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Allergenicity of *Acroptilon repens* and *Juglans regia* pollen in rats

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Abstract

Pollen proteins that are located in the cytoplasm or on the surface of the exine can function as allergens and evoke immune system responses in sensitive patients, leading to allergic rhinitis and asthma. In this research, the pollen allergenicity and ability to induce IgE response of the pollen of two plant species were studied in rats. *Acroptilon repens* is an herbaceous, invasive plant with entomophilous pollen, while *Juglans regia* which is a tree crop produces anemophilous pollen. Immunoblot analysis using sera of sensitised rats revealed IgE reactivity to three protein bands including the 70, 41 and 25.12 kDa bands present in the *A. repens* pollen extract, while only one single immunogenic band of 11 kDa was detected in *J. regia* pollen extract. Both pollen extracts increased the eosinophil content and caused some clinical signs of allergy in treated rats. The results showed that both entomophilous and anemophilous pollen can be allergenic.

Keywords: allergy, anemophilous, entomophilous, immunoblotting, protein extract

Allergic asthma is a serious global health problem that affects people of all ages throughout the world (Datta et al. 2016). Allergic patients suffer from the production of IgE antibodies against harmless antigens, termed allergens. Contact with environmental allergen sources such as dust mites, insects, drugs, plant pollen and food induces IgE-mediated inflammatory responses in allergic patients (Swoboda et al. 2004). Pollen is an important trigger of seasonal rhinitis, conjunctivitis and asthma and a significant factor in atopic dermatitis (Li et al. 2011; Hinge et al. 2017).

Most prevalent pollen-expressed genes are involved in cell signalling, cell wall metabolism, vesicle transport and cytoskeleton formation (Sheoran et al. 2007). Pollen protein content is highly variable among plant species: ‘crude protein content’ (based on nitrogen measure) ranging from 2.5% to 61% of dry mass and ‘protein-bound amino acid’ (including oligopeptides and polypeptides) from 3.5% to 24.9% (Vanderplanck et al. 2014).

Pollen proteins are located in the pollen cytoplasm and on the surface of exine and intine (Suen et al. 2003; Vigh-Conrad et al. 2010). Pollen grains release proteins upon contact with the

respiratory mucosa, interacting with the human immune system to elicit an allergic response in susceptible individuals (Traidl-Hoffmann et al. 2005; Platts-Mills & Woodfolk 2011). The highest risk to sensitised individuals is during the flowering periods of plants (Erkan et al. 2011). The anemophilous pollen grains that are the major source of pollen allergens are carried by wind and can affect people who are far from the pollen producing plants. However, patients also develop sensitisation to entomophilous pollen (Bousquet et al. 2008).

Acroptilon repens (L.) DC. (Russian knapweed), an invasive herbaceous plant, is a member of the Asteraceae family and forms purple-pink capitule flowers (Nadaf et al. 2013). This species has been reported as an allelopathic agent (Quintana et al. 2008), an invader in the environment (Goslee et al. 2001) and toxic to horses (Chang et al. 2012). Its oil also inhibits the growth of Gram-positive bacteria (Norouzi-Arasi et al. 2006). So far, no scientific research has been done on its pollen allergenicity. The other plant studied in this research is *Juglans regia* L. (walnut) from the Juglandaceae family which is pollinated by

wind in spring (Mert 2010). The walnut tree is native to central Asia, the western Himalayas and China, producing seeds that are important in the food industry (Martinez et al. 2010). Severe anaphylaxis to walnut seeds has been reported (Pastorello et al. 2004). Walnut seed proteins have been recognised by the International Union of Immunological Societies (IUIS) as major food allergens (Downs et al. 2014). Its pollen is also considered to be allergenic (Wong et al. 2012), but no serologic study has been performed on it. In this study, the allergenicity of mature pollen grains of *A. repens* and *J. regia* was evaluated by the immunoblotting method in rats.

