alcian blue

periodic acid Schiff (AB-PAS) stain. The mean number of goblet cells counted at 400x. Twelve visual fields were counted per tracheal section. The slide reading carried out without knowledge of the exposure status (coded slide).

The finding caused by gasoline on the trachea and lung were classified into mild, moderate and sever changes according to this semi quantitative methods:

- Mild tracheal affection
- Focal small areas of desquamated epithelium
- Small amount of inflammatory cell infiltrate

Moderate tracheal affection

- Focal small areas of desquamated epithelium with shortening of cilia
- Moderate amount of inflammatory cell infiltrate

Sever tracheal affection

- Large areas of desquamated epithelium with loss of cilia
- Large amount of inflammatory cell infiltrate
- Retained bloody and mucous secretion

Mild affection of lung

- Focal small areas of inflammatory cell infiltrate
- Mild emphysematous changes
- No hemorrhages
- Bronchi and bronchioles showed minimal amount of inflammatory cell infiltrate and retained secretions

Moderate affection of lung

- Focal areas of inflammatory cell infiltrate
- Moderate emphysematous changes
- Small areas of hemorrhages
- Bronchi and bronchioles showed moderate amount of inflammatory cell infiltrate and retained secretions

Sever affection of lung

- Large areas of inflammatory cell infiltrate
- Sever emphysematous changes
- Multiple areas of hemorrhages
- Alveolar septal thickening and intra-alveolar exudates.
- Bronchi and bronchioles showed large amount of inflammatory cell infiltrate, retained secretions and thickening of the smooth muscle layer

Statistical analysis: The values of the various parameters (animal weight, lung weight and number of goblet cells in trachea) were calculated for each animal and then pooled to give the mean and standard deviation values for all animals within a given group. Differences between control and experimental groups were tested by student

t-test by the use of SPSS computer program. Differences among groups were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Statistical results: The present study showed that there was no significant difference in body weight between control and experimental groups (Table 1). On the other hand, there was significant increase in lung weight of experimental group subjected for 90 days to gasoline vapor (group B2) compared to the control (Table 2). The number of tracheal goblet cells, was significantly decrease in all experimental groups exposed to gasoline vapor (B1a, B2b and B2) compared to control group (Table 3).

Histological results

Trachea: Trachea of group B1b (30 days exposure of gasoline vapor) showed focal desquamation of epithelial cells, loss and shortening of cilia compared with control group (Fig. 1a). In some animals the lumen contained retained bloody mucous secretion (Table 4). There was inflammatory cell infiltration in the mucosa and sub mucosa consisting mainly of lymphocytes and eosinophils. The submucosa showed some dilated tracheal glands with degenerated lining cells (Fig. 1b and c). As regards goblet cells, there was significant reduction in number (Fig. 1g compare with Fig. 1f), which was confirmed by morphometric studies (Table 3). Trachea of group B2 animals (90 days exposure of gasoline vapor) showed an exaggerated form of the previously mentioned changes (Fig. 1d). Group B1a animals (exposed to gasoline vapor for 30 days plus ascorbic acid supply) showed slight loss of cilia in tracheal epithelium and less cellular infiltration in sub mucosa compared to group B1b animals (Fig. 1f).

Scanning electron microscopic observation: Examination of the trachea of experiment animals of group B2 (90 days exposure to gasoline vapor) revealed shortening and sometimes complete loss of cilia of the columnar epithelial cells (Fig. 2c) compared with control group (Fig. 2a, b). Disruption and detachment of both ciliated and goblet cells could also observed (Fig. 2e). The number of goblet cells was markedly decreased in comparison to control group (Fig. 2d).

The lung: The lung tissues of control groups were formed of minute air spaces called alveoli. They were lined by two types of cells; pneumocytes type I (the commonest), which were flat squamous cells with flat nuclei and type II pneumocytes, which were cubical cells with rounded nuclei (Fig. 3a, b). The wall of intrapulmonary bronchi is formed of mucosal layer surrounded by spirally arranged smooth muscle layer and adventitia. The latter contained hyaline cartilage plate, bronchial glands and lymphoid tissue. The bronchi were lined by pseudostratified columnar epithelium with few goblet cells (Fig. 4a). The wall of the bronchiole was formed of mucosa (lined by columnar ciliated epithelium), muscle layer and outer connective tissue layer. There were no inflammatory cells present in the lung tissue (Fig. 5a).

