Protective Effects of Vitamin C Against Benzene-Induced Lung Injury in Rats

DOAA A. SOUROUR, M.D.; NESRIENE M. EL MARGOUSHY, M.D. and GAMAL M. EL NEMR, M.D.
The Department of Medical Research and Radiation, Nuclear Materials Authority, Egypt

Abstract

Background: Exposures to car fuel vapor among workers result in functional alterations in the airways of exposed people and appearance of pulmonary diseases. Benzene, a well-known human carcinogen and a ubiquitous environmental pollutant, possesses a potential health threat to a broad spectrum of individuals. Benzene exerts multiple effects by generating one or more reactive oxygen species. Thus the use of natural antioxidants may be a protective strategy against benzene-induced toxicity.

Aim: The present study was designed to investigate the protective role of vitamin C, a strong antioxidant, in attenuating benzene-induced lung injury in rats.

Methods: Thirty male albino Wistar rats were divided into three groups as follows: Group I: Control group; group II: Rats exposed to benzene inhalation (6h/day, 5 days/week) for 4 weeks; group III: Rats exposed to benzene inhalation (6h/day, 5 days/week) and received vitamin C treatment concomitantly (200mg/kg/day given orally) for 4 weeks. Several biochemical parameters were estimated. Histological examinations of the lung and trachea were performed.

Results: The results of the present study showed that vitamin C treatment in benzene exposed rats resulted in significant decrease in apoptotic Bax gene expression in the lung with significant increase in lung antiapoptotic Bcl-2 gene expression compared to untreated benzene exposed group. Vitamin C treatment increased significantly lung matrix metalloproteinase-1 mRNA expression, decreased significantly lung tissue inhibitor matrix metalloproteinase-1 mRNA expression together with significant decrease in lung hydroxyproline content as well as significant increase in plasma paraoxonase-1 level in benzene treated rats compared to untreated benzene exposed group. Vitamin C also improves histological changes in the lung and trachea in benzene treated group.

Conclusion: From this study it can be concluded that co-administration of vitamin C during benzene inhalation in rats may significantly diminish the toxic effects of benzene on the lung. This beneficial effect of vitamin C may be mediated by its antioxidant effect and as a consequence results in antiapoptotic and antifibrotic effects.

Key Words: Benzene – Lung – Apoptosis – Matrix metalloproteinases – Vitamin C.

Introduction

EXPOSURE to volatile chemicals is a major occupational and environmental health concern. Benzene, a lipid-soluble volatile organic compound, is the simplest compound of aromatic hydrocarbons which is found naturally in the environment at low concentrations. Benzene occurs naturally in crude oil and as a consequence is a constituent of petrol. It is also a commercially important intermediate in the manufacture of many chemicals [1].

A considerable amount of the current environmental exposure is related to all combustion processes, including emissions from automobiles and tobacco smoking [2].

Exposure to benzene can occur via the oral, and/or dermal route but the inhalation route is the most important especially in occupational environments. Acute inhalation exposure of humans to benzene causes systemic toxicity such as narcosis, and at high concentrations, respiratory depression [3]. Chronic inhalation exposure has been linked to various hematologic disorders such as aplastic anemia, leukopenia, thrombocytopenia or pancytopenia in occupational setting [4].

Benzene is a known human carcinogen in which human subjects exhibit increased incidence of leukaemia, increased risks for adenocarcinoma and squamous cell carcinoma of the lung when occupationally exposed to benzene [5].

Inhalation of benzene in rats induced apoptotic changes in the parenchymal components in the lungs that significantly exceeded the events of programmed cell death in normal control tissues [6]. Benzene also altered the physiologic profile
of matrix metalloproteinases (MMPs) on human lung cells in vitro [2].

MMPs are zinc-dependent endopeptidases, known for their ability to cleave one or several constituents of the extracellular matrix (ECM). They have been implicated in lung pathological conditions including fibrosis. The activities of MMPs are controlled at several levels including their interactions with specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). It has been suggested that abnormal alteration of MMPs/TIMPs balance could lead to disruption of lung tissue, and/or accumulation of ECM without adequate repair, leading to impairment of lung function [7].

Beside its direct toxicity, benzene exerts multiple effects after being converted to reactive metabolites such as hydroquinone and benzoquinone by generating one or more reactive oxygen species (ROS) such as superoxide anion (O\(^{2-}\)), hydrogen peroxide (H\(_2\)O\(_2\)), and hydroxyl radicals (OH·) [8].

Vitamin C, the most abundant and effective antioxidant in the human body, is a potent water-soluble antioxidant that scavenges reactive oxygen and nitrogen species. It prevents oxidative modification of both cytosolic and membrane component of cells [9]. Vitamin C deficiency is characterized by increased oxidative stress and tissue injury including oxidant-induced necrotic cell death [10].