Material and methods

Plant material and preparation of protein extracts

The mature pollen grains of *Acroptilon repens* collected from its natural habitat in Kerman Province, Iran. Pollen grains of *Juglans regia* were collected from mature male catkins of trees planted in Sarduiyeh, Kerman Province, Iran.

The pollen extracts were prepared by incubating pollen grains in 0.1 M phosphate buffered saline (PBS) pH 7.4 in 20% *w/v* at 4 °C for 18 h. Suspensions were centrifuged at 14 500g at 4 °C for 35 min, and the supernatants were collected and stored at -20 °C until usage. The total protein content of pollen extracts was determined according to Bradford protein assay using bovine serum albumin (BSA) as a standard (Bradford 1976).

Rat sensitisation and serum collection

All experiments were carried out in accordance with the national ethical requirements for animal research and approved by the Ethics Committee of Shahid Bahonar University (Ethics Code: EC/105). Eighteen male Wistar rats, 4–6 weeks old, were purchased from the Animal Centre of the Shahid Bahonar University of Kerman, Iran. They were randomly divided into a control group and two sensitisation groups. The rats were housed in colony cages, six rats per cage, at 24 ± 2 °C with the altering 12 h:12 h light–dark cycle and fed with laboratory diet and water *ad libitum*.

The animals were sensitised by injection of pollen extracts on six occasions at weekly intervals through the intraperitoneal route. Furthermore, 75 µg and 60 µg of protein of *Juglans regia* and *Acroptilon repens* pollen extract were used, respectively, each time with alum. For the negative control group, the rats were intraperitoneally treated with PBS (Khajooie et al. 2013). The signs in

injection sites were determined for one hour after each injection. After the injection period, the animals were nasally administered pollen, 1 mg in 20 µl of PBS or 20 µl PBS alone for six consecutive days in the first and second week and four consecutive days in the third week (days 0–5, 7–12 and 14–17). The breathing mode of animals for two hours after every nasal challenge was determined clinically (Kato et al. 2014).

After the last week of nasal pollen administration, the animals were decapitated and their blood was collected. A portion of blood was used to count white blood cells using Sysmex XN1000. From the rest of the bloods, sera were obtained through centrifugation and stored at -20 °C until usage.

SDS-PAGE and immunoblotting

Pollen proteins were separated by 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and stained by Coomassie blue, or electrotransferred onto a 0.45 µm pore size polyvinylidene difluoride (PVDF) membrane. Immunoblot analysis of IgE binding protein fractions was carried out by the method outlined by Towbin et al. (1979). The membranes were blocked overnight at 4 °C in blocking buffer containing 5% non-fat milk powder in Tris Buffered Saline Tween 20 (TBST). The membranes were washed three times for 10 min in TBST and incubated for eight hours with 1:20 diluted pooled sera of allergic subjects in TBST. Then, the membranes were washed and incubated for two hours with 1:10 000 dilution of an anti-rat IgE tagged with horse radish peroxidase in TBST. The membrane was washed again and then treated by ECL chemiluminescence substrate (Amersham, UK). The allergen bands were revealed on X-OMAT Kodak film after 2 min contact with membranes (Khajooie et al. 2013).

Statistical analysis

Data on the amount of white blood cells, are presented as mean of repeats $\pm$ standard error of means. Duncan multiple range test was performed using SPSS software to compare the means at 0.05 significance level. The charts were plotted with Excel software.

