

Effect of Car Fuel (Gasoline) Inhalation on Trachea of Guinea Pig: Light and Scanning Microscopic Study under Laboratory Conditions

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Abstract: Exposures to car fuel vapor among workers result in appearance of pulmonary diseases. An evaluation of histological and scanning electron microscopic changes induced by subjecting guinea pigs to gasoline vapor under laboratory conditions was studied. Thirty males of guinea pigs were used in the experiment where it was divided into two main groups (control and experimental). Each group was subdivided into three subgroups according to the duration of exposure (30, 60 and 90 days). Experimental groups were subjected to gasoline vapor in an inhalation chamber for 6 h daily, 5 days a week. Control group were left access to fresh air under laboratory conditions. Gasoline vapor induced histological changes, which increased according to duration of exposure. There were infiltration of inflammatory cells within mucosa and submucosa of trachea, loss of cilia in tracheal epithelium and increase in size of tracheal gland in submucosa, disruption and desquamation in tracheal epithelium and infiltration and reduction in goblet cell numbers. Scanning electron microscopic findings revealed that shorting and disorientation of cilia, focal disruption and decudation of ciliated epithelial cells and decrease in number of goblet cells in comparison to control group. It was concluded that gasoline vapor can harm tracheal mucosal and submucosal components, which will reflect on its functions and predispose to lower respiratory tract diseases. Prophylactic measures must be provided during exposure to car fuel (gasoline) inhalation.

Key words: Gasoline, trachea, inhalation, guinea pigs, goblet cells

INTRODUCTION

Gasoline is one of the largest volume commercial products in the world (Cutler *et al.*, 2007). It is a complex substance of variable composition depending on the source of raw material (crude oil), the refining processes, performance specifications, season and other factors (Grebic *et al.*, 2007). Formulated gasoline contains both volatile and nonvolatile components covering a wide distillation range. Under normal conditions of consumer use and in gasoline production facilities, exposure is primarily via inhalation of the vaporized material (Benson *et al.*, 2001).

Gasoline can be modified to reduce pollution and improve the fuel combustion by adding components containing oxygen. The most widely used oxygenate is Methyl Tertiary-Butyl Ether (MTBE), whereas Ethyl Tertiary-Butyl Ether (ETBE), Tertiary Amyl Ethyl Ether (TAME), methanol and ethanol are possible substitutes

(Nihle'n *et al.*, 1998; Lin *et al.*, 2005; Zhang *et al.*, 2006). The amount of MTBE added to gasoline varies with season, among gasoline companies and between regions and countries. Typically, concentrations up to 15% MTBE by volume (2.7% oxygen by weight) are being used in oxygenated gasoline (Graham *et al.*, 1993; Upton *et al.*, 1996).

The respiratory tract is the first target for atmospheric pollutants. Chronic exposure of female rats to gasoline vapors for 2 years resulted in an increase in the incidence of mild multifocal pulmonary inflammatory response (compared to respective controls) that was thought to be due to the irritant effect of gasoline (MacFarland *et al.*, 1984). Al-Saggaf *et al.* (2008) previously reported that, guinea pigs exposed to gasoline vapor resulted in focal collapse in the lung parenchyma, infiltration of inflammatory cells, intra-alveolar hemorrhage, blood congestion, disruption of alveolar septa causing focal emphysema, marked lymphoid aggregation within parenchyma.

Several studies were performed to determine the hazardous effect of inhaling gasoline vapor on lung were performed (MacFarland *et al.*, 1984; O'Regan and Turgeon, 1986; Vyskocil *et al.*, 1988), while little attention had been paid to the effect of gasoline on trachea, which is an important airway. So, the aim of this study was to determine the histological and scanning electron microscopic changes of guinea pigs trachea induced by inhalation of gasoline vapor in laboratory.

MATERIALS AND METHODS

Animals and animal care: Thirty Dunkin Hartley males guinea pigs weighing between (400-600 g) were purchased from animals house in King Fahd Medical Research Center. Animals were housed in a polycarbonate cage with stainless steel wire lids under constant conditions of temperature and humidity, with 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.

Inhalation process: The animals were randomized into two main groups control and experimental (15 animals each). Experimental group was subdivided into three groups (5 animals each) according to the duration of gasoline exposure (30, 60 and 90 days). Two experimental animals were placed in each cage with a total of four cages inside the inhalation glass chamber (160×130×70 cm). The cover of the chamber has four circular openings (10 cm in diameter) for fresh air to enter. A container filled with 1 L of gasoline (octane 95) was obtained from car fuel station in Jeddah. It was put (introduced) in the center of the chamber then the lid was opened to subject the animals to gasoline vapor for 6 h daily, 5 days a week for the total period of the experiment (Sureshkumar *et al.*, 2005). Experimental animals were removed from the inhalation chamber and left to recover in a different room with fresh air before returned back in the next day. The gasoline was changed twice per week to insure the presence of gasoline volatile materials during the whole duration of the experiment. Inhalation chambers were placed in the laboratory at King Fahd Medical Research Center. Control group were left in another room with access to fresh air under laboratory conditions.