Compared to available literature, the protective effect of vitamin C against benzene-induced lung toxicity is not well investigated. Thus the present study was designed to find out the preventive role of vitamin C in attenuating benzene-induced lung injury in rats.

**Material and Methods**

**Animals**

A total of thirty healthy male Wistar albino rats weighing 200-230g obtained from the animal house of Faculty of Medicine, Cairo University were used in this study. The animals were housed in standard stainless-steel cages at a 12h cycle of light and dark. Room temperature was kept at 24±2°C and humidity maintained at 50%. Rats were provided with standard food and water ad libitum. All animal experiments were carried out in strict compliance with the guidelines of the Institutional Animals Ethics Committee on the care and use of laboratory animals.

**Drug and chemical**

Vitamin C (Memphis CO. For Pharm. And Chemical, Astrapzeneca, Egypt) was supplied as white powder, soluble in distilled water freshly prepared as aqueous solution to be given as a single daily oral dose of 200mg/kg/day [11] via gastric tube for 4 weeks. Pure benzene was obtained from El Nasr Pharmaceutical Chemicals Company, Cairo, Egypt.

**Inhalation process**

Experimental animals were placed in cages with two animals placed in each cage with a total of four cages inside the inhalation chamber (160x130x70cm). The cover of the inhalation chamber has four circular openings (10cm in diameter) for fresh air to enter. A container filled with 1L of benzene was put (introduced) in the center of the chamber then the lid was opened to subject the animals to benzene vapor for 6h daily, 5 days a week for the total period of the experiment [12]. 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 benzene was changed twice per week to insure the presence of benzene volatile materials during the whole duration of the experiment. Control group were left in another room with access to fresh air under laboratory conditions.

**Experimental protocol:**

Rats were divided into three groups (10 rats each) as follows:

- **Group I (unexposed group):** Control rats were exposed to fresh air only.
- **Group II (Benzene exposed group):** Rats were exposed by inhalation to benzene for 4 weeks (6h/day, 5 days/week).
- **Group III (Benzene exposed+Vitamin C treated group):** Rats were exposed by inhalation to benzene for 4 weeks (6h/day, 5 days/week). Treatment with vitamin C (200mg/kg/day), given orally via gastric tube, began concomitantly with benzene inhalation and continued for 4 weeks.

At the end of the experiment (4 weeks), blood samples were taken from the rat tail vein in collecting heparinized capillary tubes. The samples were centrifuged at 1000 Xg and plasma was split and frozen in polypropylene tubes at −20°C until assayed. Then all rats were sacrificed and the lung and tracheal tissues from all groups were removed. A part of the lung was blotted with a piece of filter paper, kept in foil paper and freezed at −70°C till used for further biochemical analysis. Another part of the lung and part of trachea were fixed in 10% formal saline for histological studies.
Biochemical determinations:

- Detection of Bax and Bcl-2 gene expression by Reverse transcription-polymerase chain reaction (RT-PCR):

  Total RNA was extracted from lung tissue by the acid guanidinium thiocyanate-phenol-chloroform method [13]. RNA content and purity was measured using ultraviolet spectrophotometer (A260/A280 ratio of 1.8-2.0). For amplification of the target genes, reverse transcription-PCRs were run in two separate steps. In brief, equal amounts of total RNA (6µg) were heat denatured and reverse transcribed by incubating at 42°C for 90min with 12.5U (avian myeloblastosis virus) reverse transcriptase (Promega, Madison, WI, USA), 20U ribonuclease inhibitor RNasin (Promega Corp.), 200nM deoxyribonucleotide triphosphate mixture and 1nM oligo-dT primer in a final volume of 30ul of 1x avian myeloblastosis virus buffer. The reactions were terminated by heating at 97°C for 5min and cooled on ice. Complementary DNA samples were amplified in 50ul of 1x PCR buffer in the presence of 2.5U Taq DNA polymerase (Promega), 200nM deoxyribonucleotide triphosphate mixture and appropriate primer pairs (1nM of each primer). PCR consisted in a first denaturing cycle at 97°C for 5min, followed by a variable number of cycles of amplification defined by denaturation at 96°C for 1.5min, annealing for 1.5min and extension at 72°C for 3min. A final extension cycle of 72°C for 15min was included.

    RT-PCR for β-actin, a housekeeping gene, was performed in the same PCR run to confirm integrity of RNA and for relative quantitation of PCR products. All PCR products were electrophoresed on 2% agarose stained with ethidium bromide and visualized by ultraviolet transilluminator (Fig. 1). Primer sequences were as follows: beta actin gene: forward primer: 5´TGTTGTCCCTGTATGCCTCT-3´, reverse primer: 5´-5´ TAATGTCACGCACGATTTCC-3´, Bax gene: forward primer: 5 CT-GAGCTGACCTTGAGGAGC-3, reverse primer: 5-GACTCCAGCCAAAGATG-3; Bcl-2 forward primer: 5-GAGGACCACCTTCTCAGAG-3 and reverse primer: 5-GGCGTGGCATTCCAGACT-3.

