A comparative study of grape seed extract and vitamin E effects on silica-induced pulmonary fibrosis in rats

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ABSTRACT

Due to the production of reactive oxygen species (ROS), oxidative stress has been implicated in the pathogenesis of silica-induced lung fibrosis. So it is hypothesized that grape seed extract (GSE) or vitamin E (Vit E) as antioxidants may ameliorate some symptoms of the disease. Male Wistar albino rats were divided into 7 groups: rats in group I instilled intratracheally (IT) with a single dose of silica suspension (50 mg/rat) as positive control (PC), Treatment groups (II–IV) received Vit E (20 IU/kg/day), GSE (150 mg/kg/day), or Vit E+GSE simultaneously orally 1 day after instillation of silica. Groups V and VI were given oral GSE or Vit E after instillation of the equivalent volume of saline (IT) as controls for GSE or Vit E. Rats of group VII only instilled saline (IT) as negative control. After 90 days animals were sacrificed and plasma-malondialdehyde (p-MDA) and lung tissue hydroxyproline (HP) were quantified. The lungs were also investigated for histopathological changes. The mean concentrations of p-MDA and HP in studied groups (I–VII) were 1.95, 2.77, 0.72, 0.81, 0.64, 0.94, 1.02 m molMDA/Lplasma and 27.85, 22.83, 22.64, 15.40, 18.31, 18.51 mgHP/gtissue, respectively. Silica caused a significant increase in HP content of lungs and MDA levels in the plasma except in GSE-treated groups (III and IV). According to the results of this study GSE could reduce the fibrogenic effect of silica. However; no synergistic effect was observed after co-administration of GSE and Vit E.

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1. Introduction

Silicosis, of all pneumoconiosis, has probably claimed the largest number of victims, either alone or in combination with tuberculosis. Classically it is a chronic progressive disease of pulmonary parenchyma [1]. The crystalline silica, alpha quartz, is the major cause of silicosis worldwide. The incidence of overt disease is greatly increased in industrial operations by the mechanization and use of sand blasting, drilling, pulverizing, cutting, grinding tools and other pneumatic equipments [2]. Clinically there are three types of silicosis: (1) Simple chronic silicosis which results from long-term exposure (more than 20 years) to low amounts of silica dust. Inflammatory responses caused by the silica dust form in the lungs and chest lymph nodes. This disease may cause people to have trouble breathing and may be similar to chronic obstructive pulmonary disease (COPD). (2) Accelerated silicosis usually occurs after exposure to larger amounts of silica over a shorter period of time (5–15 years). Swelling in the lungs and symptoms occur faster than in simple silicosis. (3) Acute silicosis results from short-term exposure to very large amounts of silica. The lungs become very inflamed and can fill with inflammatory fluid, causing severe shortness of breath and low blood oxygen levels. Progressive massive fibrosis can occur in either simple or accelerated silicosis, but is more common in the accelerated form. Progressive massive fibrosis is caused by severe scarring and destroys normal lung structures [3].

The molecular basis for cell injury by silica has been the subject of considerable studies. Destabilization of the cell’s plasma membrane and membranes of the phagosomes containing silica particles can provoke the generation of reactive oxygen species (ROS) and the release of hydrolytic enzymes. However, the actual molecular mechanisms of cell injury in silicosis have not yet been defined [4,5]. The fibrogenic features of silica in lung is unique and it is different from other types of pulmonary fibrosis, e.g. due to bleomycin. It excites a foreign body reaction with morphages, giant cells, etc., which is later, followed by dense fibrosis. The fibrotic nodules seperated by emphysematouse lung produce the typical picture of silicosis.

