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Research Article

Effect of Cadmium on the allergenicity of pollen grains of *Petunia hybrida* Juss

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Abstract

Purpose: Cadmium is a heavy metal and is an important part of Diesel Exhaust Particles (DEPs). Cd in high concentrations exhibited detrimental effects and cause also increase the allergenicity of pollen grains. The aim of the research was to study the effects of Cd on the proteins and allergenicity of *Petunia hybrida* pollens.

Methods: Petunia planets were cultured in a greenhouse and treated with various concentrations of Cadmium Chloride (400, 800 & 1200 μmol/L). The pollen was daily collected from the flowers of both experimental and control plants. Pollen extracts were prepared in PBS from control and Cd-treated plants and changes in protein banding profiles were compared by SDS-PAGE. Mice were sensitized by treatment of pollen extracts and $AlOH_3$, as an adjuvant, three times. The allergenicity of pollen extracts was evaluated by skin test and the amount of IgE in the experimental animals.

Results: Gel electrophoretic studies revealed that in the Cd-treated pollen grains, two new protein bands, 46 and 51 kDa, were observed that may have allergenicity. Results of serological tests showed the mean of wheal diameter and IgE level in animals that were sensitized to Cadmium chloride-treated pollen grains are more than the control.

Conclusion: In recent years pollinosis is increased in polluted areas, especially DEP-polluted areas; the reason may be that Cadmium, as an important part of DEP, acts as an effective agent in the induction of the formation of detoxifying proteins which can also act as new allergens.

Introduction

Epidemiological studies show an increasing trend in allergies in recent years. Reasons discussed for this phenomenon include a westernized lifestyle and air pollution [1-4]. Recent studies suggest that environmental pollution has a major role in the phenomenon [5,6] and air pollutants are effective in the formation of allergens, especially pollen allergens [7]. They can affect pollen grains during growth periods on the plant or directly by means of contamination of anthers or pollen grain flight [8].

Several components of air pollution are associated with asthma. In addition to major air pollutants including sulfur dioxide, nitrogen dioxide, and ozone, diesel exhaust particulates (DEPs) and diesel exhaust play an effective role in allergic diseases [9-11]. The coarse particulate matter was decreased in the atmosphere in the last years but the amounts of fine particles (less than 2.5 μm) (PM2.5), such as DEPs, are still high [12]. DEPs are made of a carbon core surrounded with organic chemical components (NO, SO_2, NO_2, CO, hydrocarbons) and heavy metals that are deposited [13], of which cadmium is an important part.

Cadmium is a well-known environmental pollutant with numerous adverse health effects [14]. Cadmium enters the cells using calcium channels or transporters of iron, manganese, and zinc due to its chemical and physical properties similar to these

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plant nutrients [15]. Researchers have studied the toxic effects of cadmium on various plants, including the general symptoms of cadmium toxicity, including reduced plant growth ((Singh et al. 2014), interference with the absorption, transport, and utilization of several nutrients [16].

Heavy metals can be useful to study the link between air pollution and allergy because they are known to alter the plant proteome) [17]. In particular, some heavy metals can change the expression of several proteins related to plant defense [18]. Exposure of plants to cadmium (Cd2+) resulted in the expression of several allergen-like proteins by *Arabidopsis thaliana* (Roth et al. 2006) [19]. In addition, class I PR-3 chitinases that are produced in response to heavy metals, have also allergenic potency because of their chitin-binding domain [20].

Materials and tests

Plant materials and treatments

In this research work, the allergenicity of *Petunia* pollen grains was evaluated following exposure to Cadmium as an important part of DEP. Detection of changes in pollen proteins is the other goal because they are associated with pollen allergenicity.

The seeds of petunia plants (*Petunia hybrida* juss. from Solanaceae) were purchased from Pakan Bazr Company (Isfahan, Iran). The seeds were grown in pots and planted in four groups in a greenhouse. Forty pots (40 cm high and 40 cm diameter) were filled with soil containing about 30% perlite, 20% humus, and 50% loam. Each group included 10 pots and each pot contained two plants. They were kept in stable conditions (25 ± 5 °C, humidity 60% and daylight for 15 hours) in the greenhouse. The experimental groups were classified as follows: groups treated with (1) 400 µmol/L, (2) 800 µmol/L, (3) 1200 µmol/L cadmium chloride, and (4) control ones treated with water. The plants growing in the pots were treated with cadmium solutions fifty days prior to flowering and continued for two weeks, during their pollen formation. Organs of experimental plants were sprayed with different concentrations of cadmium chloride and the control group was with water. Each plant was sprayed with about 20 ml of the above solutions every day.