Gasoline vapor inhalation resulted in many histopathological changes in the lung. In experimental group B1b (30 days exposure to gasoline vapor) the alveoli showed focal inflammatory cell infiltrate, intra-alveolar hemorrhage and in some region emphysematous changes (Fig. 3c, d).

In Perl's stain sections, the alveoli showed deposition of hemosiderin blue granules (indicating associated hemorrhage) (Fig. 3g). Alveolar septal thickening and accumulation of intra-alveolar exudate was observed in experimental group B2 (90 days exposed animals to gasoline vapor) (Fig. 3e).

Bronchi and bronchioles of gasoline exposed animals showed sloughing of their epithelial lining, elongation of the mucosal folds, increased luminal secretion and inflammatory cell infiltrate (Fig. 4b-d) and (Fig. 5b-d). In some animals, the bronchial wall showed thickening (hypertrophy) of the muscle layer (Table 4). Similar changes were observed in the media of the accompanying blood vessels (Fig. 4e and 5c). On the other hand, animals of group B1a (received simultaneous administration of ascorbic acid during exposure to gasoline for 30 day) showed less pathological changes compared to group B1b that subjected only to gasoline vapor for 30 days (Fig. 4e and 5e).

Table 1: Effect of gasoline vapor exposure on body weight (gm)

Groups	30 days +		
	30 days (B1b)	Vit. C (B1a)	90 days (B2)
Control	712.8±28.4	712.8±28.4	727.6±42.4
Experimental	743.3±48.6	697.2±24.3	732.5±40

Student t-test: no significant difference between experimental and control groups

Table 2: Influence of gasoline vapor exposure on lung weight (g)

Groups	30 days (B1b)		30 days + Vit. C (B1a)		90 days (B2)	
	Right lung	Left lung	Right lung	Left lung	Right lung	Left lung
Control	2.8±0.3	2±0.2	2.8±0.3	2±0.2	3±0.1	2.4±0.18
Experimental	3±0.2	2.3±0.2	2.6±0.2	2.4±0.3	4.2±0.4*	3.3±0.3*

