

## The effects of air pollution on structures, proteins and allergenicity of pollen grains

Ahmad Majd<sup>1</sup>, Abdolkarim Chehregani<sup>2,\*</sup>, Mostafa Moin<sup>3</sup>, Mansour Gholami<sup>2</sup>, Shigekatsu Kohno<sup>4</sup>, Takeshi Nabe<sup>4</sup> & M.A. Shariatzade<sup>5</sup>

<sup>1</sup>Laboratory of Cytology, Department of Biology, Teacher Training University, Tehran, Iran; <sup>2</sup>Department of Biology, Bu-Ali Sina University, Hamadan, Iran; <sup>3</sup>Asthma and allergy research center, Tehran, Iran; <sup>4</sup>Department of Pharmacology, Kyoto Pharmaceutical University, Kyoto, Japan; <sup>5</sup>Faculty of Health, Urmia University, Iran. (\*Author for correspondence: E-mail: chehregani@basu.ac.ir)

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### Abstract

The prevalence of allergic disease has increased world wide during the last decades. Pollen allergy is the most typical form of allergic disease. The increase in its frequency during recent years is the most evident. Environmental factors play an important role in the problem of pollen allergy in large cities. The aim of this research is determination of allergenicity of *Canna* pollen in polluted and non-polluted conditions, detection of their allergenic proteins and also elucidation of some microscopic effects of air pollutants on pollen structure and proteins. Mature and immature pollen grains of *Canna indica* were collected from polluted and non-polluted areas. Pollen grains were studied by scanning electron microscopy. Mice were sensitized by injection of pollen extract and an adjuvant for five times. Allergy potency of different pollen extracts were compared by means of skin test, blood eosinophils number and IgE levels in sensitized and treated animals. Pollen proteins were studied by SDS-PGE and allergenic proteins were detected by immunoblotting techniques. Scanning electron microscope study of the pollen grains showed that in polluted areas, air born particles accumulated on the surface of pollen and changed both pollen's shape and pollen's tectum. Also many vesicles were released out of polluted pollen and the pollen material agglomerated on the surface of pollen. SDS-PAGE showed that different proteins exist in mature and immature pollen. In pollen collected from polluted area, some of protein bands between 22 and 45 kDa were disappeared. Also in all polluted pollen grains, protein content of pollen decreased in response to air pollution causing the release of pollen proteins. According to our experiments and regarding induction of allergic symptoms, the polluted pollen is more effective than non-polluted one, and mature pollen has more allergy potency than immature one.

### 1. Introduction

Over the centuries, pollen allergy has increased from a local nuisance to a global problem (Emberlin, 1998). The reasons for this increase are unknown. Among many hypotheses, the idea that environmental pollutants may play a role has gained substantial public and scientific attention (Ishizaki et al. 1987; Braun-Fahrlander et al., 1992;

Ring and Behrendt, 1993; Rusznak et al., 1994; Behrendt et al., 1997). Air pollutants have detrimental effects on organisms and many researchers are working on the effects of air pollution on plants, animals and people. Air pollution may affect pollen grains indirectly via stress on the growth on the plant or directly either through contamination of the anthers on the plant or during the flight of pollen grains through the air

when it dispersed (Emberlin, 1998; Chehregani et al., 2004). Airborne pollen grains can be attacked directly by air pollutants and changes in pollen shape and tectum occur under polluted condition (Behrendt et al., 1992; Knox and Suphioglu 1996; Knox et al., 1997). Changes of the structure, ultra-structure, chemical compound and allergenicity of some pollen were reported in polluted areas (Ghanati and Majd 1995; Majd and Kiabi 1997). Laboratory experiments have shown that NO<sub>2</sub>, SO<sub>2</sub> and CO treatments can cause changes in the soluble protein composition of pollen grains. Some studies showed that air pollutants could induce the breakage of proteins or the formation of new proteins in the pollen that exposed to pollutants (Ruffin et al., 1983; Chakraborty et al., 1996). A decrease of total pollen protein in polluted areas was reported by Behrendt et al. (1997).