Pollen protein extraction

Mature anthers (before anthesis) were collected from control and Cd-treated plants. The anthers were crushed in a microtube to release the pollen grains. Large pieces of anthers were removed and pollen grains were purified by passing through a mesh with a diameter of 40 µm. Protein extracts were done by immersion of pollen grains in 0.1 M phosphate saline buffer, PBS (pH 7.4) at a ratio of 17% for protein analysis and 8% for injection into mice, using a stirring unit at 5-7 °C for 4 hours. Centrifugation was done at 8000 g for 30 min [21].

Electrophoresis

The buffer including glycerol, sodium dodecyl sulfate, mercaptoethanol, and bromophenol blue was used as a loading buffer and added to pollen extract samples, vortexed, and then heated in a water bath for 3-4 min. Pollen extracts (25 µl) and a molecular ladder (15 µl; Sigma St. Louis MO) were run using discontinuous SDS-polyacrylamide gels (modified Laemmli method [22] (4% stacking, 12% resolving) were performed. Electrophoresis was done at 80 V twice in a plate gel apparatus (Bio-Rad), then the bands were stained using 0.2% Coomassie brilliant blue R250.

Animals

Mice variety of Male Balb/C (40-50 days old) were purchased from Razi Institute and kept in a room at a temperature of 23 °C – 27 °C, fed a laboratory diet. The mice were treated by intradermal injection of 40 ml extract (containing approximately 15 mg protein in PBS, prepared from control and Cd-treated plant pollen [23]. $Al(OH)_3$ (in the same amount) was used as an adjuvant in each case. The treatments were repeated four times for 4 weeks, one each week. They were divided into five groups (n = 6). First group as the control group received PBS and Aluminum hydroxide (Alum). The second group was treated with control plant pollens extract plus Alum and other groups were treated with pollens extract plus Alum of Petunia plants that were treated with three different concentrations of Cadmium Chloride (400, 800 & 1200 µmol/L) respectively.

Skin tests

A week after the last sensitization, animals were used for skin tests. Each mouse was injected with 40 ml of extract (20 mg protein added as an adjuvant) diluted in 0.05 M PBS (pH 7.4). The negative control was buffered saline. Skin reactions in the abdominal area were read after 60 minutes from the start of the experiment and measured based on the diameter of the weal. A positive skin test was considered a wheal diameter of 3 mm or more [24].

Blood analysis

Blood samples were obtained directly from the heart [25]. The mice were anesthetized with chloroform, then their blood was obtained directly from the heart. The serum from each animal was individually tested for IgE reaction.

The serum IgE level was expressed as ng/ml. Blood smears of control mice were compared with those of experimental ones. The numbers of various kinds of blood cells, including eosinophils and neutrophils, were determined in control and experimental animals.

Statistical analysis

The results were analyzed using ANOVA in SAS (9.1 version) and Duncan's multiple range test to compare the means of the experimental groups. Data are presented and illustrated as mean ± standard error.

Results

Skin test

The results of the skin tests analysis showed that pollen

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extracts prepared from normal plants do not have significant sensitizing effects, but pollen grains from plants exposed to cadmium chloride can cause skin reactions (Figure 1). Plants exposed to 1200 µmol/L of cadmium chloride showed maximum allergenic activity. Rats receiving this preparation showed a mean weal diameter of 10.4 mm. Allergic reactions in plants exposed to 400 and 800 µmol/L CdCl were also relatively high (average wheal diameters, 5.5 and 7.5 mm, respectively). In contrast, the injection of pollen extract prepared from untreated plants did not cause obvious skin reactions. Statistical analysis showed that the effect of pollen extract prepared from plants treated with 1200 µmol/L cadmium chloride was about 10 times higher than PBS and about 3 to 4 times higher than pollen extract of normal plants. $p \leq 0.05$).