Semiquantitation was performed using gel documentation system (BioDO, Analyser) supplied by Biometra. According to the amplification procedure, relative expression of each studied gene (R) was calculated following the formula:

\[
R = \frac{\text{Densitometrical Units of each studied gene}}{\text{Densitometrical Units of β-actin}}.
\]

- Detection of MMP-1 and TIMP-1 gene expression by RT-PCR:

  RNA extraction:

  Total RNA was extracted from lung tissue by the acid guanidinium thiocyanate-phenol-chloroform method [13]. RNA content and purity was measured by a UV spectrophotometer.

  RT-PCR experiments:

  RT-PCR was done using the extracted RNA for detection of MMP-1 and TIMP-1 genes. For amplification of the targets genes, reverse transcription and PCR were run in two separate steps. Briefly, Reaction mixture of RT reaction containing 1µg total RNA, 0.5µg random primer, 5xRT buffer, 2.5mmol/L dNTP, 20U RNase inhibitor and 200U MMLV reverse transcriptase in a total volume of 25µl was incubated at 37°C for 60 minutes, then heated to 95°C for 5 minutes to inactivate MMLV.

  PCR was carried out with 1.5µl RT products, 1 0x PCR buffer (without Mg$^{2+}$) 2.5µl, 2.0µl dNTP (2.5mmol/L), 2.0µl MgCl$_2$ (25mmol/L), 0.5µl each primer (20µmol/L) of β-actin, 0.5µl each primer of gene to be tested (20µmol/L) and 1U of Taq DNA polymerase (Promega Corp.), in a final volume of 25µl. Thermal cycler conditions were as follows: A first denaturing cycle at 97°C for 5min, followed by a variable number of cycles of amplification defined by denaturation at 96°C for 1.5min, annealing for 1.5min and extension at 72°C for 3min. A final extension cycle of 72°C for 15min was included. The specific primers for beta actin gene: forward primer: 5´ TGTGTCCCTGTATGCCTCTGCTCTCT-3´, reverse primer: 5´-5´ TAATGTCACGCACGATTTCC-3´, rat MMP-1 was: 5´ CG-GAGCAGCGGGGGACGGGGAT-3´ (forward) and 5´ AAGACGAAGGGGGGACGGGAT-3´ (reverse) and rat TIMP-1 was: 5´-CTTGGCATCCTTTTGCTGCT-3´ (forward) and 5´-CAGCAGCAGCAGAGTAGGTCTTT-3´ (reverse).

Agarose gel electrophoresis:

All PCR products were electrophoresed on 2% agarose stained with ethidium bromide and visualized by UV transilluminator (Fig. 2).

Semi-quantitative determination of PCR products:

Semi-quantitation was performed using the gel documentation system (BioDO, Analyser) supplied...
by Biometra. According to the following amplification procedure, relative expression of each studied gene (R) was calculated following the formula:

\[ R = \frac{\text{Densitometrical Units of each studied gene}}{\text{Densitometrical Units of } \beta\text{-actin}} \]

\( \beta\)-actin gene, a housekeeping gene, was used as an internal control such that data were standardized according to \( \beta\)-actin values.

- Hydroxyproline content assay:

To estimate the total amount of collagen in the lung, hydroxyproline was measured by a colorimetric assay as described previously by Nakamura et al. \([14]\). Briefly, lung sections (0.5g) were hydrolyzed for 20h in 6mol/L HCl at 100°C, redissolved in water and centrifuged to remove any impurities. Samples were incubated for 10min in 0.05mol/L chloramine-T (Fisher, Fair Lawn, NJ, USA) at room temperature, followed by 15-min incubation in Ehrlich's perchloric acid solution at 65°C. Sample absorbencies were assessed at 561nm and resulting values compared to a hydroxyproline standard curve. Each sample was assayed in duplicate. The hydroxyproline content was expressed as micrograms per gram of wet lung.

- Paraoxonase-1 (PON1) assay:

PON1 was estimated spectrophotometrically by a modified technique described by Schiavon et al. \([15]\). Briefly, the assay mixture consists of 500µl of 2.2mmol/l paraoxon substrate in 0.1mol/l Tris-HCl buffer, pH 8.0 containing 2mmol/l CaCl\(_2\) and 50µl of fresh plasma. After mixing the contents, kinetic measurements were taken immediately at every minute for five minutes, at 405nm at 25°C. First absorbance reading is taken as 0-minute reading and subsequent absorbance readings were taken as one-minute to four-minute readings. Corrected absorbance readings were obtained by subtracting 1 minute reading with 0 minute reading; likewise, the latter minute reading was subtracted with the previous minute readings. The mean absorbance was calculated. Mean absorbance was used to determine PON1 level, and standard graph plotted using 1mM P-Nitrophenol. PON1 level was expressed in international units (IU). One IU was defined as 1 \( \mu \text{mol of p-nitrophenol formed/min} \) /L at 25°C.