There are some evidences today that air pollution may be responsible for the increase in pollen-induced allergies and asthma in highly polluted areas (Emberlin, 1998; Behrendt et al., 1997; Ruffin et al., 1983; Behrendt and Becker, 2001). Aberg (1989) had reported increasing of asthma in children due to SO<sub>2</sub>, cigarette smoke and air pollution, while Riedel and Kramer (1988) and Jae-Kyoung et al. (2001) had suggested that air contaminants such as SO<sub>2</sub> and other chemical pollutants are responsible for developing asthma in industrial regions. Additionally, irritant gases and/or diesel exhaust particles have been shown to express both adjuvant activity for sensitization against common allergens and enhancing effects on allergic symptoms in sensitized individuals (Matsuramara, 1970; Holt et al., 1986; Riedel and Kramer, 1988; Kainka-Staniche et al., 1989; Ito et al., 1996; Helander et al., 1997).

In this report allergenicity of *Canna* pollen grains was examined in different condition. We

tried detecting of the allergenic proteins. Also the complex interaction between allergy potency of pollen and air pollutants is investigated.

## 2. Materials and methods

### 2.1 Sampling

We chose Tehran as a highly-polluted city and as a real condition, for our studies. In Tehran, the amount of air pollutants is several times more than the standard amounts (Table 1). *Canna indica* belonging to Cannaceae family is one of the popular plants which use for landscaping in Tehran. We selected samples planted in the central region of Tehran as a polluted region and also out of Tehran as a non-polluted region. Passing through mesh with pores 30–40 µm in diameter, the fresh pollen was being purified. We collected immature pollen from immature anthers before releasing their pollen grains.

### 2.2 Scanning electron microscopy

Polluted and non-polluted pollen were studied by the scanning electron microscope. After coating with gold, samples were analyzed using a scanning electron microscope model SEM-EDS, EX 300 Link, England. The purified pollen was fixed by glutaraldehyde, post fixed with Osmium, dehydrated and dried prior to gold coating.

### 2.3 Protein studies

Pollen protein extraction of non-polluted and polluted pollen grains was carried out separately at 4 °C in Tris-HCl buffer, pH 7.6. Finally, 12% SDS-polyacrylamid gel electrophoresis was performed for the total soluble protein according to the

Table 1. Comparison of average amount of air pollutants in Tehran and standard amounts

| Pollutants      | Carbon monoxide (ppm) | Sulfur dioxide (ppb) | Nitrogen dioxide (ppb) | Hydro carbons (ppm) | Respirable particle (µg/m <sup>3</sup> ) |
|-----------------|-----------------------|----------------------|------------------------|---------------------|------------------------------------------|
| Average in 2000 | 5.89                  | 56                   | 81                     | 3.6                 | 135                                      |
| Average in 2001 | 4.39                  | 72                   | 78                     | 2.47                | 112                                      |
| Standard amount | 9.5                   | 35                   | 54                     | –                   | <10 µm 51<br>>10 µm 15                   |

Data evaluated at during spring and summer in 2 years, 2000 and 2001. Data obtained from 6 to 8 air pollution measurement stations. Standard amount of air pollutants adopted from Nation Ambient Air Quality Standards, USA. This table shows that the amount of some pollutants are several times more than standard in Tehran.

method of Laemmli (1970). The extraction of soluble proteins was made in sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10%  $\beta$ -mercaptoethanol, 0.1% bromophenol blue dye) with heating for 3–4 minutes at 100 °C before loading. The amount of protein was 10  $\mu$ g per channel and the total current was 14 mA. The gel run in Tris-glycine buffer (pH 8.3) with 0.1% SDS and calibrated with a marker protein was obtained from 'Sigma Company'. The evaluation of protein concentration of the pollen extract was accomplished by using the method of Lowry et al. (1951).

#### 2.4 Animals

We used sensitized Mice as experimental models. Male Balb/C Mice (8–10 weeks of age) were sensitized and treated with *Canna indica* pollen. The animals were housed in an air-conditioned room at a temperature of  $25 \pm 2$  °C; they were fed a standard laboratory diet and given with water added Vitamins. The experimental animal research section at Razi Institute provided these animals. Animals were sensitized and treated by intraperitoneal injection of 50  $\mu$ l pollen extract (containing about 20  $\mu$ g protein in phosphate saline buffer) of immature, non-polluted and polluted pollen separately, with added the same dose  $AL(OH)_3$  as an adjuvant. Treatments were repeated five times, for 5 weeks, one in each week.