Blood analysis

Evaluation of total IgE levels in the mice (blood) indicated all pollen extracts of the plants were able to increase the levels of IgE (Figure 2). The data and results showed that IgE was increased considerably in the animals injected with the pollen extract of Cadmium-exposed plants. The quantity of IgE in these animals was about 20–25 times more than the mice treated using PBS only, and was also 9–10 times more than the group treated with non-polluted pollens Table 1.

Protein analyses

The pollen protein profiles of normal plants and those exposed to Cadmium ranged in molecular weight from 15 to 120 kDa (Figure 3). SDS-PAGE showed that the pollen grains of plants exposed to different concentrations of Cadmium have different bands that were not observed in the extract of normal plants. They showed two extra bands (46 and 51 kDa). It means that two new proteins were formed in the pollen grains of Cd-treated plants.

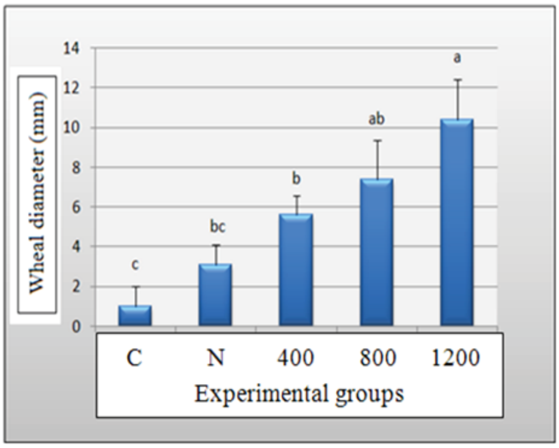

Figure 1: The mean values of the wheal diameter of the intradermal skin test in five experimental groups. The data showed that the skin reaction increased significantly in all groups that were treated with plant pollen extract. C: control group; N: group treated with pollen extract of normal plants. 400, 800, and 1200 are groups that were treated with pollen extract of plants that were exposed to 400, 800, and 1200 µmol/L of cadmium chloride, respectively. The difference between the 400, 800, and 1200 groups are significant with the control group, but not significant between the normal group and the control group ($p \leq 0.05$). Each column represents the mean ± SE of 6 animals.

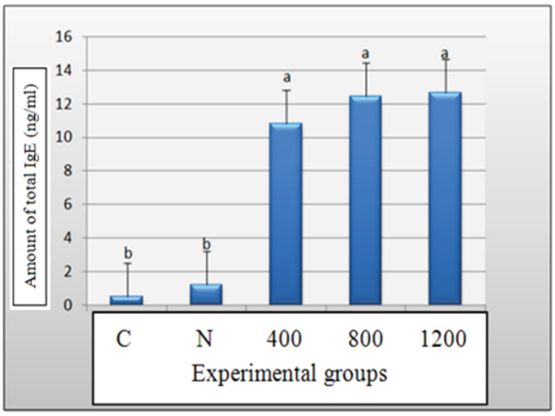

Figure 2: Amount of IgE (ng/ml blood) of the control and experimental mice. Cadmium-exposed plant pollen extracts cause to increase in blood IgE as allergic characteristics. C: the mice injected with saline; N: the mice received non-polluted pollen; 400, 800, and 1200 are the groups that were treated with pollen extract of plants that were exposed to 400, 800 and 1200 µmol/L of Cadmium Chloride respectively. Each column represents the mean ± SE of at least 6 animals.

Table 1: Comparing allergy reactions by means of skin test and IgE levels in control and pollen-treated animals.

Sampels	C	N	400	800	1200
Wheal diameter (mm)	1.00±0.5	3.10±0.8	5.58±1.3*	7.37±2.35*	10.40±3.55*
IgE (ng/ml)	0.525±0.25	1.20±0.52	10.82±3.1*	12.41±4.7*	12.66±3.45**

Data indicated that in all groups of Cadmium-exposed plants, skin reaction, and IgE levels were more than normal ones significantly. C: the group treated with saline; N: the group treated with none-polluted pollen; 400, 800, and 1200 the groups treated with pollen extract of plants that were exposed to 400, 800, and 1299 µmol/L of Cadmium Chloride respectively. Each datum represents the mean data ± SE of at least 6 mice.

