

## Influence of high-pressure treatment on allergenicity of rDau c1 and carrot juice demonstrated by *in vitro* and *in vivo* tests

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The aim of our study was to detect the influence of high-pressure treatment (HPT) on the allergenicity of recombinant allergen rDau c1 and carrot juice using *in vitro* and *in vivo* tests. The buffer solution of recombinant main carrot allergen rDau c1 for the basophil activation test (BAT) and Western blot (WB) was used. Dau c1 was pre-treated by pressure 500 MPa for 10 min and different temperatures (30 °C, 40 °C, 50 °C) and pressure from 400 to 550 MPa for 3 and 10 min. Neither the HPT from 400 to 550 MPa for 3 and 10 min nor the HPT at 500 MPa for 10 min and temperatures 30 °C, 40 °C, and 50 °C had the influence on basophil activation by rDau c1. Serum samples of birch pollen allergic patients reacted in WB with solution of rDau c1 treated by HP 500 MPa for 10 min at temperature 30 °C, 40 °C, and 50 °C. This pressure procedure did not influence the immune reactivity of rDau c1 in WB test. The structural changes of rDau c1 caused by HPT studied by circular dichroism spectra were found. Mild increase of the beta-sheet structure was observed. The main changes were seen in rDau c1 samples treated 10 min at 500 MPa and temperature 50 °C. The influence of HPT on the allergenicity of carrot juice was studied afterward. The reactivity in skin prick tests and BAT did not show any influence of HPT on allergenicity of carrot juice. Pressure of 500 MPa for 10 min and temperature 30 °C, 40 °C, and 50 °C did not inactivate allergen Dau c1 in carrot juice in WB. HPT from 450 to 550 MPa for 3 and 10 min at temperature 30 °C had no influence on the immune reactivity of Dau c1 in carrot juice. Nineteen patients underwent the double-blind, placebo-controlled food challenge. Thirteen of them reacted to placebo and were excluded, one patient did not react to any material (placebo, HPT material and fresh frozen carrot juice), three patients had positive test (reacted on HPT material and non-treated fresh frozen carrot juice), and two patients had negative reaction (reacted only on fresh frozen material). We could not confirm the influence of HPT on allergenicity of rDau c1 and carrot juice using *in vitro* and *in vivo* tests.

**Keywords:** carrot; Dau c1; allergen; high-pressure treatment

### 1. Introduction

Birch pollen allergens belong to the most wide-spread inhalant allergens in Central, Northern and Eastern Europe, and have a high potential to cause pollen allergy symptoms. The number of

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patients sensitive to this pollen has constantly increased during the last few years [1]. Nearly 50% of patients also have symptoms of oral allergy syndrome (OAS) [2], which manifests immediately after the consumption of food of vegetable origin (fresh fruit, vegetables, or tree-nuts) by itching, burning or swelling of the oral cavity, tongue, or lips. This is due to the cross reactivity between the main birch allergen Bet v1 and homologous food allergens from a group of highly preserved proteins, so-called pathogenesis related protein 10 (PRP-10) [2]. Twenty-five percent of patients with pollen and food allergy describe OAS after consumption of fresh carrots [3]. The major carrot allergen Dau c1 causes a reaction in 98% of patients. Thirty-eight percent of patients react to minor profilin allergen Dau c4, which is homologous to the birch allergen Bet v2 [4]. IgE reactivity to the cross-reacting carbohydrate determinants was found in nearly 20% of patients [5].

Scheibenzuber [6] and Meyer-Pittroff et al. [7] described a study suggesting that the allergenicity of apple slices can be inactivated by high-pressure treatment (HPT). Meyer-Pittroff et al. [8] published a method using HPT on apple slices to remove symptoms of OAS in allergic patients. Houska et al. [9] studied the capability of HPT to modify the allergenicity of the main apple allergen Mal d1 and apple juice. We extended this approach to study the influence of HPT on the allergenicity of rDau c1 and carrot juice by *in vitro* and *in vivo* tests. We assume this method may lead to the production of hypoallergenic food variants with the preserved content of vitamins suitable for allergic patients.

## 2. Materials and methods

### 2.1. *Dau c1* detection in processed carrot juice (semi-quantitative electrophoresis)

Semi-quantitative SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) protocol was used for Dau c1 protein separation in differently processed carrot juices.

### 2.2. *rDau c1* solution preparation

Two milligrams of lyophilized recombinant carrot allergen (rDau c1) (Biomay, Austria) were diluted in 10 mL of buffer solution. The diluted samples were stored in aliquots at  $-30^{\circ}\text{C}$ . Before use, aliquots were carefully thawed at  $5^{\circ}\text{C}$  in a refrigerator.

