Impacts of Air Pollution Exposure on the Allergenic Properties of Arizona Cypress Pollens

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Abstract: Epidemiological studies have demonstrated that urbanization and high levels of vehicle emissions correlated with the increasing trend of pollen-induced respiratory allergies. Numerous works have investigated the role of pollutants in the pathogenesis of respiratory diseases but impacts of anthropogenic pollution on pollen allergenic properties are still poorly understood. The objective of this survey was to evaluate impacts of the traffic-related pollution on the structure and allergenic protein content of Arizona cypress (\textit{Cupressus arizonica}, CA) pollens, recognized as a rising cause of seasonal allergy in various regions worldwide. According to our results, traffic-related air pollution by its direct effects on the elemental composition of pollens considerably increased the fragility of the pollen exine, causing numerous cracks in its surface and facilitating pollen content liberation. Pollen grains were also covered by numerous submicronic orbicules which may act as effective vectors for pollen-released components into the lower regions of respiratory organs. On the other hand, this study provides us reliable explications about the low efficiency of standard commercial allergens in the diagnosis of the Arizona cypress pollen allergy in Tehran. Although traffic related pollution affects the allergenic components of CA pollens, the repercussions on the respiratory health of urban populations have yet to be clarified and need further investigations.

1. Introduction

Pollen grains are the male gametophyte generation of seed plants. Anemophilous pollens (wind pollinated) dominate the pollen rain of any area, representing major seasonal carriers of allergens. Of all the sources of allergy, pollen is undeniably one of the most pervasive; carried on the wind, it can travel over very long distances and interact with numerous biological and chemical agents present in the atmosphere.

The importance to study the interaction between pollens and environmental pollution has been put forward two decades ago by Ishizaki \textit{et al.} [1], who demonstrated the relationship between pollutants and a higher prevalence of pollinosis in urban areas. Since then, effects of pollution on the molecular and developmental biology of pollen have been the subject of interesting studies revealing the accumulation of numerous inorganic elements such as sulphur, cadmium, and lead in pollen grains [2-5] and the acidification of pollen surfaces by the absorption of acid gases such as nitric acid in polluted areas [6]. Several studies also proved that pollutants by attaching to the surface of pollen...
grains and sub-pollens particles can modify the morphology of these antigen-carrying agents and alter their allergenic potential [4-10].

The city of Tehran with more than 2 millions cars emitting about 1.5 million tons of pollutants annually is a good model to study interactions between pollens and traffic-related pollution. The geographic situation of this town, hemmed in by the Alborz Mountains to the north, favors the absence of wind, causing the increasing volume of pollutants to become trapped.

In winter, the inversion phenomenon frequently happens and the amount of gaseous and particulate air pollutants attains critical levels. These peaks of pollution correlate with the early pollination period of Arizona cypress trees (mid-winter time), abundantly planted for reforestation, wind barrier and landscaping programs in numerous parts of the city [11]. These facts have prompted us to investigate the impacts of the traffic-related pollution on the structure and allergenic protein content of Arizona cypress (Cupressus arizonica, CA) pollen, currently recognized as a rising cause of winter allergies in various regions worldwide.

2. Materials and Methods

2.1. Sampling
Polluted areas of the city were selected according to data provided by Air Quality Control Company related to the National Department of Environment (table 1).

In the first sampling, pollens were collected just after microsporangia (male cones, figure 2) bursting, and the second sampling was performed two weeks later, in order to permit a natural pollen exposure to the urban air pollution.

Pollens were sifted by passage through mesh and the purity checked by light and scanning electron microscopy. The mode of sampling and storage was exactly the same for both samples.

2.2. Scanning electron microscopy (SEM)
Pollen grains were dried prior to gold coating. Samples were coated by physical vapor deposition (Sputter coater SCDOOS, BAL-TEC. Co, Switzerland) and then, were observed by using a scanning electron microscope (Philips XL30).

Elemental composition of exposed (EP) and non-exposed (NEP) pollens to air pollution were compared by using Energy Dispersive X-ray microanalysis (EDX). The percentage of different elements was determined by ZAF quantification method.