There is an exquisite balance between production and destruction of ROS. When this equilibrium is destroyed, ROS are produced excessively and all tissues are exposed to oxidative injury [4]. Antioxidants, including vitamins, carotenoids and tannins provide protection against oxidative damage. Such agents are now gaining big attention as potential chemo-preventive agents. Grape seeds are rich in antioxidant compounds, including phenolic compounds (predominantly tannins), and it has been...
demonstrated that these compounds reduce the risk of chronic disease by protecting against free radical-mediated damage [4]. Grapes (*Vitis vinifera*) are one of the most widely consumed fruits in the world. Grape seeds are rich in dimers, trimers and other oligomers of flavan-3-ols (the major are catechin, epicatechin and epicatechin-3-O-gallate), named proanthocyanidins (PAs) [6]. There is a growing interest in the utilization of PAs for their dietary and pharmacological properties, especially positive effects on vascular injury [7], capillary protective action [8], free radical scavenging [9,10] and antimutagenic activity [11]. PAs present in grape seeds are known to exert anti-inflammatory, anti-arthritic and anti-allergic activities, prevent skin aging, scavenge oxygen-free radicals and inhibit UV radiation-induced peroxidation activity [11]. Oral administration of grape seed PAs at a dose of 2 mg/kg three times daily for 6 days inhibits carrageenin or dextran-induced hind paw edema, stabilizes the capillary wall and prevents the increase in capillary permeability caused by local cutaneous application of xylene [12]. The antioxidative activities of PAs were found to be much stronger than Vitamin C or E (Vit C or E) in aqueous systems [13]. The potential influence of grape seed extract (GSE) on the silica-induced lung fibrosis has not been previously reported. The aim of the present study was to examine the effects of orally administered GSE in a rat model of lung injury produced by endotracheal silica by comparing it with that of Vit E.

In the present study crystalline quartz used in the ferrosilicon production factory in Iran was investigated. We investigated the in vivo effect of GSE or Vit E on silica-induced lung injury in rats. The alterations in state of oxidative stress were determined by measuring the plasma levels of malondialdehyde (MDA), and hydroxyproline (HP) as an index of collagen synthesis were determined in rat lung tissue and histopathological evaluation of lung tissue was performed.

2. Materials and methods

2.1. Quartz particle samples

The quartz stones were obtained from the Iranian Ferrosilicon Factory (Semnan-Iran). The stone samples were delivered to the Iranian Geological and Mine Exploring Organization, which crushed, processed and then milled them in a rotary ball mill to produce respirable silica particles (< 5 μm). The surface characteristics of the particles were determined with an Oxford scanning electron microscope (Fig. 1). The particles were angular or rounded.

2.2. Hydroalcoholic extract of grape seed

Grape seeds were a gift from Sasan Shahd Dietary Industries (Uromnia, Iran). Seeds were obtained from red grapes (*V. vinifera*) in the process of grape juice production. Grape seeds were dried in drying oven at 50 °C for 72 h. Then, dried seeds were ground to fine powder by a grinder. About 500 g of the powder was mixed with 1000 ml of 70% ethanol in distilled water and kept for 3 days at room temperature. The extract was then filtered through a Büchner funnel. Solvent (ethanol/water) was removed using rotary evaporator under vacuum at 50 °C. The residue (dried extract) obtained and kept in refrigerator for further experiments. Enough amounts of the dried extract were suspended in water and administered orally to rats for 90 days (150 mg/kg/day).

2.3. Vitamin E

DL-α-tocopherol (Darmstadt, Germany) was dissolved in liquid oil and administered at a dose of 20 IU/kg/day for 90 days.

2.4. Animals

Male Wistar albino rats weighing 180–200 g were purchased from the Animal house and research center, Jundishapur University of Medical Sciences, Ahwaz, Iran. The animals were kept on standard food pellet and tap-water *ad libitum*. The rats were housed in polycarbonate cages (5 animals per cage) and kept in an air-conditioned animal room at a temperature of 23 ± 3 °C, with a relative humidity of 50 ± 5%. The animal room was on a 12 h photo-period cycle.

2.5. Intratracheal instillation of silica

The rats were anesthetized by IP injection of ketamine hydrochloride (50 mg/kg) and instilled intratracheally (IT) with silica suspension (50 mg in 0.1 ml saline/rat), while normal animals received an equal volume of sterilized saline instead of silica suspension. The rats were sacrificed 90 days after silica injection. Pulmonary fibrosis and silicosis were assessed as described in the following sections.

2.6. Experimental groups

Thirty five rats were randomly divided into the 7 groups as follows; group I: a single IT instillation of silica (positive control (PC)); group II: single IT silica and oral Vit E (20 IU/kg/day) [32]; group III: single IT silica and oral GSE (150 mg/kg/day) [32]; group IV: single IT silica and GSE+Vit E at doses mentioned above simultaneously for probable synergistic effects; group V: single IT saline and oral GSE (control for GSE); group VI: single IT saline and oral Vit E (control for Vit E); and group VII: only single IT saline (negative control). The day of IT injection of silica or saline was designated as day 0.