Tissue processing and histological techniques: At 30, 60 and 90 days animals from each group with its counter partner control were euthanized by neck dislocation after anesthesia by ketamine and seton. The chest was opened and trachea was injected via insulin syringe with normal saline followed by 10% neutral buffered formalin for light microscopy and gluteraldehyde (3%) in phosphate buffer

for scanning electron microscopy to insure good washing of surface epithelium. Samples for light microscopy were fixed, dehydrated, cleared before embedding in paraffin wax, then sectioned 5µm thickness. For light microscopic examination sections were stained with Hematoxyline and Eosin (H and E). For counting goblet cells we chose Alcian Blue-Periodic Acid Schiff (AB-PAS) stain. 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. To quantify number of goblet cells, the mean number of all goblet cells counted at 400×/field was determined and twelve visual fields, six from the right side and six from the left side were counted per tracheal section (Guo *et al.*, 2003). Samples from trachea of guinea pigs, which exposed to gasoline vapor for 90 days were prepared to be examined by Scanning Electron Microscope (SEM). They were fixed in 2% glutaraldehyde and osmium tetroxide. Then they were dehydrated in a graded series of ethyl alcohol (70-100%), then immersed in acetone and infiltration in a mixture of acetone and hexa methylsilazana 1: 1. The dried samples were fixed on the specimen stubs and coating with gold stain.

Statistical analysis: The values of the various parameters (animal weight, number of goblet cells) 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 using SPSS computer program. Differences among groups were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

The present results showed that during exposure to car fuel (gasoline) vapor within inhalation chamber, the animals looked calm, with sluggish movement. After they were removed they showed aggressive behavior, restlessness and tend to bite each other.

Effect of car fuel inhalation on body weight of adult male guinea pig: As regards body weight, there was no significant difference between control and experimental groups during the whole length of the experiment (Table 1).

Table 1: Influence of gasoline vapor exposure on animal's weight

Groups	Days		
	30	60	90
Control	697.7±31.4	782.2±14.9	850±20
Experimental	726.8±12.5	784±37.3	869.8±30.6

Student t-test: No significant difference between experimental and control group

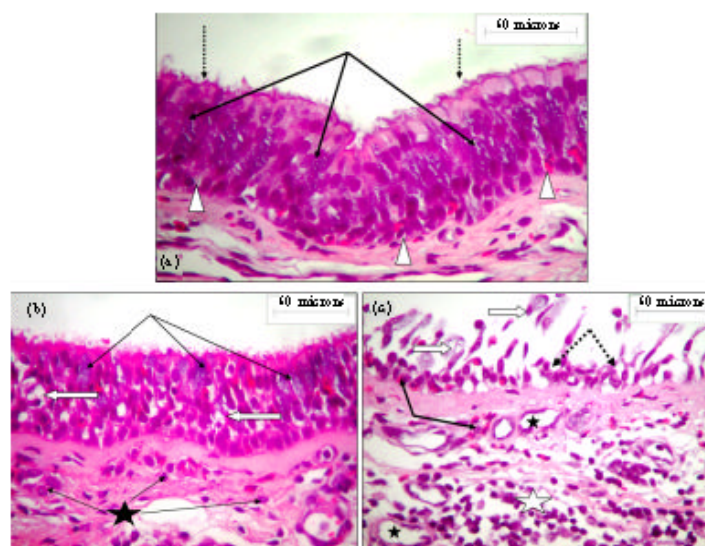


Fig. 1: a) Control guinea pig tracheal epithelium showing normal pseudo stratified columnar epithelium with goblet cells showing basophilic stained mucous content (arrows). The surface cilia could be seen as a fuzzy layer (dotted arrows). Few eosinophil and lymphocytes are seen in the basal layer (white arrow heads). b) Thirty days following careful (gasoline) inhalation, guinea pig tracheal epithelium showing degeneration and shrinkage of epithelial cells leaving empty spaces (thick arrows), notice the decrease of number and size of goblet cells (thin arrows). The underlying lamina propria showed few mononuclear cells infiltrate (star and arrows). c) Ninety days after careful inhalation showing marked destruction and desquamation of tracheal epithelium (dotted arrows). Goblet cells are seen fallen into the lumen (white arrows). The lamina propria is heavily infiltrated with mononuclear cell (lymphocytes (white star) and Eosinophils. The latter are seen migrating to damaged epithelium (black thin arrows). Dilated vessels (black stars) are seen among cellular infiltrate. Scale bar = 60 μ m (Hx and E stain)