Results

Clinical studies

After 40–60 min, swelling was observed at the injection site with stiffening but, there was no trace of redness of the skin (erythema). Swelling

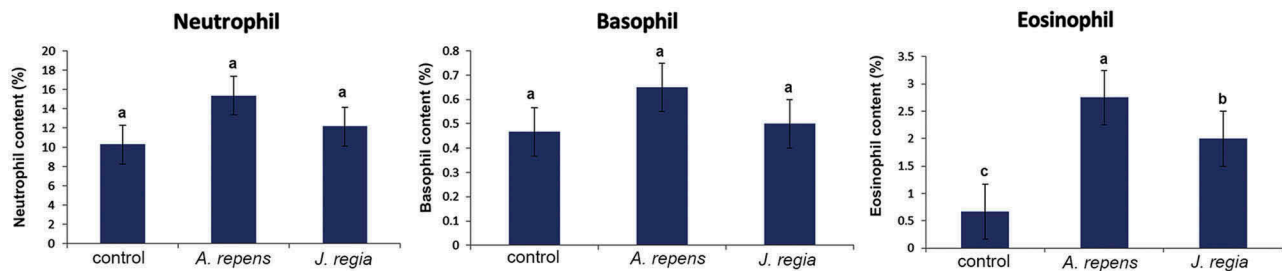


Figure 1. The content of white blood cells in sensitised and control rats. **A.** Neutrophils. **B.** Basophils. **C.** Eosinophils.

diameter of rats treated with extract of *Acroptilon repens* pollen and extract of *Juglans regia* pollen was about 2.5 and 1.2 cm, respectively, but no significant changes were observed in the control rats. Some treated rats showed ascites during the injection period. Breathing mode studies at nasal challenge duration showed that the animals contracted sneezing.

White blood cells content

It was shown that the treated animals had a significantly higher amount of eosinophils compared to the control. The increase in neutrophils and basophils content was not statistically significant (Figure 1).

Western blot analysis

SDS-PAGE separations of *Acroptilon repens* pollen extract revealed different protein bands with relative molecular masses of 90, 70, 53, 47.81, 41, 35.89, 31 and 25.12 kDa (Figure 2A), while *Juglans regia* pollen extract exhibited protein bands with relative molecular masses of 91.77, 85.31, 66.79, 58.29, 52.88, 36, 27.32, 18.10, 17, 13.46 and 11 kDa (Figure 3A).

The immunoblot analysis was carried out in order to detect the IgE binding proteins in pollen extracts. Three immunogenic bands at 70, 41 and 25.12 kDa were observed in *Acroptilon repens* (Figure 2B). High IgE reactivity to the 11 kDa protein band present in the *Juglans regia* pollen extract was also observed (Figure 3B).

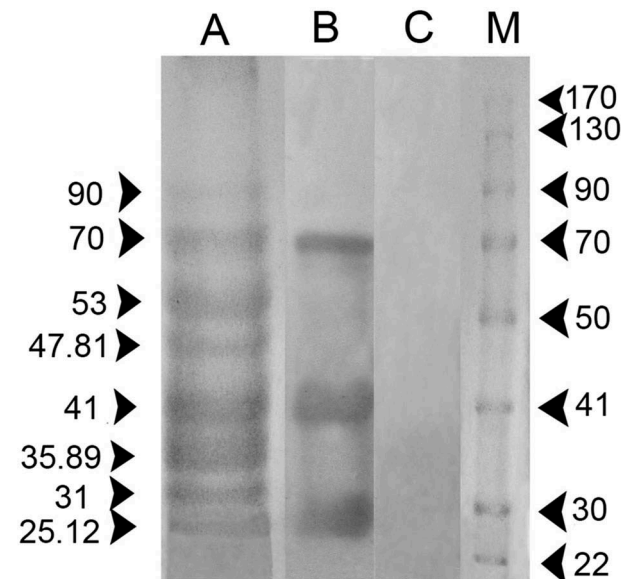


Figure 2. Pollen protein profile of *Acroptilon repens*. **A.** The stained SDS-PAGE profile. **B.** Immunoblots using the pooled sera of six sensitised rats. Proteins with molecular weight of 70, 41 and 25.12 kDa were allergenic components of *A. repens* pollen. **C.** No protein with IgE reactivity was observed in immunoblots using the pooled sera of control rats. **M.** Marker.