**Histological examination:**

Animals were sacrificed by an overdose of ether vapor anesthesia. Light microscopic specimens from lung and trachea were put in 10% neutral buffered formalin. Paraffin blocks were prepared and sectioned. Serial sections of 5µm thickness were obtained and stained by:

1. Haematoxylin and Eosin (H&E): For histological examination of both lung and trachea \([16]\).
2. Masson trichrome to reveal lung collagen \([16]\).

**Quantitative morphometric measurements:**

The area percent of collagen deposition in Masson trichrome stained sections was estimated by using “Leica Quin 500” image analyzer computer system (Leica image system Ltd; Cambridge, England). Measurements were done within 10 non overlapping fields for each animal at X400 magnification. Each field consisted of alveoli and inter-alveolar septa but no bronchi, bronchioles or blood vessels were encountered in the manually selected areas.

**Statistical analysis:**

Data were summarized using mean and standard deviation (SD) for the quantitative variable using the statistical package The Instat version 2.0. Comparisons between groups were done using ANOVA (analysis of variance) followed by Tukey-Kramer test for intergroup comparisons. The results were considered significant when \( p \)-value <0.05.

**Results**

**Biochemical results:**

Effects of benzene on lung Bax and Bcl-2 mRNA expression:

In this study, the levels of Bax gene are significantly increased \((p<0.001)\) with significant decrease \((p<0.001)\) in the levels of Bcl-2 gene in the lungs of rats exposed to benzene inhalation (group II) compared to control rats (Table 1, Figs. 3,4 respectively). Coadministration of vitamin C during benzene inhalation for 4 weeks (group III) led to significant decrease \((p<0.001)\) in lung Bax mRNA expression with significant increase \((p<0.001)\) in lung Bcl-2 mRNA expression as compared to untreated benzene exposed rats (group II) but there was significant difference \((p<0.001)\) in group III compared to control group (Table 1, Figs. 3,4 respectively).

Effects of benzene on lung MMP-1 and TIMP-1 mRNA expression:

The present study showed alterations in lung MMP-1 and TIMP-1 gene expression, by RT-PCR in benzene exposed rats compared to control rats. There was significant decrease \((p<0.001)\) in lung MMP-1 mRNA expression with significant increase \((p<0.001)\) in lung TIMP-1 mRNA expression in
benzene exposed rats (group II) compared to control group (Table 1, Fig. 5). Concomitant administration of vitamin C during benzene inhalation for 4 weeks (group III) led to significant increase ($p < 0.001$) in lung MMP-1 mRNA expression with significant decrease ($p < 0.001$) in lung TIMP-1 mRNA expression compared to untreated benzene exposed group (Table 1, Fig. 5). Meanwhile, levels of MMP-1 and TIMP-1 genes in the lung did not reach control value by vitamin C treatment as indicated by significant difference ($p < 0.001$) in group III as compared to control group (Table 1, Fig. 5).

**Effects of benzene on lung hydroxyproline content**

In the present study, rats exposed to benzene inhalation for 4 weeks (group II) showed significant increase ($p < 0.001$) in lung hydroxyproline contents as compared to control group (Table 1, Fig. 6).

Vitamin C treatment in benzene exposed rats for 4 weeks (group III) led to significant decrease ($p < 0.001$) in lung hydroxyproline content as compared to untreated benzene group, but this decrease did not reach control value indicated by significant difference ($p < 0.001$) in group III compared to control group (Table 1, Fig. 6).

**Effects of benzene on plasma PON1 level**

The present study showed significant decrease ($p < 0.001$) in plasma PON1 level in benzene exposed rats compared to control group (Table 1, Fig. 7). Treatment of benzene exposed rats with vitamin C (group III) for 4 weeks led to significant increase ($p < 0.01$) in plasma PON1 level compared to untreated exposed benzene group (group II) but with significant difference ($p < 0.001$) compared to control group (Table 1, Fig. 7).

![Fig. (1): An agarose gel electrophoresis showing PCR products of Bcl-2 gene (A), Bax gene (B) & beta actin gene (C) in rat lungs of the studied group.](image1)

Lane M: DNA ladder with 100bp.
Lane 1: PCR products in control group.
Lane 2: PCR products in benzene exposed group.
Lane 3: PCR products in benzene exposed + vitamin C treated group.