#### 2.5 Skin test

We used sensitized Mice for skin tests. Pollen extracts were prepared from all samples (including immature, mature and polluted pollen) by using the method of Sheldon et al. (1967), and the skin prick tests performed on Mice. Each animal was tested with 50  $\mu$ l of the total extract diluted with 0.05 M phosphate buffered saline, pH 7.4, and containing 25  $\mu$ g protein without adjuvant. The negative control was buffered saline and the positive control was histamine acid phosphate. The skin reactions were read after 4, 8, and 24 hours from the start of the test and quantified on the basis of wheal diameter.

#### 2.6 Clinical and serological tests

Sensitized Mice were used in serological tests. Blood samples were directly drawn from the heart,

one week after skin test (Aberg, 1989). At the end of each experiment, the animals were anaesthetized with Ether and blood samples were directly obtained from the heart. Up to the time they were needed, the sera of all samples were stored at  $-76$  °C. Serums of 10 Canna-allergic sensitized animals were individually tested for IgE reaction to Canna pollen grains. Serums of 10 sensitized animals were tested for polluted pollen, and 10 animals were tested for immature pollen. Sera from 10 animals, which were not sensitized to Canna pollen, were tested as the control sera poll. The total serum IgE was evaluated by using ELISA for the blood of mice. The standard Pharmingen mouse IgE protocol for sandwich ELISA was used to quantify the total amount of IgE. The serum IgE level was expressed in nonogram per milliliter after comparing with mouse IgE standards (Pharmingen, Calif, USA). Peripheral blood smears of healthy controls were compared to experimental animals (Mondal et al., 1997). We determined blood cells in each group and compared them by using statistical analysis.

#### 2.7 IgE-specific immunoblotting

Pollen samples were subjected to SDS-PAGE and then electrophoretically transferred to PVDF membrane (Robtom et al., 2002). After SDS-PAGE, proteins were transferred to a 0.2  $\mu$ m PVDF membrane (Bio Rad, USA) 1 hour at 100V through use of an Electro-Transfer unit (Bio Rad). The membranes were blocked over night at 4 °C in PBS/5% non-fat dry milk/0.05%  $NaN_3$ . Diluted sensitized and treated mice sera, 1:5 (v:v) in PBS/1% non-fat dry milk/0.01%  $NaN_3$ , were added to membrane and incubated over night at 4 °C. The membrane were then washed for 20 minutes 3 times in PBS/0.1% Tween 20 and incubated over night at 4 °C with polyclonal Goat anti-Mouse IgE (Abcam, Cambridge, UK), diluted 1:400 in non-fat milk buffer. The membranes were washed for 20 minutes 3 times and incubated 2 hour at RT with peroxidase-conjugated polyclonal Rabbit anti-Goat IgG(H+L)(Jackson Immunol research, USA) diluted 1:2000 in non-fat milk buffer. The membrane were washed for 20 minutes 5 times and incubated 1 minutes in enhanced chemiluminescence plus (Amersham Bioscience). The membrane were exposed to Kodak medical X-ray film 3 minutes and developed by Fuji X-ray film

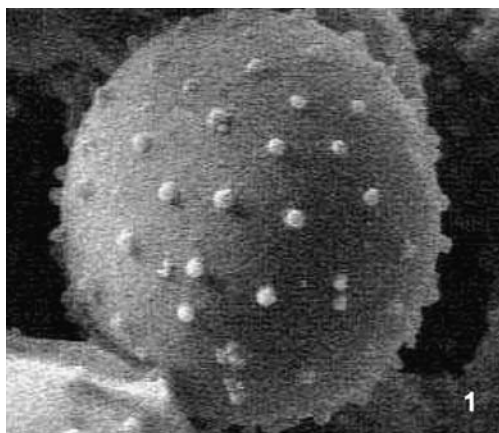


Figure 1. Scanning micrograph of *Canna* pollen grains that collected from non-polluted regions. Pollen grains are spherical having small mast as the tectum (1700 X).

processor model FPM 700. The prestained protein ladder for western blot was purchased from New England Biolabs.