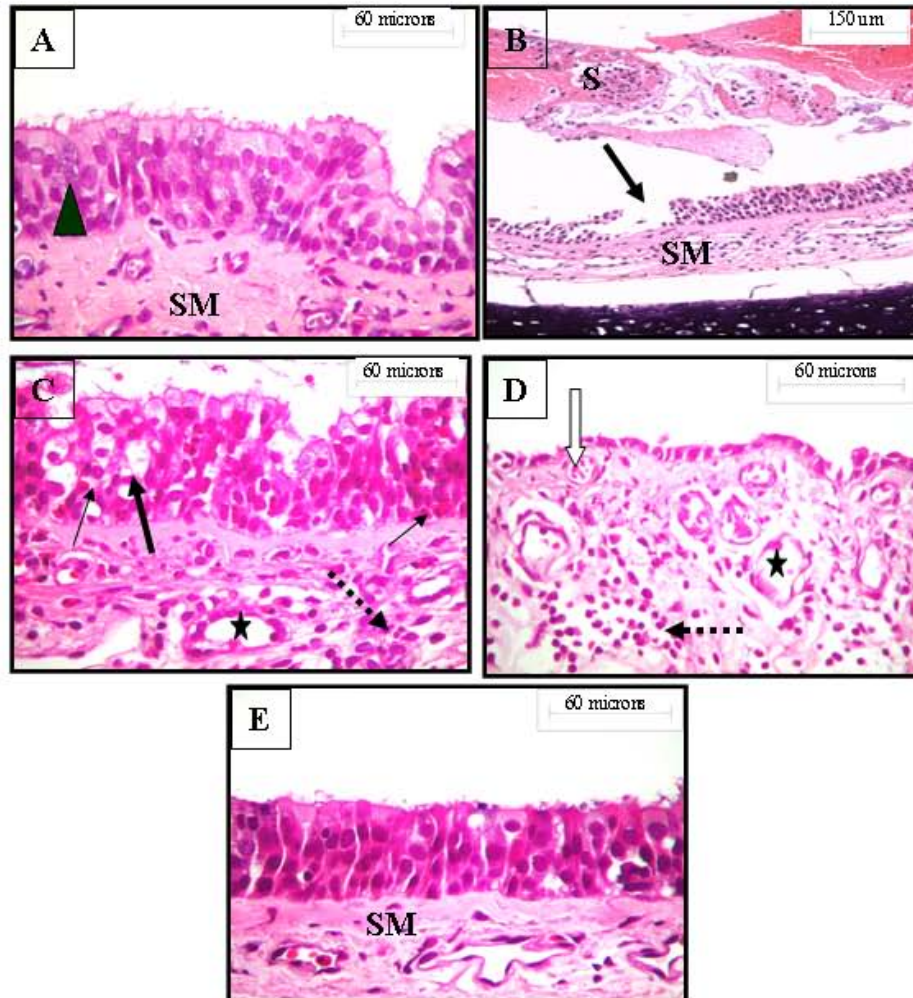


Fig. 1: Light microscopic sections of trachea from control group, (a) group B1b, (b, c) group B2, (d) group B1a, (e) group B1a. S= Secretion, SM = Submucosa. Eosinophils (thin arrow), inflammatory infiltrate (interrupted arrow), dilated gland (asterisk) and desquamated epithelium (white arrow). Lost cells (black thick arrows) and goblet cells (arrow head) (a-e stained with H and E) (E and G stained with Alcian blue and PAS)

Table 3: Influence of gasoline vapor inhalation on number of tracheal goblet cells

Groups	30 days (B1b)	30 days + Vit. C (B1a)	90 days (B2)
Control	41.05±1.5	41.05±1.5	43.2±1.1
Experimental	23.6±1.21*	20.6±0.92*	21.06±1.23*

Student t-test: *p< 0.05 compared to control groups

Environmental airborne pollution has been reported to affect multiple aspects of brain function, leading to cognitive and behavioral changes. Pronounced inflammatory response in the respiratory airways with subsequent cardiopulmonary disorders was also among health hazards (Grebix *et al.*, 2007).

In the present study, animals were exposed in car fuel station to gasoline vapor for 30 and 90 days. Car fuel (gasoline) inhalation produced marked irritative and

Table 4: Histological effect of gasoline vapor inhalation on trachea and lung of guinea pigs

Groups	30 days (B1b)		30 days + Vit. C (B1a)		90 days (B2)	
	n	%	n	%	n	%
Mild	2	40	3	60	1	20
Moderate	2	40	2	40	1	20
Sever	1	20	-	-	3	60

inflammatory changes in tracheal mucosa. Shortening and loss of cilia as well as desquamation of ciliated cells were observed. Some animals showed accumulation of bloody mucous secretion within the tracheal and bronchial lumen. Bronchi showed elongation of epithelial folds, sloughing of epithelial cells and an increased mucosal secretion. Increased mononuclear cell infiltrate was observed in