![Fig. (2): An agarose gel electrophoresis showing PCR products of MMP-1 gene (A), TIMP-1 gene (B) & beta actin gene (C) in rat lungs of the studied group.](image2)

Lane M: DNA ladder with 100bp.
Lane 1: PCR products in control.
Lane 2: PCR products in benzene exposed group.
Lane 3: PCR products in benzene exposed + vitamin C treated group.

![Fig. (3): Mean levels of lung Bax gene expression in the studied groups.](image3)

<table>
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<th>Parameters</th>
<th>Control Group I</th>
<th>Benzene Group II</th>
<th>Benzen + VitaminC Group III</th>
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<td>TIMP-1 gene</td>
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<td>Hydroxyproline content (ug/g protein)</td>
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<td>7.16±0.76#</td>
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<td>PON1 (U/L)</td>
<td>25.16±3.00</td>
<td>12.06±1.34*</td>
<td>17.08±0.11#</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 10 each group).
*Significant difference from control group.
#Significant difference from benzene exposed group.
Levels of Bcl-2 gene

Levels of MMP-1 & TIMP-1 genes

Levels of lung hydroxyproline

Levels of PON1 (U/L)

- Histological results:

A- Trachea:

H&E:

Sections stained with H&E showed that the trachea in the control group (group I) consists of mucosa, submucosa and fibro-cartilagenous layer. Tracheal mucosa is lined by pseudostratified ciliated columnar epithelium with goblet cells (Fig. 8).

Examination of tracheal H&E stained sections in rats exposed to benzene (group II) revealed shortening, clumping and even loss of cilia in some epithelial cells (Fig. 9). Some tracheal epithelium showed degeneration of epithelial cells leaving empty spaces with disruption of the epithelium in certain areas (Figs. 9,10). Both lamina propria of mucosa and submucosa of trachea showed heavy infiltration with inflammatory cells (Fig. 10).

H&E stained sections of rats exposed to benzene vapor for 20 days and treated with vitamin C (group III) showed diminished pathological changes in tracheal epithelium and diminished inflammatory cellular infiltration in mucosa and submucosa in comparison with non treated rats subjected only to benzene (group II, Fig. 11).

B- Lung:

H&E:

In control group (group I) H&E stained sections revealed normal lung architecture with spongy appearance of the lung. The alveoli and alveolar sacs attained thin walls or septa (Fig. 12).

Examination of lung H&E stained sections in rats exposed to benzene (group II) revealed separation of bronchial epithelial cells from the basement membrane (Fig. 13) as well as sloughing of
the bronchial epithelial cells (Fig. 14) in certain areas. Nodular aggregations of inflammatory cells appeared in the wall of both bronchi and bronchioles (Figs. 13, 14) together with alveolar septum thickening which is disrupted in some areas with focal emphysematous changes (Fig. 14). The bronchial wall showed thickening (hypertrophy) of the muscle layer (Fig. 13). Blood vessels congestion were also observed (Fig. 13) with hypertrophy of the arterial muscle layer (Fig. 14).

On the other hand, rats exposed to benzene vapor for 20 days and treated with vitamin C (group III) showed diminished both alveolar septum thickening and aggregations of inflammatory cells in the wall of bronchi and bronchioles compared to group II subjected only to benzene (Fig. 15).

**Masson trichrome stained sections:**

Masson trichrome stained sections in control group revealed minimal amount of collagen in the lung interstitium (Fig. 16).

Rats exposed to benzene vapor showed extensive collagen deposition in lung interstitium affecting most of examined lung tissues (Fig. 17).

While rats exposed to benzene vapor for 20 days and treated with vitamin C showed moderate collagen deposition in the lung interstitium (Fig. 18).

**Morphometric results of area percent of collagen deposition in lung tissues:**

Table (2) showed that the mean area percent of collagen deposition in Masson trichrome stained sections was significantly increased ($p<0.001$) in rats exposed to benzene inhalation for 4 weeks (group II) compared to control group. Vitamin C treatment in benzene exposed rats (group III) significantly inhibited ($p<0.001$) collagen contents in lung tissues as compared to untreated benzene exposed rats (group II) but there was significant difference ($p<0.001$) in group III compared to control group (Table 2).

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**Fig. (8):** A photomicrograph of tracheal H&E stained section from control group showing that the trachea consists of mucosa, submucosa and fibro-cartilagenous layer. Tracheal mucosa is lined by pseudostratified ciliated columnar epithelium with goblet cells. Columnar ciliated cells possess long healthy cilia (arrow) (H&E X400).