### 3. Results

#### 3.1 SEM analysis

Ultra-structural studies of pollen grains of *Canna indica* showed that they were spherical apolar, 30–35  $\mu\text{m}$  in diameter, which had a thick exin with one visible pore. SEM analysis showed that tectum on pollen grains was small mast (Figure 1). On the surface of non-polluted pollens neither atmospheric fine dust nor agglomeration was shown. After contamination by polluted air, the color of the pollen grains changed dramatically and darkened considerably. Pollen grains became folded when they exposed to polluted air and vermicular particles accumulated on the surface of pollen grains (Figure 2). Pollen grains collected from polluted regions were covered by high amounts of pollutants too; the tectum of polluted pollen grains was disrupted. The interaction of pollen and particulate pollutants caused the pollen constituents to be released and agglomeration of organic particles on to pollen surface to be formed (Figure 3).

#### 3.2 Protein analysis

The SDS-PAGE protein profiles of different pollen samples (mature, immature, polluted and non-

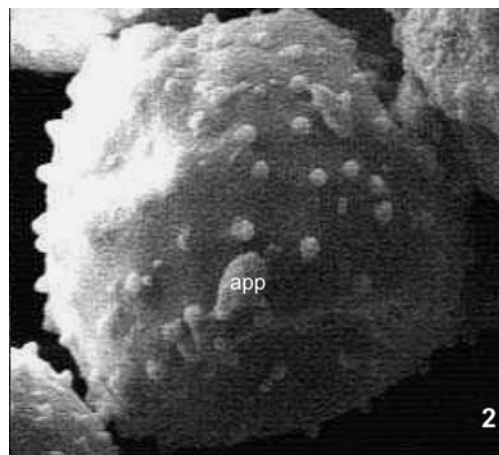


Figure 2. Scanning micrograph of *Canna* pollen grains that collected from polluted regions. Pollen grains be came folded, the tactum disrupted and air born particles accumulated in the surface of pollen grains. app, air pollutant particles (1700 X).

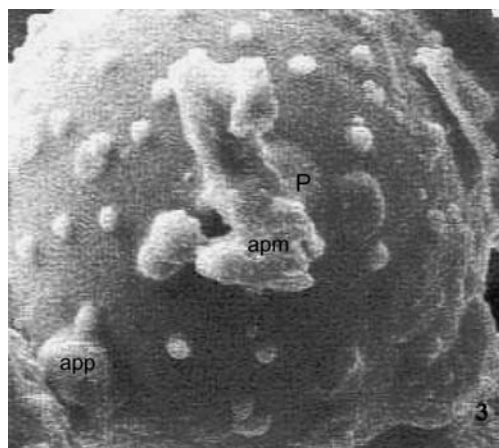


Figure 3. Scanning micrograph of *Canna* pollen grains that were exposed to Tehran polluted air for 20 days. Pollen grains became folded; air born particles accumulated on the surface of pollen and pollen material are released out of the pollen and agglomerated on the surface of pollen. P, pore; amp, agglomerated pollen's material (2000 X).

polluted) are shown in Figure 4. The bands are present in the molecular weight range between 12 and 67 kDa. SDS-PAGE showed that several proteins exist in immature pollen grains, which are different from bands observed in mature pollen grains. Some bands between 22 and 45 kDa disappeared and there was an increased quantity of another protein band with 22 kDa weight in pollen grains which were collected from polluted areas. Also in all polluted pollen, protein content was

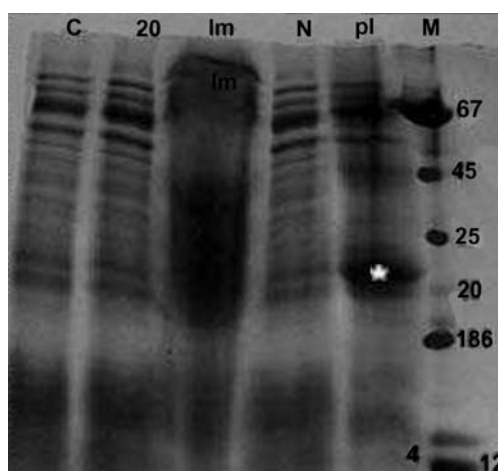


Figure 4. Protein profile of different pollen grains. Immature pollen grains have several different bands from mature ones. Some bands disappeared and increased the quantity of band 22 kDa(\*) in plants which were grown in polluted area. M, markers; Pl, the bands of pollen grains that collected from polluted area; N, the bands of normal pollen grains that collected from non-polluted area; Im, the bands of immature pollen.