Figure 1. Arrows indicate the ranges of cypress trees planted alongside one of the most important highways of the city (Chamran highway). Cupressus arizonica and Cupressus sempervirens represent the most common species used in Tehran green spaces belonging to the Cupressaceae family.
Table 1. Average amounts of air pollutants in the highest polluted areas and a non-polluted area situated outside the city. Data have been obtained from 3 air pollution measurement stations situated in the polluted areas of the city and a control station localized outside the city. Standard amounts of air pollutants have been adopted from Nation Air Quality Standards, US.

| Pollutants | Carbon monoxide (ppm) | Sulfur dioxide (ppb) | Nitrogen dioxide (ppb) | Hydrocarbons (ppm) | Respirable particles (µg/m³) |
|------------|------------------------|----------------------|-----------------------|--------------------|----------------------------|
| Polluted areas | 8.5 | 52 | 63 | 4.8 | 124 |
| Non-polluted area | 1.1 | 7.6 | 16 | 2.2 | 50 |
| Standard amounts | 9.5 | 35 | 54 | - | 51 |

2.3. Protein studies
For the preparation of pollen extracts, an equal amount of each samples, 50 mg per ml, were extracted in 0.01 M PBS, pH 7.4 for 14 h at 4 °C under stirring. The suspensions were centrifuged at 14,000 g for 1 h at 4 °C and the supernatant were dialyzed against distilled water.

Total protein contents of *C. arizonica* var. arizonica (var. a) and *C. arizonica* var. glabra (var. g) pollens were determined and compared before and after air pollution exposure according to Bradford protein assay [12] by using bovine serum albumin (BSA) as standard. Extracted proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (90 µl of each pollen extract per well) and detected by Coomassie blue staining. The molecular weight was calculated by comparison with known markers. Protein bands of each extract were quantitatively compared by densitometric analysis.

2.4. Immunoblotting for the detection of IgE reactivity
After electrophoresis, proteins were transferred from the gel onto membrane polyvinylene difluoride [13] and membranes were blocked for 1 h at room temperature with 0.05% Tween20 and 3% Bovine Serum Albumin (BSA) in PBS. The membrane was washed three times for 10 min in PBS-Tween20 and incubated overnight with 1:15 diluted serum of allergic subject. After incubation, the membrane was washed and incubated for 1 hour with 1:20000 dilution of anti-human IgE tagged with horse radish peroxidase in PBS. The blots were immersed in an enhanced chemiluminescence’s solution (Amersham Kit) and the specific IgE-binding bands were revealed after 1-min exposure to a X-OMAT Kodak film.
3. Results and Discussion

3.1. Pollen

Pollen grains are sealed in a double-layered wall. Its outer layer, the exine, is composed out of sporopollenin, a lipophilic polymer of carotenoid, which is extremely resistant to enzymatic and chemical degradations. Our SEM analysis illustrated that exposure to the ambient air pollution considerably increased the fragility of the pollen exine, causing numerous cracks and collapses in its surface (figure 4) and facilitating pollen content liberation (Figure 5).

**Figure 3.** Scanning micrograph of a non-exposed pollen to pollution (NEP). Pollen is spherical with approximately 24µm in diameter. Numerous orbicules (Or), sat over the pollen surface.

**Figure 4.** Scanning micrograph of a CA pollen exposed to air pollution (EP). Exine degradations are shown by black arrows.

**Figure 5.** CA pollen exposed to pollution (EP): before their dispersion from male cones, many of polluted pollens were empty of their content.

Due to their sizes (> 5 µm), intact cypress pollens can be easily removed by the protective mechanisms of nasal and upper tracheobronchial passages and they are unable to reach the bronchial tree. That is probably the reason why cypress pollinosis tends to particularly affect conjunctivae and upper respiratory tracts. In polluted areas, pollutants by affecting the exine structure and permeability of cypress pollens may modify two important physical factors influencing their clinical significance:
the particle sizes, by increasing the premature release and dispersion of pollen derived particles smaller than pollen in the atmosphere, and the rapidity with which antigen molecules diffuse or leach out the pollen. Rapidly released allergens may induce immediate allergic reactions and may be adsorbed through the respiratory mucosa before the pollen grains are removed or swallowed. One of the major mechanisms of allergen release in CA pollens is described in the figure 6.

![Figure 6](image)

**Figure 6.** In contact with any source of humidity, which can be in the atmosphere, on a female flower, or on the mucosal surface of the upper respiratory tract, pollen metabolic activity is reengaged. Pollen hydration rapidly results to an important dilatation of the pollen intine (In), the layer situated beneath the exine (Ex) and essentially made of hydrophilic components such as pectin. Following pollen hydration, the exine is progressively pushed out (A). The dilatation of the intine may continue till its rupture (B) causing the liberation of the cytoplasm (Cy).