Discussion

Respiratory allergy is induced by nasal allergen exposure that results in IgE-mediated inflammation of nose membranes. The allergic response is divided into two phases: early and late. Allergen-specific IgE is bound to membrane of mast cells and basophils. When they are crosslinked by cognate allergen, mast cells immediately secrete a preformed mediator, histamine, which induces early-phase responses such as sneezing, itching and watery nasal discharge. Early-phase responses occur within minutes after allergen exposure, while late-phase responses are evoked later. Inflammatory cells, eosinophils, mediate the late-phase response. Eosinophils-derived mediators induce epithelial damage resulting in nasal mucosal swelling (Galli et al. 2008; Wallace et al. 2008). A diagnosis of respiratory allergy is mainly based on nasal symptoms and the detection of serum antigen-specific IgE. Generally, patients with respiratory allergy show increased serum total and antigen-specific IgE levels (Bousquet et al. 2008; Wallace et al. 2008). Respiratory allergies can also be accompanied by ascites which is the accumulation of protein-containing fluid within the abdomen (Adema et al. 2010).

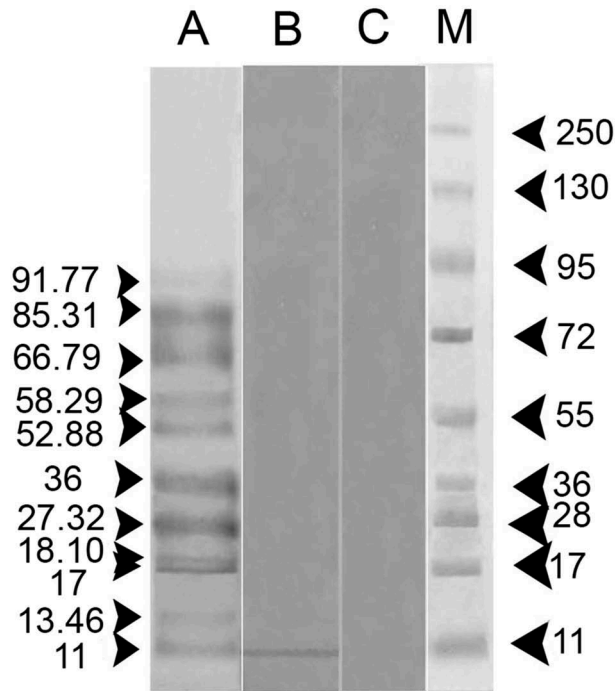


Figure 3. Pollen protein profile of *Juglans regia*. **A.** The stained SDS-PAGE profile. **B.** Immunoblots using the pooled sera of six sensitised rats. A protein with molecular weight of 11 kDa was the allergenic component of *J. regia* pollen. **C.** No protein with IgE reactivity was observed in immunoblots using the pooled sera of control rats. **M.** Marker.

Pollen grains released from weeds belonging to the Asteraceae family can cause respiratory allergy and represent a major source of seasonal allergies among susceptible people in many countries (Himly et al. 2003). So far, pollen extract analysis of some Asteraceae species through IgE immunoblotting by sera from allergic patients has identified some major allergenic components. Amb a 1 represents the most important allergenic compound of the Asteraceae family with a sensitisation frequency of > 95% among ragweed (*Ambrosia*) allergic individuals. It comprises 6% of the total protein in a ragweed pollen extract and is an acidic, non-glycosylated, single-chain protein with a molecular weight of 38 kDa (Gadermaier et al. 2004). Mugwort (*Artemisia vulgaris* L.) pollen triggers allergic reactions in 10–14% of pollinosis patients in Europe and in 11.3% of asthma and/or rhinitis patients in China (Gadermaier et al. 2004; Li et al. 2009). Art v 1 is the major allergen of mugwort pollen with a molecular weight of 13–16 kDa. Among *A. vulgaris* allergic patients, up to 95% demonstrate IgE-binding to Art v 1 (Himly et al. 2003; Gadermaier et al. 2004). Many other allergens with varied molecular weights have also been identified in ragweed and mugwort pollen extracts (Gadermaier et al. 2004).