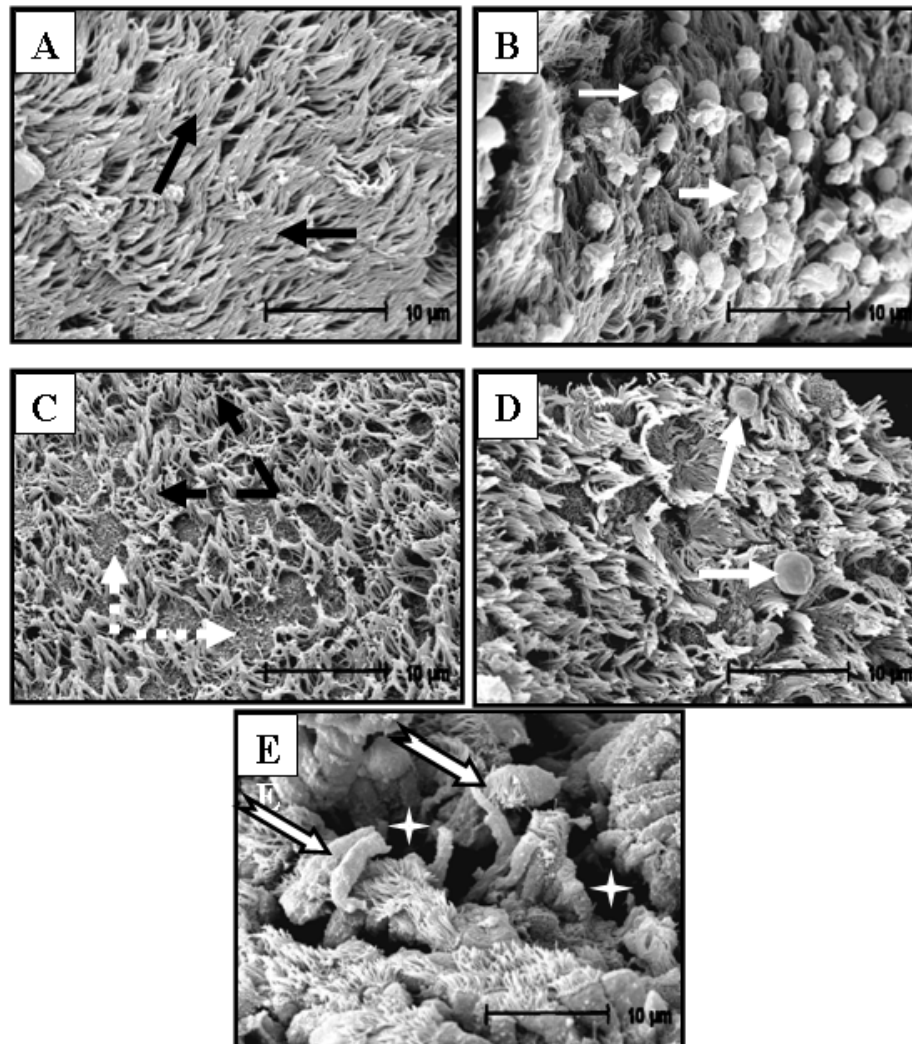


Fig. 2: Scanning electron microscopic sections of the trachea from control (a, b) and group B2 (c-e). Normal cilia (thick black arrow), lost cilia (interrupted white arrow), short cilia (interrupted black arrow) goblet cells (thick white arrow) and desquamated cells (bifid arrow) and site of lost cells (asterisk) (Gold stain)

peribronchial tissue. Similar finding were observed by (Sureshkumar *et al.*, 2005). They explained that epithelial cells lining respiratory airways act as effectors and target cells responding to exposure to a variety of inflammatory mediators and cytokines by alternating one or several of their functions such as mucin secretion, ion transport or ciliary beating. Aberrations in any of these functions can evoke local inflammatory response and compromise pulmonary defense.

This result demonstrated decrease in the number of trachea goblet cell and this was in accordance with (Kato *et al.*, 2000). They found that exposure of rat lung and trachea to diesel emissions result in increasing goblet cells acid form mucus granules without hyperplastic

changes in the goblet cell number. Inflammatory changes in the upper respiratory tract and trachea will compromise the mucociliary barriers and predispose to lung disease, with subsequent deficit in respiratory functions (Kumar *et al.*, 2003).

Lung parenchyma changes include scattered perivascular or peribronchial lymphoid aggregation, inflammatory and hemorrhagic exudate within affected alveoli. Emphysematous changes of the alveoli were observed.

It could be due to damage of alveolar walls. This damage might be attributed to an increase in interstitial collagenase and elastase activity. Collagenase was primary secreted by inflammatory cells in case of irritative