**Fig. (9):** A photomicrograph of tracheal H&E stained section from rat exposed to benzene (group II) revealing shortening, clumping and even loss of cilia in some epithelial cells (arrow). Tracheal epithelium shows degeneration of epithelial cells leaving empty spaces in certain areas (spiral arrow) (H&E X400).
Fig. (10): A photomicrograph of tracheal H&E stained section from rat exposed to benzene (group II) revealing degeneration of epithelial cells leaving empty spaces in certain areas (spiral arrow). Both lamina propria of mucosa and submucosa of trachea show heavy infiltration with inflammatory cells (star) (H&E X400).

Fig. (11): A photomicrograph of tracheal H&E stained section from rat exposed to benzene and treated with vitamin C (group III) showed diminished pathological changes in tracheal epithelium (arrow) and diminished inflammatory cellular infiltration in mucosa and submucosa (star) in comparison with non treated rats subjected only to benzene (H&E X400).

Fig. (12): A photomicrograph of lung H&E stained section from control group showing normal lung architecture with spongy appearance of the lung. The alveoli and alveolar sacs attained thin walls or septa (arrow heads) (H&E X100).

Fig. (13): A photomicrograph of lung H&E stained section from rat exposed to benzene (group II) revealing separation of bronchial epithelial cells from the basement membrane in certain areas (bifed arrow). Nodular aggregations of inflammatory cells appeared in the wall of both bronchi and bronchioles (spiral arrow). The bronchial wall shows thickening (hypertrophy) of the muscle layer (star). Blood vessel congestion is also observed (arrow) (H&E X 100).
Fig. (14): A photomicrograph of lung H&E stained section from rat exposed to benzene (group II) revealing alveolar septum thickening (arrow head) which is disrupted in some areas with focal emphysematous changes (astrix). Sloughing of bronchial epithelial cells in certain areas is also seen (biffed arrow). Nodular aggregations of inflammatory cells appear in the wall of both bronchi and bronchioles (spiral arrow). The arterial wall shows thickening (hypertrophy) of the muscle layer (arrow) (H&E X100).

Fig. (15): A photomicrograph of lung H&E stained section from rat exposed to benzene and treated with vitamin C (group III) showing diminished both alveolar septum thickening (arrow heads) and aggregations of inflammatory cells in the wall of both bronchi and bronchioles (spiral arrow) compared to group II subjected only to benzene (H&E X100).

Fig. (16): A photomicrograph of lung Masson trichrome stained sections from control group showing minimal amount of collagen in the lung interstitium (arrow heads) (Masson trichrome X100).

Fig. (17): A photomicrograph of Masson trichrome stained section from rat exposed to benzene (group II) revealing excess collagen deposition in the lung interstitium affecting most of examined lung tissues (arrow heads) (Masson trichrome X100).

Fig. (18): A photomicrograph of lung Masson trichrome stained section from rat exposed to benzene and treated with vitamin C (group III) revealing moderate collagen deposition in the lung interstitium (arrow heads) (Masson trichrome X100).
Benzene is a prototypical human and rodent hematotoxic and genotoxic carcinogen and a ubiquitous environmental pollutant. As a constituent of gasoline, benzene possesses a potential health threat to a broad spectrum of individuals [17]. The respiratory tract is a primary organ for inhaled toxic compounds and the lung is a target of benzene toxicity in humans and animals [18].

The present study revealed that benzene inhalation (6h/day, 5 days/week) for 4 weeks in rats was associated with significant increase in lung apoptotic Bax gene expression, together with significant decrease in lung antiapoptotic Bcl-2 gene expression compared to control rats. Our results are in accordance with the findings of the following studies. Weaver et al. [6] stated that benzene inhalation in rats induces lung cell apoptosis as confirmed by DNA electrophoresis, in situ nick end labeling, and the upregulation of apoptosis-related gene products. Also, a study by Weaver and Liu [19] revealed that rats exposed to benzene via inhalation for 7 days showed a significant upregulation of proapoptotic gene expression for p53, Bax, and Bad with significant reduction in antiapoptotic Bcl-2 gene expression in the lung epithelia.

Apoptosis is a tightly regulated mechanism of cell death, a process by which cells eliminate unwanted damaged cells. A growing body of evidence supports that oxidative stress activates or induces expression of the pro-apoptotic Bcl-2 family member Bax, which in turn triggers the release of cytochrome c from mitochondria into the cytosol where it binds to apoptosis protease-activating factor 1 and participates in the activation of caspases that leads to apoptosis [20].

It is well known that the upregulation of anti-apoptotic Bcl-2 family proteins inhibits many forces of apoptosis induced by numerous stimuli and promotes cell survival by inhibiting mitochondrial cytochrome c release, thereby blocking caspase activation and subsequent apoptotic death [21].

Benzene metabolism plays a prominent role in expressing its toxicity. Benzene is metabolized by cytochrome P-450 to various phenolic metabolites, which accumulate in bone marrow [22]. A mechanism by which benzene metabolites induce their genotoxic effects may be by generating one or more ROS such as O$_2^-$, H$_2$O$_2$, and OH$^-$ [8].