Light microscopic study of tracheal mucosa

Control tracheal mucosa: Sections stained with H and E showed that trachea in control group consist of mucosa, submucosa and fibro-cartilagenous layer. Tracheal mucosa is lined by pseudostratified ciliated columnar epithelium with goblet cells. Columnar ciliated cells, basal cells and intervening goblet cells could be easily distinguished in H and E stained sections. Goblet cells could be also identified by their shape and the accumulation of purple mucus granules in their apical regions (Fig. 1a). In PAS-alcian blue stained sections, goblet cells were easily identified by their affinity of mucous granules to alcian blue, whereas the nearby columnar cells stained moderately by PAS (Fig. 2a). The sub-epithelial mucosal and submucosal connective tissue of trachea in control groups showed normal structure with no inflammatory infiltration (Fig. 1a).

Effect of different periods of exposure to inhalation of car fuel (gasoline) vapor: Exposure to of careful (gasoline) vapor for 30 days induced degeneration and shrinkage of epithelial cells, leaving empty spaces at the site of lost

cells. There was loss of normal ciliated surface (Fig. 1b). Increasing the duration of exposure to 60 and 90 days resulted in progressive damage of surface epithelium. Exposure for 90 days to car fuel vapor resulted in marked destruction and desquamation (deciduation) of tracheal epithelium. Goblet cells were seen fallen into the lumen. The lamina propria is heavily infiltrated with mononuclear cells and Eosinophil. The latter are large in size are seen migrating to overlying damaged epithelium (Fig. 1c).

Effect of inhalation of car fuel on number of goblet cells:

Sections stained with PAS-Alcian blue showed that although goblet cells were seen larger and engorged with faintly stained mucous, they were significantly decrease in number (Fig. 2b). The decrease in goblet cells observed at light microscopic examination was significant ($p < 0.05$) following car fuel exposure at all periods compared to control unexposed animals (Table 2).

Scanning electron microscopic of tracheal mucosal surface:

Scanning microscopy gives a dimensional view of tracheal surface epithelium regarding ciliated and

Table 2: Influence of gasoline vapor inhalation on number of tracheal goblet cells

Groups	Days		
	30	60	90
Control	40.05±1.2	41.1±1.3	43.2±1.1
Experimental	29.8±1.5*	29.7±1.9*	29.2±1.94*

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

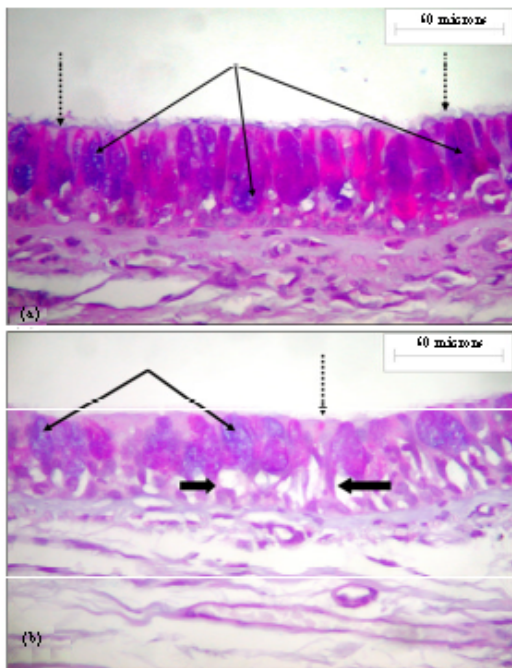


Fig. 2: a) Control guinea pig tracheal epithelium stained by PAS-alcian blue showing normal intact epithelium and goblet cells stained blue for its mucous content. Notice the intact ciliated surface (dotted arrow). b) Sixty days after car fuel (gasoline) inhalation guinea pig tracheal epithelium showing basal separation of epithelial cells (thick horizontal arrows), decrease number and staining intensity of goblet cells (black arrows) and loss of surface cilia (dotted arrows). Scale bar = 60 µm (PAS-alcian blue stain)

goblet cells, which was found to be correlated with light microscopic observation. Ciliated cells in control samples possess long healthy cilia that mask the nearby columnar non ciliated types (Fig. 3a). In trachea of animals exposed for 90 days to gasoline vapor inhalation, scanning microscopy showed, focal loss of cilia, other regions showed shortage and ciliary or clumping (Fig. 3b).