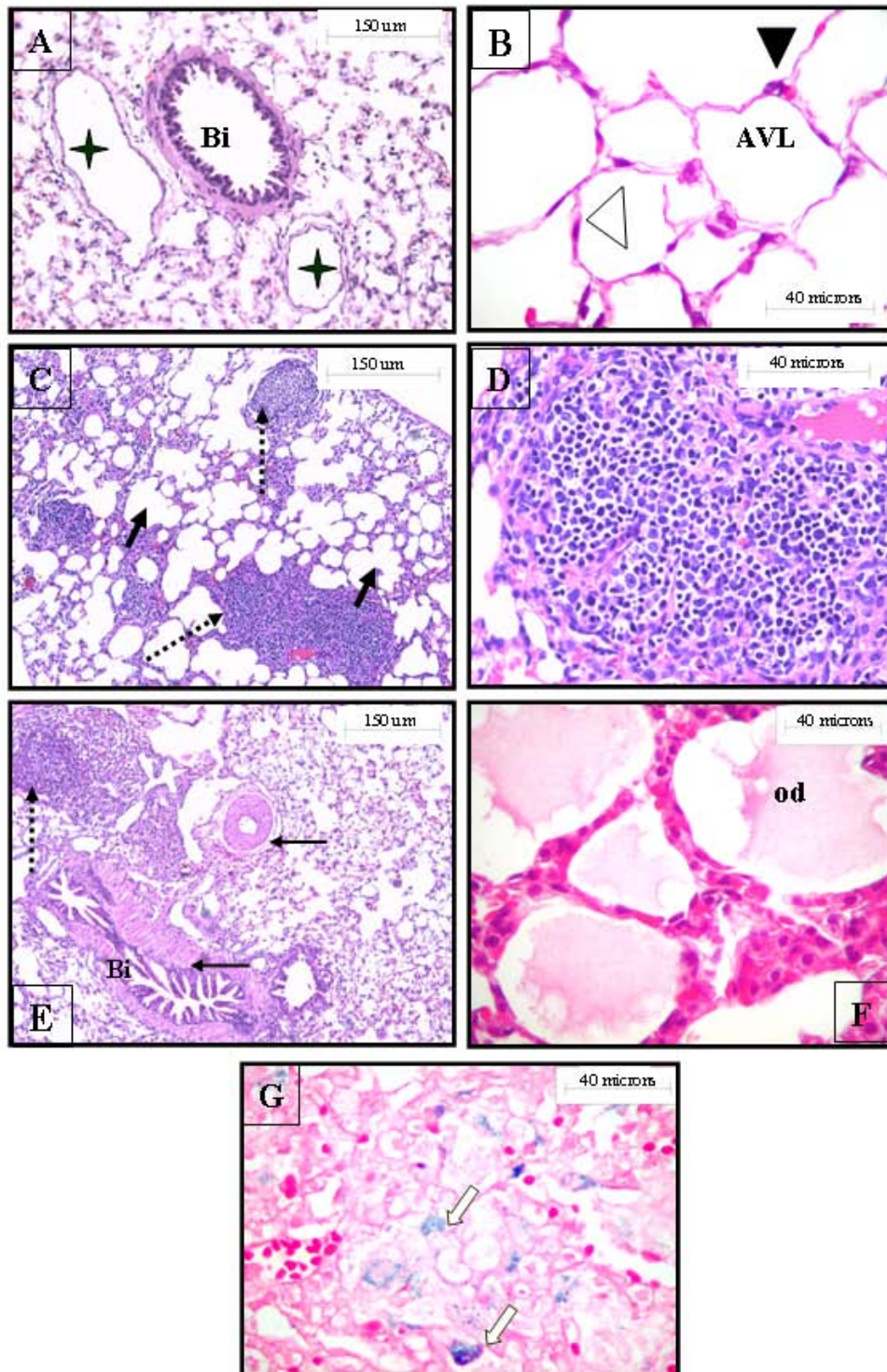


Fig. 3: Light microscopic sections of lung of control (a, b) group B1b (c, d and g) group B2 (e, f). AVL = alveoli, Bi = bronchiole, Od = edema. Pneumocyte type I (white arrow head), Pneumocyte type II (black arrow head). (Thick black arrow) indicate emphysema, (interrupted arrow) indicate lymphoid aggregation, (asterisk) indicate blood vessels. Hemosiderin granules (white arrow), thick muscle layer (thin black arrow) (A-F stained with H and E) (G) Perl's stain