Thus in the present study, benzene induced apoptosis is mediated by ROS generation. This was supported by the findings of Ibuki and Goto [23] who found that the antioxidant Nacetylcysteine treatment nearly inhibited benzene metabolites-induced apoptosis which implies that ROS generation contributes to benzene metabolites-mediated cell death.

In this study, rats exposed to benzene inhalation for 4 weeks showed significant decrease in lung MMP-1 gene expression together with significant increase in lung TIMP-1 gene expression as compared to controls. Our results are consistent with the findings of Giuliano et al. [2] who reported that benzene altered the physiologic profile of MMPs with a statistically significant decrease in MMP-1 observed when using 10$^{-8}$M benzene on human lung cells, in vitro. They demonstrated that the ratio MMPs/TIMPs is naturally altered after benzene exposure. Meanwhile, they found that TIMP-1 was downregulated at the end of the experiments (48h) which is on the contrary to the results of the present work.

The current work showed increased lung collagen deposition in benzene inhaled rats demonstrated by significant increase in the mean area percent of collagen in Masson trichome stained sections as well as by significant increase in lung hydroxyproline content in benzene inhaled rats compared to controls. Hydroxyproline level is a good indicator of collagen amount since it is a major component of the collagen protein as proved by Nelson and Cox [24]. The assay of this compound could be used as a direct measure to quantify the severity of lung fibrosis.

The previous findings reflect alterations in both the synthetic and degradative pathways of collagen homeostasis as stated by Peters et al. [25]. MMPs are a family of zinc and calcium-dependent endopeptidases that play a key role in ECM remodeling in the lung. MMPs have the combined ability to degrade the various components of connective tissue matrices, specifically directed to ECM components. MMP-1 (also known as collagenase-1)
degrades fibrillar interstitial collagens, preferring type III collagens which are the most abundant proteins in fibrotic tissues [26].

However, the activities of MMPs are controlled at several levels including their interactions with specific inhibitors, the TIMPs. Selman et al. [27] stated that disequilibrium of some MMPs and TIMPs participates in the development of lung fibrogenesis and might be involved in the extensive structural disorganization/remodeling that characterize the fibrotic response.

Thus in the present work, benzene inhalation leads to increased expression of lung TIMP-1 gene expression which lead to decreased lung MMP-1 gene expression resulting in increased lung collagen and as a consequence lung fibrosis. Supportive of our findings, Madtes et al. [28] found that TIMP-1 gene expression was consistently upregulated in the injured rats evolving to fibrosis and it was spatially restricted to areas of lung injury. This inhibitor has been shown to be markedly upregulated during experimental lung fibrosis.

In the current study, lung apoptosis might be a mechanism of lung fibrosis since alveolar epithelial loss, putatively caused by apoptosis, is a common finding in pulmonary fibrosis as stated by Uhal [29]. He mentioned the following mechanisms by which epithelial apoptosis might be important in the pathogenesis of a fibrotic response. The epithelium of the lung has important antifibrotic functions as epithelial cells are involved in the production of mediators as prostaglandin E2 (inhibit fibroblast proliferation), plasminogen activators (degrade interstitial fibrin) and metalloproteinase (degrade collagen). In addition, intact lung epithelial cells provide a physical barrier which may prevent profibrotic molecules, e.g. cytokines from activated alveolar macrophages, from reaching underlying tissue. Therefore if epithelial cells are removed by increased apoptosis there could be a collapse of barrier function, which allows profibrotic cytokines from activated alveolar macrophages access to the underlying fibroblasts. At the same time, a reduction in the production of prostaglandin E2, less fibrinolysis and less matrix metalloproteinase production could lead to a profibrotic microenvironment.

In this study, benzene exposed rats showed significant decrease in plasma PON1 level compared to control rats. PON1, one of antioxidant proteins to protect low density lipoprotein from the oxidation, is known to lose its activity in the oxidative environment. Cigarette smoke extract was found to inhibit human plasma PON1 activity as well as aromatic hydrocarbons [30]. Thus, benzene being the simplest compound of aromatic hydrocarbons depressed PON1 level by generation of ROS.

Our biochemical results were supported by the histological results found in the present study, in which examination of trachea and lung using light microscope revealed structural changes in benzene exposed rats compared to control group. Consistent with our histological results, Al Saggaf et al. [31] found similar histological changes in the trachea and lung of guinea pigs exposed to gasoline vapor inhalation for 30 days as compared with control group.

The histological changes present in benzene exposed rats in the present study were explained by Sureshkumar et al. [12] where they stated that the general pathological response to an inhaled toxicant is said to be epithelial cell injury and the triggering of acute inflammatory immune processes. They reported that these changes were most probably due to irritative effect of gasoline vapor, recruitment of inflammatory cells and release of many inflammatory mediators, reactive oxygen species or free radicals.