3.2. Orbicules
Cypress pollen grains are covered by numerous submicronic orbicules (350-600 nm) which may act as effective vectors for pollen-released components into the lower regions of respiratory organs (figure 7). Aerobiological studies demonstrated that small pollen-derived particles (< 2.5µm) can bind to fine aerosols in polluted air, such as DEP (diesel exhaust particles), forming an association of inhalable particles and facilitating allergen transmission [14-15]. The interactions between these cypress nanoparticles and pollutants may explain the increasing frequency of asthma reported in sensitized patients to cypress pollens, reaching 18% in Italy [16] and 20% in Izmir, Turkey [17].

3.3. EDX analysis
EDX analysis showed that Si, S, F, Co, Ni, Cu, Zn, Br, Ag, Mg, Al, Ca, K, Cl and Pb represent 15 essential elements composing the pollen tectum. The comparison of the elemental composition of pollens collected before and after exposure to air pollution showed that most important augmentations were in the ratios of nickel, sulfur, iron and copper respectively. In polluted pollens, sulfur (49%) and copper (11%) were the dominant elements and amounts of nickel and iron were respectively fourteen-fold and fourfold higher than control pollen (table 2).
Figure 7. Scanning micrograph of orbicules (Or), A: magnified 8000x. The surface of these spherical nanoparticles of sporopollenin is irregular and gemmate, similar to the pollen exine B: Orbicules magnified 15000x.

Table 2. Energy dispersive X-ray microanalysis: elemental composition of exposed (EP) and non-exposed pollens (NEP) to the urban pollution. Levels of silica (Si), sulphur (S), cobalt (Co), nickel (Ni), zinc (Zn), bromide (Br), argent (Ag), copper (Cu), iron (Fe), magnesium (Mg), aluminium (Al), calcium (Ca), potassium (K), chloride (Cl) and lead (Pb) were compared.
3.4. Protein Studies
Bradford protein assay showed an important decrease of the total protein content in polluted pollens, from 220 μg/ ml to 180 μg/ ml for var. a and from 250 μg/ ml to 150 μg/ ml in var. g.
The comparison of the protein components of CA mature pollens collected just after microsporangia bursting with those collected 2 weeks later (exposed to the urban environment) revealed that in both var. a and var. g pollens, the amount of Cup a 1 (a pectate lyase protein with an approximate molecular weight of 45 kDa which is currently recognized as the major allergen of Arizona cypress pollen extracts), considerably decreased to only 14.6% and 14.9% of all protein content, respectively, and a new protein of 35 kDa represented more than 50% of the total protein content (figure 8).

3.5. Immunoblottings
Western blotting revealed that serum specific IgE of our CA allergic subject showed a high reactivity to the new 35 kDa major allergen in all tested extracts. On the other hand, no IgE-binding to Cup a 1 (45 kDa) has been detected in the lanes 3 and 4, representing extracts of pollens exposed to the traffic-related pollution of Tehran (figure 9).
The lack of this new 35kDa allergen in the established commercial extracts intended to in vivo and in vitro clinical tests considerably brought into question their performance in the diagnosis of the CA pollen allergy in Tehran.

![Figure 8](image1.png)

**Figure 8.** SDS-PAGE profile of CA pollen extracts obtained just after microsporangia bursting (A) and 2 weeks later (B). MW indicates molecular weights, M, marker, lanes 1 and 2, extracts of respectively var. a and var. g pollen collected from trees planted in Tehran.
Figure 9. Immunoblotting: specific IgE-binding protein bands to respectively var. a and var. g pollen extracts obtained just after microsporangia bursting (lane 1 and 2) and after 2 weeks exposure to the urban pollution (lane 3 and 4). Immunoblots were probed with the serum of a CA allergic patient.

4. Conclusion
According to our results, traffic-related air pollution by its direct effects on the elemental composition of pollens considerably increases the fragility of the pollen exine, causing numerous cracks and collapses in its surface and facilitating pollen content liberation. Cypress pollen grains are also covered by numerous submicronic orbicules which may act as effective vectors for pollen-released components into the lower regions of respiratory organs.

The present study also demonstrates that in polluted areas, Arizona cypress pollens are not only important carriers of allergens but also of pollutant elements, such as sulphur and copper, able to increase mucosal hyper reactivity in susceptible subjects.

Although the influence of the traffic related pollution on the allergenic components of CA pollens is evident, the repercussions on the respiratory health of urban populations have yet to be clarified and need further investigations.

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