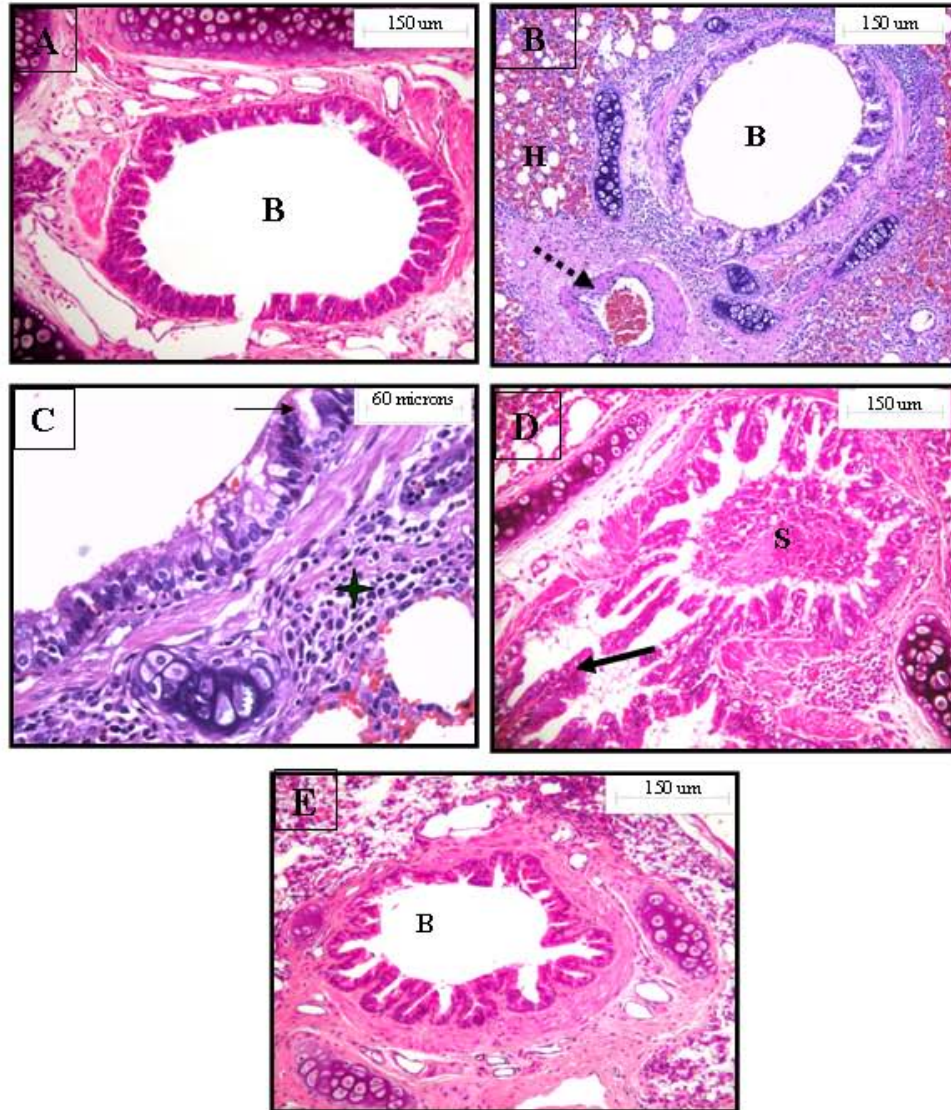


Fig. 4: Light microscopic sections of intrapulmonary bronchus from control (a) group B1b (b, c) group B2 (d) and group B1a (e) B = bronchi, S = secretion and H = hemorrhage. Inflammatory cell infiltrate (asterisk), elongated mucosal folds (thick arrow) thickened arterial wall (interrupted arrow) and lost cells (thin arrow) (H and E stain)

lung disease as this enzyme was also secreted by type II alveolar cells, when guinea pigs were exposed to tobacco smoke (Selman *et al.*, 1996). Cutler and Washinton, 2007 reported that workers, who are exposed to gasoline every day in their jobs have suffered throat irritation, breathing difficulties and decreased respiratory functions. On the other hand, at very high levels of exposure to unleaded gasoline vapors continuously for 2 years, workers developed liver, kidney and lung tumors (Benson *et al.*, 2001).

The non-neoplastic changes were most probably due to irritative effect of gasoline vapor, recruitment of

inflammatory cells and release of many inflammatory mediators, active oxygen species or free radicals (Mossman, 2003; Sureshkumar *et al.*, 2005). The thickening of bronchiolar muscular wall is among signs of increased air way resistance (asthma), which is a well known consequence of exposure to air pollutants (Jeffery, 1991).

Thickening of arterial wall are one of signs of pulmonary hypertension, which could eventually result in cardiopulmonary disorders known as cor pulmonale (Miller *et al.*, 2007). *In vitro* studies have documented the antioxidant effect of low concentration of vitamin C, which