decreased in response to air pollution. The protein content of extracts from pollen collected on the same day from plants which were growing in non-polluted and polluted area are shown in Table 2. Significant changes were found in concentration of total soluble protein per gram of pollen, between polluted and non-polluted pollens.

Table 2. Total protein content of polluted and non-polluted pollen grains of *Canna indica*

| Specimen             | Pollen grains collected from non-polluted area | Pollen grains collected from polluted area | Pollen grains that 20 days exposed to air pollution |
|----------------------|------------------------------------------------|--------------------------------------------|-----------------------------------------------------|
| Protein content mg/g | 6.9 ± 2.6                                      | 4.6 ± 0.8                                  | 2.8 ± 0.7                                           |

Data shows that pollen protein content are decreased in polluted condition.

Table 3. Comparison of allergic reaction by means skin test (wheal diameter), blood eosinophil, neutrophil and IgE levels in control and treated Mice

| Samples                                             | C          | Im          | N          | Pl         |
|-----------------------------------------------------|------------|-------------|------------|------------|
| Wheal diameter (cm)                                 | 0.4 ± 0.2  | 1.6 ± 0.9   | 3.1 ± 0.7  | 5.6 ± 1.03 |
| Eosinophil number (×10 <sup>4</sup> cells/ml blood) | 5.5 ± 1.3  | 9.8 ± 2.2   | 38.6 ± 3.9 | 57.7 ± 5.6 |
| Neutrophil number(×10 <sup>4</sup> cells/ml blood)  | 35 ± 1.9   | 68.5 ± 3.4  | 84.2 ± 2.8 | 93.4 ± 3.7 |
| IgE (ng/ml)                                         | 0.56 ± 0.2 | 2.32 ± 0.47 | 7.82 ± 1.2 | 11.7 ± 0.9 |

Data shows in all groups that have treated by pollen extracts, skin reaction, amount of eosinophils and IgE increased considerably. C, control group that treated by saline; Im, the group that treated by immature pollen extract; N, the group that treated by mature non-polluted pollen extract; Pl, the group that treated by mature polluted pollen extract.

### 3.3 Skin tests

Results of skin tests for different samples are shown in Table 3. Pollen extract had allergenic effect. While performing the skin-prick test, the maximum allergenic sensitivity was seen for the pollen which were collected from polluted areas, with an average wheal diameter about 6 cm. Allergy reactions were relatively large in normal pollens which were collected from non-polluted regions, with average 3 cm wheal diameters. Statistical analysis showed that the effect of polluted pollen is about two times more than normal pollen extract. Injection of these extracts caused the formation of deep wound in the place of injection after 24 hours whereas normal pollen caused only small and superficial wound. Mature non-polluted pollens were also observed to be more effective in causing allergic symptoms than immature pollens.

### 3.4 Clinical and serological tests

Results of blood smears from different groups are illustrated in Table 3. Study of blood smears showed that the amount of eosinophiles and neutrophils were increased in animals which were treated with pollen extracts. The quantity of eosinophiles in animals which were treated with polluted pollen was about two times more than those treated with non-polluted pollens and ten times more than the control group (Figure 5). The