It has also been reported that pollen grains of other members in Asteraceae such as *Helianthus annuus* L. (Asturias et al. 1998), *Chrysanthemum maximum* Ramond (Sharif Shoushtari et al. 2013), *Zinnia elegans* Jacq. (Chehregani et al. 2004), *Xanthium strumarium* L. (Jaggi & Gangal 1987) and *Dendranthema grandiflorum* (Ramat.) Kitam. (Lee et al. 2007) are allergenic.

Therefore, current knowledge of Asteraceae pollen allergens is concentrated on major allergen sources in the world. In this study for the first time, the ability of *Acroptilon repens* pollen to induce respiratory allergy and to increase serum IgE in sensitised rats was evaluated. The signs of allergy such as ascites, sneezing and increase in eosinophil content were observed in treated animals. Western blotting results showed three allergenic bands indicating pollen allergenicity of this species. Regarding molecular weight, the 41 kDa band detected in *A. repens* immunoblot is most close to Amb a 1 (38 kDa) in ragweed or Sal k 1 (43 kDa) in Russian thistle (*Salsola kali* L.) among the identified pollen allergens in Asteraceae. The 70 kDa band which has a relatively high molecular weight is most close to Art v 2 (60 kDa) of mugwort pollen, while 25.12 kDa band is relatively close to Par h 1 (31 kDa) pollen allergen in feverfew (*Parthenium*).

Walnut (*Juglans regia*) seed proteins have been recognised by the IUIS as major food allergens. Jug r 1 (a 2S albumin), Jug r 2 (a 7S vicilin-like globulin), Jug r 3 (a non-specific lipid transfer protein), and Jug r 4 (a 11S legumin-like globulin) and series of peptides from the N-terminal region of the 7S seed storage globulin proproteins were identified and characterised as low molecular weight allergens (Downs et al. 2014).

It has also been known that a number of people suffer from allergy and respiratory diseases resulting from *Juglans regia* pollen grains (Wong et al. 2012). However, no scientific research has been performed to identify its pollen allergenic components so far. In this research, the allergenicity of *J. regia* pollen in rats was clinically determined through signs such as ascites, sneezing and eosinophil enhancement. Immunoblot analysis revealed a high specific IgE reactivity to the 11 kDa protein band present in pollen extract of *J. regia*.

According to their migration, pollen grains are classified as anemophilous or entomophilous. Anemophilous species often causing allergic diseases produce a huge quantity of readily airborne pollen grains. They disperse in the air and are carried a long distance. Those of entomophilous species are usually bigger and heavier, so that they rapidly drop onto a soil surface, where they are usually inactivated (Bartkova-Scevkova 2003). However, it is known that people develop sensitisation to both pollen types (Bousquet et al. 2008). The results of this study also show that both entomophilous and anemophilous pollen can be allergenic.

More complete identification, structure characterisation, isolation and cross reactivity of these important allergen molecules are needed to improve the diagnosis of these allergens in patients.

Conclusion

The entomophilous pollen of *Acroptilon repens* from Asteraceae and the anemophilous pollen of *Juglans regia* from Juglandaceae were both allergenic in rats. However, the entomophilous pollen had three protein bands with IgE reactivity to sera of sensitised rats, while the anemophilous pollen had only one protein band with IgE reactivity. The higher number of allergenic proteins in entomophilous pollen comparing anemophilous pollen could be a trait related to the migration behaviour of pollen. It can be assumed that animals (and perhaps humans) have developed resistance against some allergenic proteins of anemophilous pollen during evolution because this pollen type is more experienced when compared with entomophilous pollen that is less produced and less inhaled. This issue must be studied in detail.

Disclosure statement

No potential conflict of interest was reported by the authors.

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