The thickening of bronchiolar muscular wall observed in H and E stained sections in benzene exposed rats in this study is among signs of increased airway resistance (asthma) which is a well known consequence of exposure to air pollutants as explained by Jeffery [32]. Also, thickening of arterial wall in benzene group is one of the signs of pulmonary hypertension which could eventually result in cardiopulmonary disorders known as cor pulmonale as stated by Miller et al. [33].

The present study revealed that vitamin C treatment given concomitantly during benzene inhalation for 4 weeks resulted in significant decrease in lung apoptotic Bax gene expression together with significant increase in lung antiapoptotic Bel-2 gene expression in benzene treated rats compared to untreated benzene group. This finding suggests an antiapoptotic effect of vitamin C. Supportive of our findings, Banerjee et al. [34] found that administration of vitamin C almost completely prevents protein damage, apoptosis and the lung injury in guinea pigs exposed to cigarette smoking for 21 days.

The current work showed that administration of vitamin C for 4 weeks during benzene inhalation resulted in significant increase in lung MMP-1 gene expression, significant decrease in lung TIMP-
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1 gene expression together with significant decrease in lung hydroxyproline content in benzene treated rats compared to untreated benzene exposed group. These results suggest an antifibrotic effect of vitamin C which is also confirmed by significant decrease in the mean area percent of collagen deposition in Masson trichrome stained sections in vitamin C benzene treated group compared to untreated benzene exposed rats.

The antifibrotic effect of vitamin C found in the present study was in agreement with that found in other models of lung fibrosis in which Hemmati et al. [35] found that co-administration of vitamin C reduced collagen and hydroxyproline content of lung tissue in an animal model of lung fibrosis caused by hexavalent chromium.

The results of the present study showed that vitamin C treatment resulted in significant increase in plasma PON1 level in benzene treated rats compared to untreated benzene group. Our results are consistent with the findings of the following studies. Jarvik et al. [36] found a positive correlation between the dietary and medicinal intakes of vitamins C and E and serum PON1 activity in 189 white men from the Pacific Northwest region of the United States. A study by Kunes et al. [37] revealed that vitamin C attenuates hypochlorite-mediated loss of PON1 activity from human plasma in vitro. On the contrary, Kleemola et al. [38] reported that high intake of vegetables, possibly rich in vitamins E and C was negatively correlated with serum PON1 activity.

Various in vitro and in vivo studies in animals and humans have provided initial evidence that antioxidants can increase PON1 activity, possibly by protecting the enzyme from oxidative stress-induced inactivation.

Vitamin C is the most common nontoxic essential dietary antioxidant. The possible action of vitamin C is mediated through scavenging physiologically relevant reactive oxygen and nitrogen species. These include free radicals such as hydroxyl radicals, aqueous radicals, superoxide anion, and nitrogen dioxide, as well as nonradical species such as hypochlorous acid, ozone, singlet oxygen, nitrosating species (N₂O₃/N₂O₄), nitroxide, and peroxynitrite, thereby effectively protecting other substrates from oxidative damage [39].

At cellular level, vitamin C has been reported to mitigate the deleterious effect of ROS directly by increasing antioxidant enzyme activities of cells and indirectly by reducing oxidized form of vitamin E and GSH [40]. Thus antioxidant and free radical scavenger properties of vitamin C possibly prevent the effects of oxidative stress.

Thus in the present study, the antioxidant effect of vitamin C resulted in inhibition of apoptosis and as a consequence prevents lung fibrosis in benzene treated rats compared to untreated benzene exposed rats.

The findings of the present study showed that group III receiving vitamin C treatment during exposure to benzene for 20 days had less histological changes in the trachea, bronchi and lung tissue as revealed by H&E stained sections compared to group exposed to benzene alone. In accordance with these results, Gu et al. [41] found that supplementation of vitamin C and E ameliorated laryngeal-tracheal eosinophilia in guinea pigs induced by toluene diisocyanate exposure. Also, Al Saggaf et al. [31] found that simultaneous administration of vitamin C during exposure to gasoline for 30 days showed less pathological changes in the lung and trachea of guinea pig compared to group subjected only to gasoline vapor.

From this study it can be concluded that co-administration of vitamin C during benzene inhalation in rats reduced the structural changes of trachea and lung and diminished the toxic effects of benzene on the lung. These beneficial effects of vitamin C may be mediated by its antioxidant effect and as a consequence its beneficial antiapoptotic and antifibrotic effects.

Prophylactic measures must be provided during exposure to benzene inhalation especially workers employed in industries that frequently use or make benzene such as oil refineries, chemical plants, shoe, plastics or rubber manufacturers, and gasoline companies who face the greatest risk of high levels of benzene exposure.

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References