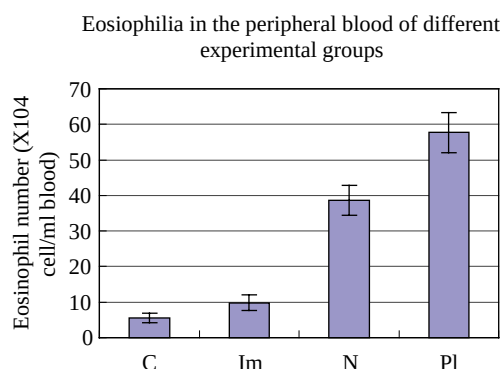


Figure 5. Eosinophilia in the peripheral blood of Mice that were treated by different pollen extract or saline injection. Polluted and non-polluted pollen extracts cause to increase in eosinophil cells as allergic characteristics, but polluted pollen grains were more effective. C, control group that treated by saline; Im, the group that treated by immature pollen; N, the group that treated by non-polluted mature pollen; Pl, the group that treated by polluted mature pollen. Each column represent the mean  $\pm$  SE of 8–10 animals  $p < 0.05$ .

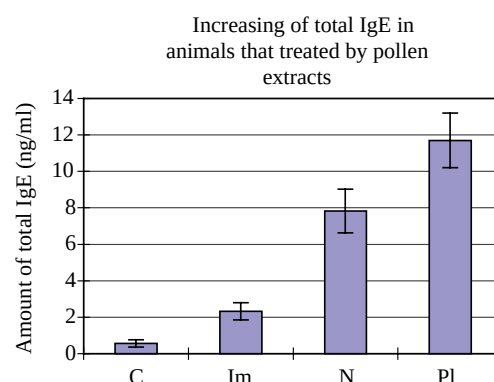


Figure 6. Amount of IgE in blood of Mice that were treated with different pollen extract or saline. Result shows that mature non-polluted and mature polluted pollen extracts induce increasing of blood total IgE as an allergy inducer. Effect of polluted and non-polluted pollen grains was significantly different. C, control group that treated by saline; Im, the group that treated by immature pollen grains; N, the group that treated by non-polluted mature pollen grains; Pl, the group that treated by polluted mature pollen grains. Each column represent the mean  $\pm$  SE of 8–10 animals  $p < 0.05$ .

data of the determination of total IgE in different groups are shown in Table 3. All pollen extracts caused an increase in IgE levels in the blood of treated animals. Serum of animals, which were treated with non-polluted pollen extracts, have more IgE than the control animals. The results indicate that the amount of IgE increased significantly in animals which were treated by polluted pollen extract. The amount of IgE in serum of animals which were treated by immature pollen extracts were more than the control group, but less than other experimental groups. Statistical analysis indicates that differences between polluted and non-polluted groups are significant (Figure 6).

### 3.5 IgE-Specific Immunoblotting

To detect the allergen by allergen-sensitive sera, immunoblotting was carried out on the pollen extracts. Figure 4 shows the different bands of pollen proteins. About 15 distinct protein bands were detected by Coomassie Brilliant blue staining. To identify bands corresponding to the allergen(s), IgE-binding bands were visualized by immunoblotting which were using sera from animals that sensitized and treated with Canna pollen extracts. After immunoblotting, two different bands with molecular mass of about 22 and 50 kDa were demonstrated (Figure 7). Intense reactivity was seen with the sera of 17 animals from a total of 20 sensitized

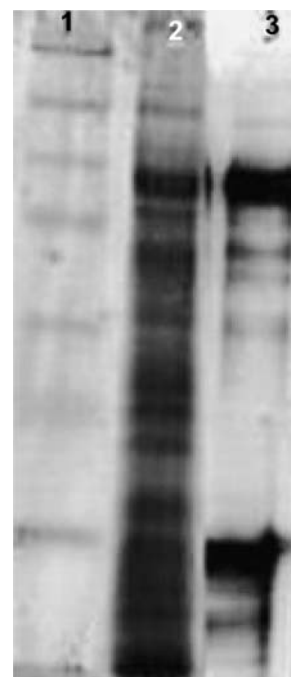


Figure 7. Results of immunoblotting by the using sensitized and treated Mice sera and pollen proteins. Results shows that Canna have two allergenic proteins with molecular mass of 22 and 50 kDa. M, pre-stained molecular mass markers; line 1, protein bands of pollen extract that stained by Coomassie Brilliant blue; line 2, protein bands that probed with pooled positive sera. The strong bands indicate reactivity of IgE antibodies with corresponding protein bands that act as allergens.

animals. There was not any difference between polluted and non-polluted animals. We could not detect any allergen bands in immature pollen extracts.