$^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

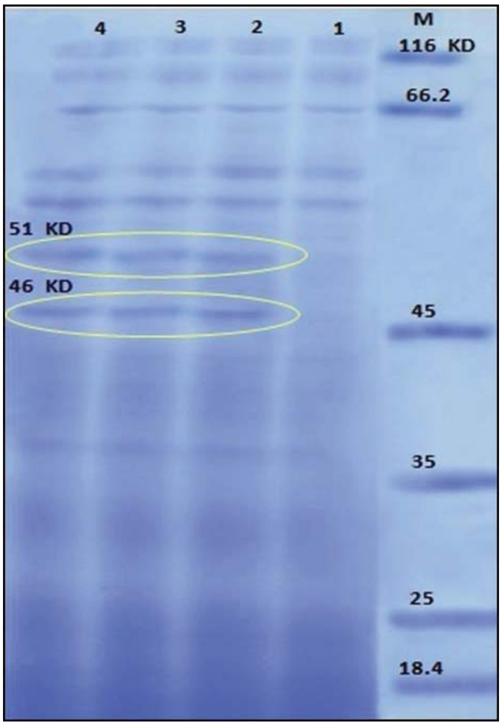

Figure 3: Protein profile of experimental pollens. Pollens of the plants that are treated with Cadmium Chloride have two extra bands (46 and 51 kDa) that are different from normal pollen. M, molecular weight standard markers; 1: Pollen grains of normal plants; 2, 3, and 4, the Plants that were exposed to 400, 800 and 1200 µmol/L of Cadmium Chloride.

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Discussion

The allergenic potential of pollen of plants treated with cadmium and normal plants was investigated using induction weals. The total IgE was evaluated in the blood of experimental mice. Observation and serological tests showed the mean diameter of weals and amount of IgE level in animals treated with normal plant pollen extract was close to the levels of the control group. It means that we can conclude that *P. hybrida* is not a real allergen species. However, the results clearly showed that cadmium could induce the allergenic potential of pollen grains. In Cd-treated plants, pollen grains could induce relatively high allergic reactions. The weal diameter was increased considerably and the amount of IgE was increased more than ten times in Cd-treated pollens than in normal ones. Similar reports are available about other air pollutants [26].

On the other hand, the results of SDS-PAGE analysis for soluble proteins (Figure 3) showed different bands in the pollen grains of treated and untreated plants. It seems that cadmium chloride treatment can affect the pollen development process. Therefore, we can assume that this allergenic potential is apparently due to the formation of new pollen allergen proteins (46 and 51 KD). This finding is consistent with some of the previous research of other researchers [26-28] but it is not consistent with the findings of some others [1,29].

Scientific evidence shows that pollen in heavily polluted areas, compared to areas characterized by less pollution, expresses a higher amount of proteins described as allergenic [30,31]. Some previous reports showed electrophoresis profiles of pollen collected from polluted and nonpolluted regions are similar, without any significant differences [25,32]. However, studies of pollen protein analysis (belonging to polluted and non-polluted areas) showed conflicting results.

In recent years, research has been done on the effects of environmental pollution on the allergenic power of pollen. Air pollutants have been shown to adhere to pollen surfaces [33] and also interact with pollen allergens and can act as adjuvants to the immune system, thus enhancing the allergenic properties of pollens [34].

Allergenic containers are derived from pollen called Ubish bodies. They are spherical and cause to grow the pollen exine. They are visible in the anther of different plant taxa. Allergenic proteins may also be a major part of the structures. Some suspended non-biological particles, e.g., DEPs, act as carriers and react also with them, as do other pollen-derived paucimicronic particles [28,33]. On the other hand, stronger Ig-mediated responses to such particles and inflammation of the respiration system may increase the frequency of allergic diseases in air-polluted areas [33,35].

Conclusion

The frequency of allergies has increased dramatically in recent years. Air pollution seems to be responsible for this phenomenon. Considering that Cadmium is an important part of diesel exhaust particles (DEPs) and DEP is the most important pollutant in polluted cities. The results showed that in Petunia plants normal pollen grains do have not any allergic activity but they are recognized as allergen pollens when treated with different amounts of Cadmium as an environmental pollutant. It is possible that Cadmium can be an effective agent that requires the formation of detoxification proteins that can also act as an allergen.

Ethical approval

The experiments were carried out according to the Guidelines of the National Institutes of Health on the principles of laboratory animal care (NIH Publication 80-23, 1996). The Local Ethical Committee approved all planned experimental procedures.

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