In Control group, regions where goblet cells dominating they could be identified from their apical rounded surface scattered among ciliated cells (Fig. 4a). Goblet cells in exposed animals to gasoline for 90 days

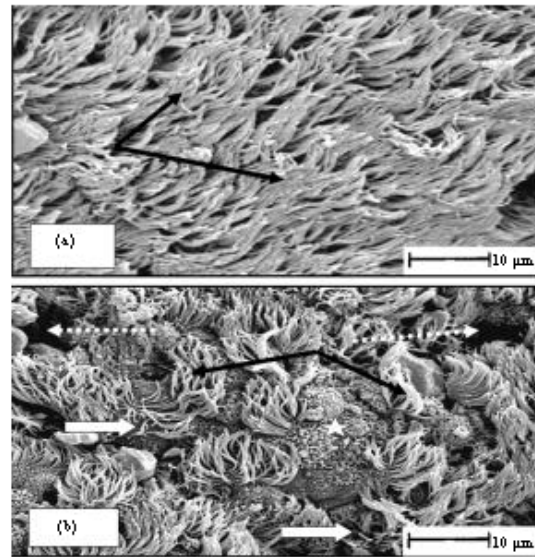


Fig. 3: Scanning microscopy of Guinea pig tracheal surface showing: a) Control normal surface cilia (black arrows). b) Ninety days after car fuel (gasoline) inhalation tracheal surface showing damage and loss (white arrows), shorting and clumping of cilia (black arrows). Non ciliated cells having short microvilli (star). Empty spaces of lost cells (dotted white arrows) could be seen. Scale bar = 10 µm

were engorged with mucous and seen detached (deciduation) and protrude above the surface (Fig. 4b).

Gasoline; the main constituent of car fuel is a volatile, highly flammable liquid mixture of hydrocarbons. It is used in internal combustion engines. It is a mixture of over five hundred hydrocarbons with varied composition according to its place of origin. Because of the wide-spread use of gasoline and oxygen containing additives, such as MTBE, the number of people exposed is very large (Lin *et al.*, 2005). Gasoline gains entrance into the body by many routes. The inhalation route would appear to be more important because more persons may be affected (Zhang *et al.*, 2006). Employees in gasoline companies may be exposed during production and transportation processes. In addition, gasoline station attendants and the general public may be exposed during refueling (Guo *et al.*, 2003). Toxic effects of inhaling gasoline vapor and additives via inhalation route range from affection of respiratory, cardiovascular, gastrointestinal, haematological, musculoskeletal, hepatic, renal and neurological systems to induction of cancer in different organs (Hartle, 1984; Wong *et al.*, 1993).

In the present study, the histological and scanning microscopic changes of guinea pigs trachea

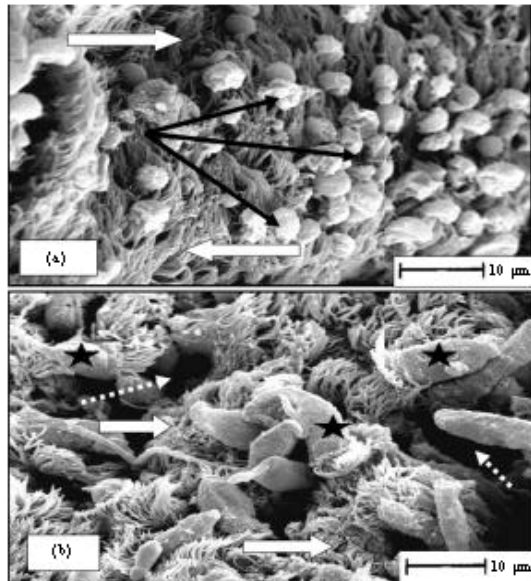


Fig. 4: Scanning microscopy of goblet cells in guinea pig tracheal epithelium showing, a) control shows normal rounded shape cells (black arrows) among ciliated cells (white arrows). b) Ninety days after car fuel (gasoline) inhalation tracheal surface showing desquamated or sloughed engorged goblet cells (black stars). Notice the clumped short cilia (white arrows). The empty spaces left by degenerated cells (dotted white arrows). Scale bar = 10 µm

induced by inhalation of gasoline vapor in laboratory for different periods (30, 60 and 90 days) were investigated.

Intermediate and chronic-duration exposures to gasoline vapors have been reported to cause significant decreases in body weight gain in rats and mice (MacFarland *et al.*, 1984; Vyskocil *et al.*, 1988). In another study, depressed body weight gain was observed in female rats exposed for 4 weeks to ethanol/gasoline (Smith *et al.*, 1993). On the contrary, in this study as there was no significant difference between control and experimental groups during the whole length of the experiment (30, 60 and 90 days).