#### 4. Discussion

Air pollution has different detrimental effects on organisms. Ultra-structural studies (Figures 1–3) showed that air pollution has affected pollen grains (Figures 1–3). On the surface of non-polluted pollens neither atmospheric fine dust nor agglomeration was shown (Figure 1). Pollen grains became folded when they exposed to polluted air and vermicular particles accumulated on the surface of pollen grains. Pollen grains collected from polluted regions were covered by high amounts of pollutants (Figure 2). Agglomeration of air borne particles were seen on the surface of pollen collected from polluted regions, this is in agreement with observations which were reported by some researchers (Behrendt et al., 1992; Behrendt et al., 1997; Knox and Suphioglu, 1996).

The results of 12% SDS-PAGE analysis for soluble proteins (Figure 4), demonstrated the different bands in polluted and non-polluted pollen grains. Some bands between 22 and 45 kDa disappeared and there was an increased quantity of another protein band with 22 kDa weight in pollen grains which were collected from polluted areas (Figure 4). It seems that air pollution could affect the developmental process of pollen that is accordance with finding of previous reports (Rusznak et al., 1994; Majd and Kiabi, 1997; Ghanati and Majd, 1995; Ruffin et al., 1983), but that is different than the reports of Behrendt et al. (1992, 1997). According to our results, total protein of pollen grains decreased significantly in all polluted pollen (Table 2). This is in accordance with the findings of Behrendt et al. (1997), but not with the findings of Ruffin et al. (1983). The releasing of pollen material (Figure 3), culminate to decrease the pollen protein content.

Allergenic potential of polluted and non-polluted pollens were measured as different size of wheal, leukocyte number changes and amount of total IgE. Our clinical and serological results showed that abundance of eosinophils, average wheal diameter and amount of total IgE in animals that were treated with extracts of fresh pollen are

more than the control group (Table 3). Statistical analysis indicates that *Canna indica* is an allergenic plant and immunoblotting studies showed that Canna have two allergenic proteins with molecular mass of about 22 and 50 kDa that reacted with serum IgE strongly (Figure 7). These bands were seemed in both polluted and non-polluted pollen grains. It means that air pollutants could not affect pollen allergenic proteins. We did not find any reports regarding to this observation, therefore we will try to get separation and more analysis of these allergenic proteins. Also the allergenic potential of polluted pollen is more than that of non-polluted pollen grains (Figures 5 and 6). Our results indicated that mature pollens are more allergenic than immature pollens. We could not detect any allergenic bands in the immature pollen proteins by using immunoblotting. It means that most of the allergenic proteins were produced in the developmental process of pollen formation and were accumulated in mature pollen grains that finished their development.

Our different results including skin tests, evaluation IgE and determination blood cells showed that allergic potential of polluted pollen is more than of non-polluted pollen grains. That is accordance with some prior researches (Rusznak et al., 1994; Ito et al., 1996; Chakraborty et al., 1996; Behrendt et al., 1997; Knox et al., 1997; Majd and Kiabi, 1997; Emberlin, 1998; Jae-Kyoung et al., 2001). According to our observations organic substances absorbed to air pollutants mediate the agglomeration of particles on to the pollen surface followed by local preactivation of the coated pollen grains. Under appropriate conditions, i.e., humidity, water-soluble compounds of air pollutants may induce local allergen release and agglomeration of pollen material including pollen proteins on the surface of pollens (Figure 3). A consequence of the release of allergen molecules as aerosols is that the molecules are free to interact with other types of air born particles, such as diesel exhaust particles, that are associated with air pollution. Findings of several researchers support this idea (Knox and Suphioglu 1996; Behrendt et al., 1997). In this condition, sensitive individuals are in more contact with pollen proteins (allergens) than in the non-polluted condition. This phenomenon is one of the reasons of increase allergy and asthma frequency in polluted cities. On the other hand, one has to take into consideration that

in regions with high pollution, air pollutant particles are not only a carrier of pollutants, but also a carrier of allergens and probably altered allergens, and that pollens are not only carriers for allergens, but also pollutants (Behrendt et al., 1992).

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