2.7. Biochemical measurements

The plasma MDA levels were determined by the method described by Buege and Aust [14] using fluorescent measurements as described by Yagi [15]. Values obtained were compared with a series of standard solutions (1,1,3,3-tetraethoxypropane). The plasma samples were obtained by collecting 1 ml blood from left ventricle, centrifuging at
1000g for 5 min, and diluting 20 times with phosphate buffer solution (pH = 7.4). Results were expressed as µmol/L.

The left lobe of lung was used for HP determination. HP content of lung tissue was determined by the colorimetric method as described by Edwards and O’Brien [16]. HP was extracted from collagen and oxidized to pyrrole by chloramine-T, it can then produce color with para-dimethylbenzaldehyde. Tissue samples were homogenized and processed according to the earlier discussed method. The absorbance was measured at 500 nm to determine the HP content (mg/g tissue).

2.8. Histological studies

When the animal was sacrificed the lung was removed, the left lobe was kept for HP determination. Right lobes were perfused with 10% formalin in an inflation pressure not more than 20 cm of H2O. Then the whole tissue was placed in formalin for at least 48 h. Lung tissues were embedded in a paraffin block and 5-µm thick sections were made and stained with hematoxylin and eosin (H&E). The sections were examined by light microscopy and assessed for the presence of fibrosis and severity of lesion. The structural alterations of tissue due to silica or treatment with GSE and Vit E were assessed based on the degree of cellular proliferation, alveolar wall thickening, inflammatory lesions and collagen deposition or fibrosis. Such changes were graded in terms of severity and distribution. The grading system adopted is as follows and was utilized for each group of animals.

For distribution of lesion over the tissue

0 absent
± rare/occasional
+ sparse/limited
++ moderate
+++ extensive/widespread
++++ very extensive/predominant

For severity of lesions

0 nothing/zero
± marginal
+
++
++++ very severe

2.9. Statistical analysis

Statistical comparison was made by one-way ANOVA. Significant F-values were tested with Tukey’s test. Data are presented as mean ± SEM. In all cases P<0.05 was considered significant.

3. Results

3.1. Lipid peroxidation

The MDA levels in the lung of each experimental group at 90th day are summarized in Fig. 2. MDA levels in the plasma of the GSE or GSE/Vit E treatment groups were not significantly different from the control groups, while the levels for positive or Vit E-treated groups were very significant (p<0.001) (Fig. 2). Interestingly, MDA levels in plasma samples of Vit E-treated group was also significant in comparison to the PC group (p = 0.005) (Fig. 2).

3.2. Hydroxyproline

Collagen production in the pulmonary tissue was assessed by the HP content of the lung at 90th day of treatment. The HP contents of the lungs obtained from each experimental group are summarized in Fig. 3. A significant increase in HP of the lung tissue was observed in the group that received silica (PC) and the Vit E-treated group in comparison to the control groups (p<0.001); however, no significant decrease was observed in HP contents in GSE treated and GSE+Vit E-treated groups in comparison to the GSE, Vit E or negative controls.

![Fig. 2. Comparison of plasma MDA levels (µmol/L) of silica-treated, silica+Vit E-treated, silica+GSE-treated, silica+GSE/Vit E-treated, saline+GSE-treated, saline+Vit E-treated and saline-treated rats. (●) A significant difference between the silica (pos. control) and silica+Vit E groups and the other control groups (neg. control, controls for Vit E or GSE) (p<0.001). (▲) A significant difference between the silica (pos. control) group and silica+Vit E group (p = 0.005).](attachment:image.png)
3.3. Histopathology

Histopathological evaluation of the pulmonary tissue was performed with light microscopy for the seven different experimental groups 90 days after treatment. Lungs of rats in control group, which received IT saline and GSE, Vit E, or none showed normal lung structure and no lesion was evident (Figs. 4–6). After 90 days of instillation, in silica group, there were mainly perivascular and peribronchial fibrosis associated with silicotic nodules and distributed hyalinized collagen fibers were observed in the nodules (Figs. 7 and 8). The Vit E-treated group also showed a considerable change in tissue structure due to interstitial inflammation and fibrosis (Fig. 9). At 90 days after instillation, in GSE/Vit E-treated groups, cellular nodules were observed, composed mainly of mononuclear phagocytes, fibroblasts and tiny collagen fibers (Fig. 10). In the GSE-treated group such events were less pronounced and the numbers of nodules were reduced (Fig. 11).