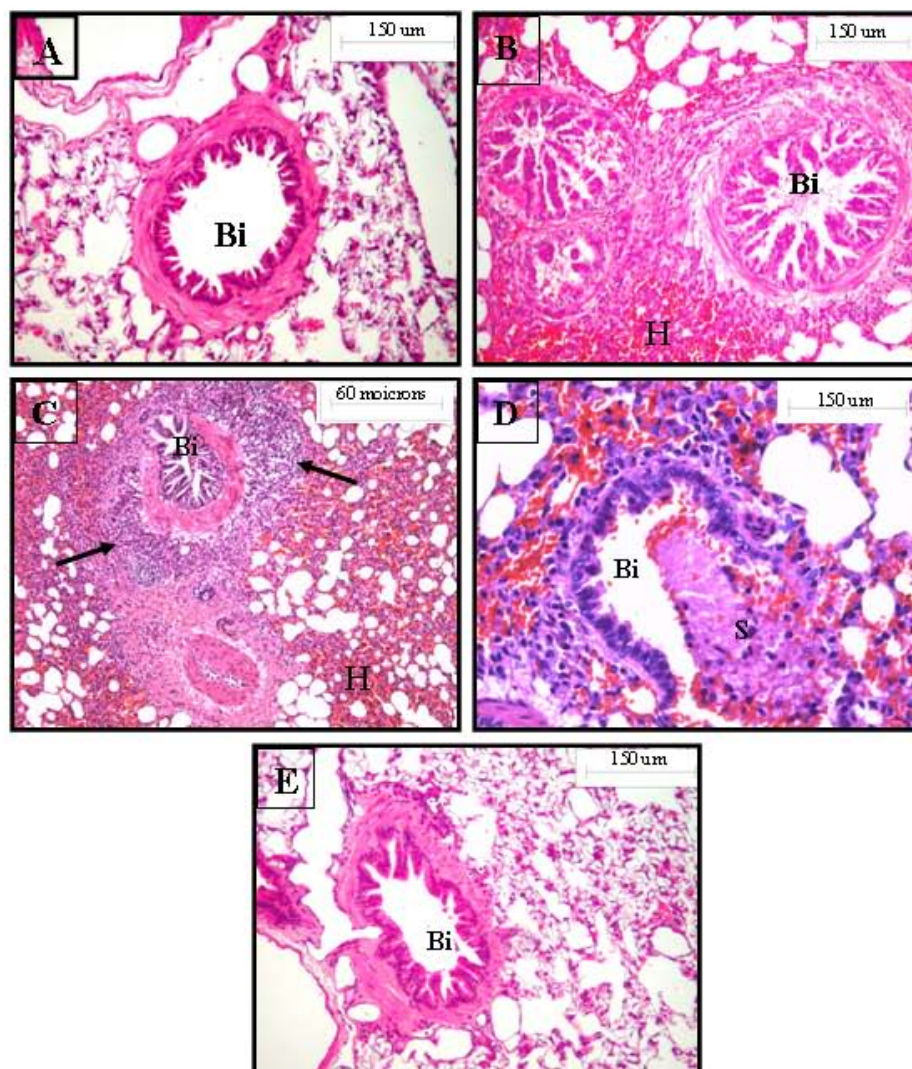


Fig. 5: Light microscopic sections of lung containing bronchiole of control (a) group B1b (b) group B2 (c, d) and group B1a (e) H=hemorrhages, Bi=bronchiole, S=secretion and (arrow) indicate inflammatory cell infiltration. The lung and bronchiole from group B3 appear near to control group (H and E stain)

revealed its important role in the antioxidant defense system (Peter *et al.*, 2003). Vitamin C had an antioxidant property *in vivo* in guinea

igs. Accordingly, the guinea pigs are similar to humans with respect to vitamin c metabolism and this offer a more appropriate experimental model to study its effects (Chen *et al.*, 2000).

The findings in this study showed that experimental group received vitamin C during exposure to gasoline for 30 days had less histopathological changes in the trachea, bronchi and lung tissue, when compared to group exposed to gasoline alone.

The use of antioxidants like vitamin C act as potent scavengers of free radicals and terminators of

free-radical chain reactions, Gu *et al.* (2003) found that supplementation of vitamin C and E ameliorates laryngeal-tracheal eosinophilia induced by toluene diisocyanate exposure. It can be concluded that exposure to gasoline vapor inhalation alter normal structure of trachea and lungs, which can be reduced by vitamin C supplement. Vitamin C for those being subjected to gasoline vapor inhalation could be of value in protecting their way.

Reducing the duration of gasoline exposure in car fuel station must be an international recommendation for occupational MTBE exposure, (8 h working day, ranging from 90-180 mg m⁻³) (ACGIH and BEIs, 1998; Chen *et al.*, 2000).

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