As regards animal's behavior during the experiment, experimental group experience periods of restlessness and violet behavior as they bite each others. Inhaling gasoline vapor may affect their nervous system as (Chu *et al.*, 2005) reported in his study on neurological effects of gasoline on rabbits. All the exposed animals exhibited restlessness, equilibrium disturbances, convulsions and narcosis.

Gasoline has been shown to be irritating at the portal of entry (i.e., the eyes, the lungs after inhalation, or the

gastrointestinal mucosa after ingestion). MTBE and ethanol vapors deposit substantially in the upper airway structure with a marked enhancement of dose at local sites (Zhang *et al.*, 2006). Several studies reported that inhalation of gasoline induced irritation and histological changes at the epithelial lining of the nasal cavity and upper respiratory tract (Przybylowski, 1971). In the present study, as a reaction to inhaling gasoline vapor in laboratory for 30 days, infiltration of inflammatory cells (lymphocytes, eosinophils) within mucosa and submucosa. When exposure period to gasoline was increased to 60 days loss of cilia in tracheal epithelium and increase in size of tracheal gland in submucosa were occurred. Animals exposed to gasoline vapor for 90 days had disruption and desquamation in tracheal epithelium and infiltration of submucosa with lymphocytes.

The acute respiratory effects of gasoline inhalation in rats were exposed to 0.1 mL gasoline in a closed container either once or intermittently for 5-7 days. In both exposure scenarios, widespread hemorrhage of the lungs was noted at necropsy (O'Regan and Turgeon, 1986). On contrary rats and monkeys failed to exhibit any consistent adverse respiratory effects following intermediate-duration exposure to 1,552 ppm gasoline vapors (Mann *et al.*, 2002). When, the lungs of rats exposed to 100 ppm of gasoline vapors for 12 weeks were examined by electron microscopy, a progression of lesions characteristic of fibrosing alveolitis (interstitial fibrosis and alveolar collapse) was observed (Lykke *et al.*, 1979). These lesions were not apparent at the light microscopic level. These results indicated that gasoline-induced pulmonary changes may occur at levels previously thought to cause no effect because the tissues were not examined ultrastructurally and/or other sensitive parameters of pulmonary function were not measured.

Previous study reported that, guinea pigs exposed to gasoline vapor for 30, 60 and 90 days in laboratory resulted in focal collapse in the lung parenchyma, infiltration of inflammatory cells, intra-alveolar hemorrhage, blood congestion, disruption of alveolar septa causing focal emphysema, marked lymphoid aggregation within parenchyma, alveolar thickening with increased interstitial reticulin disposition, enhanced proliferation of bronchus associated lymphoid tissue, sloughing of epithelial cell, increased mucosa secretion and elongation of epithelial folds lining the bronchus and bronchiolar tissue (Al-Jahdali *et al.*, 2007).

Trachea as a part of respiratory airways is lined by pseudostratified columnar ciliated epithelium that contains many mucus-secreting goblet cells. Goblet cells (30% of cell population) secrete mucus that coats the epithelial cells. This mucous coating protects the

epithelial cells from desiccation and traps inhaled particulates that are then transported up the airways and out of the lungs by the ciliated cells (Pocock *et al.*, 2006). The regulatory mechanisms that govern goblet cell proliferation and differentiation or the expression and release of characteristic secreted products are poorly understood. In the present study, results revealed that inhalation of gasoline vapor significantly decreased the number of goblet cells in experimental groups for 30, 60 and 90 days, when compared with the corresponding control group.

As regards the scanning changed induced by inhalation of gasoline vapor for 90 days, scanning electron microscopic examination of tracheal epithelium revealed that shorting and disorientation of cilia, focal disruption and decudation of ciliated epithelial cells and decrease in number of goblet cells in comparison to control group. These findings confirmed the histological observation. In a study on rats, which exposed to 100ppm of gasoline vapors for 12 weeks had fibrosing alveolitis (interstitial fibrosis and alveolar collapse), which were detected by electron microscopy. These lesions were not apparent at the light microscopic level (Kuna and Ulrich, 1984).

CONCLUSION

Gasoline vapor induced histological changes in trachea, which increased according to duration of exposure. It remains uncertain what compound(s) in gasoline caused the observed harmful effects on tracheal epithelium. Further researchs would be required to determine what constituent(s) may be responsible for these effects. Also, perform further studies on car fuel station to compare between laboratory and station results.

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