The severity and distribution of histological changes in the silica-treated group (PC) was highest among all the groups. Such changes include the area of lung damages and their locations, mainly around bronchial tree and vascular bed. Vit E treatment could reduce the severity of damages 1 (Fig. 11). However the
distribution of changes was similar to the PC group. GSE caused a big reduction in severity and distribution of lung damages. Combination of Vit E and GSE could diminish the severity of damages but their effect was less than GSE alone (Table 1).

4. Discussion

Pulmonary fibrosis is a frequent response to different insults or injuries to the lung. Although there are various initiating mechanisms, the terminal phases of fibrosis are characterized by proliferation and progressive accumulation of connective tissue replacing normal functional parenchyma. The pathogenesis of pulmonary fibrosis includes endothelial and epithelial cell injury, influx of the inflammatory cells and production of their chemical mediators leading to the proliferation and activation of the fibroblasts [17–19]. Silica-induced pulmonary toxicity is somehow different from other types of pulmonary fibrosis, e.g. by bleomycin. Silica particles are insoluble, once deposited in lung parenchyma are subsequently ingested by macrophages. Further damage may result from internal reactions in these cells. A hypothesis by Heppleston and Styles [20] claims that on the surface of silica particles, silicic acid groups interact with amide groups in proteins and phospholipide in the cell membrane of
macrophages and eventually destroy the cell membrane. As a result, the digesting enzyme and silica (quartz) crystals are released. Other macrophages proliferate and migrate to the reaction site, where they may release inflammatory mediators which stimulate the synthesis and accumulation of collagen fibers [20]. It is well known that exogenous agents such as fibrogenic minerals can cause pulmonary fibrosis through production of ROS in animal models [21]. Also it has been suggested that the generation of ROS by dusts intrinsically or via the inflammatory process is important in the initiation of the disease [22–24]. In silica toxicity, cell damage by oxidant radical is mediated by iron ions. Sialon groups (SOH), which are common to all silicates, react with ferric ions forming a complex which is referred to as a silicato-iron complex. Iron in the complex can mediate an electron exchange when it is reduced from ferric (Fe$^{3+}$) to ferrous (Fe$^{2+}$) state by superoxide anions. Hydroxyl radicals which may be produced by this reaction at the surface of silica are potent oxidizing agents. They can initiate lipid peroxidation of the cell membrane and oxidatively inactive cell proteins. The generation of oxidants initiated by silica particles leads to activation of the immune system and interaction with other cells, e.g. T and B lymphocytes. Macrophages and lymphocytes seem to have a close co-operation in the process of silicosis. Histological study of silica-exposed lung tissue shows a large increase in the numbers of these cells. Interaction between these cells may lead to the production of a number of mediators such as, platelet-derived growth factor (PDGF), interleukin 1 and 6 (IL1, IL6), transforming growth factor-beta (TGF$\beta$) and tumor necrosis factor-alpha (TNFs). Such mediators may provoke the proliferation of fibroblasts and myofibroblasts which produce collagen fibers and finally cause fibrosis [25].

It has been suggested that tetrandrine an alkaloid from Stephania tetrandra has antisilicotic effects [26]. But other studies state that this alkaloid has toxic effect on macrophages accompanied by overproduction of prostaglandins [27,28]. In many experimental studies GSE components as antioxidants have been employed [29–31]. The present study has been designed based on the free-radical scavenging actions of GSE or Vit E ($\alpha$-tocopherol) on the ROS generated by silica particles intrinsically or via the inflammatory process that has not been investigated previously.

The microscopic and biochemical findings of this study indicate that GSE treatment after silica instillation rendered relatively therapeutic effect against silica toxicity in rat. MDA value in the GSE treated group has decreased significantly compared to PC Vit E-treated group. This means that GSE components are able to suppress the rate of lipid peroxidation due to silica in the lung tissue. GSE caused a big reduction in severity and distribution of lung damages. The exact mechanisms of GSE compounds cannot be verified by this work but in association with other reports we may give some suggestions. Based on the oxidative stress hypothesis of pulmonary fibrosis, it is offered that GSE, a potent antioxidant, can exert anti-fibrosis effects by scavenging ROS; although it cannot suppress the harmful effect of silica on lung. However, more studies are required to verify whether GSE or other antioxidants are effective chronically in the immunological process of silicosis or other types of pulmonary fibrosis.

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**References**