### 2.3. Carrot juice preparation for skin prick test (SPT), basophil activation test (BAT) and Western blot (WB) test

Fresh carrot variety MAXIMA F1, CZ, from the harvest year 2007, was stored in a modified refrigerated atmosphere. After washing and peeling the carrot, a juicer (Champion, USA) was used for juice preparation. The final temperature of juice was  $22.2^{\circ}\text{C}$ . It was immediately placed into polyamide/polyethylene (PA/PE) pouches which were heat sealed. Samples were either pressure treated and frozen, or frozen and left as untreated control samples.

### 2.4. High-pressure treatment

An isostatic press (Zdas CYX 6/0103) with a chamber volume of 21 was used. Drinking water served as the pressure-transmitting medium. Aliquots with rDau c1 solution were treated with high pressure in a range between 400 and 550 MPa and held for 3 and 10 min. The final temperature of samples after HPT was  $22-26.5^{\circ}\text{C}$ . Aliquots were then stored at  $5^{\circ}\text{C}$  before structure changes of the allergen were tested by circular dichroism (CD) electron spectroscopy. Some of the samples

were frozen before BAT was performed. Other rDau c1 solutions were treated at 500 MPa and held at 30 °C, 40 °C, and 50 °C for 10 min. These parameters were chosen to avoid over processing and to mimic the existing high-pressure cold pasteurization of the carrot juice. Samples were then frozen before the CD spectra analysis and BAT and WB tests were performed.

### **2.5. Carrot juice and double-blind placebo-controlled food challenge (DBPCFC)**

The same procedure of carrot juice preparation as for SPT, BAT, and WB was used. Three different samples were prepared for the oral test – (i) positive control with allergen untreated, (ii) test sample pressure treated, and (iii) placebo sample. The carrot juice was mixed 1:1 with Sinlac premix (Nestle, Vevey, Switzerland) as a positive control. Sinlac is a dry gluten free food for allergic babies containing rice flour, carob flour, and other components). Sinlac premix was prepared by mixing 50% of the Sinlac powder and 50% of the water.

The pressure-treated samples were prepared in the same way, but the carrot juice was packed in PA/PE pouches and pressure treated at 500 MPa for 10 min at 30 °C. After HPT the samples were frozen. Packs were carefully thawed and mixed 1:1 with Sinlac before use.

Placebo samples needed to have a similar appearance and taste to the other samples. Therefore, the same carrot juice was used for placebo preparation. Carrot juice was boiled twice, then cooled down at 20 °C and frozen. Before use it was thawed and mixed 1:1 with Sinlac. All samples had to be acidified before mixing with Sinlac at the same pH adding water–sugar–citric acid solution. Mixed samples were then placed into pouches containing 10, 20, and 80 g of mixture. Pouches were coded and delivered frozen to the clinic. The day before oral testing, samples were carefully thawed in a refrigerator at 5 °C.

In addition, fresh carrot of the same variety was also used during testing. The schedule of DBPCFC was applied. The experimental group consisted of 19 patients (age range = 18–55 years) with an allergy to birch pollen and carrot. The first part of the procedure involved labial testing: 10 g of the specimen was placed onto a piece of filtration paper and placed on the lip of each patient. After 15 min the reaction was evaluated, then a 20 g sample was swallowed by the patient and again the reaction was evaluated. If there was no reaction, an 80 g sample was orally administered and the reaction was evaluated. If no reaction was noticed during an additional 15 min, the test was evaluated as negative. Due to ethical reasons, we did not use objective tests for oral mucosa reactivity (mucosal biopsy for example); we relied on reports of subjective symptoms only. All three samples (10, 20, and 80 g) were administered in three sessions within a single day. All patient reactions were evaluated by a board certified clinician. Patients enrolled to the study signed an informed consent and the protocol was approved by the local review board.

### **2.6. Skin prick test (SPT)**

Commercial diagnostic carrot allergen extract (Alyostal, Stallergenes), fresh carrot and different carrot juices either pressure treated or untreated (control) were used for the SPT. All juices were stored frozen and thawed in a refrigerator just before skin testing. Wheals of diameter  $\geq 3$  mm were regarded as a positive reaction (EAACI standards for skin testing).

### **2.7. BAT (BAT-CD63 expression)**

Activation of sensitized peripheral blood basophils stimulated by rDau c1 (10  $\mu$ L 0.01 mg/mL), carrot juice and 10 times diluted commercial carrot allergen extract (Alyostal, Stallergenes) was measured by flow cytometry (FC 500, Beckman-Coulter). The cells were identified by spontaneous CD 203c expression, then activation molecule CD63 expression measured, both by

binding of fluorochrome conjugated relevant monoclonal antibodies. The mean proportion of CD 203c+/63+ cells was registered, and activation for at least 15% was considered as positive.

## 2.8. WB test

The serum of patients sensitized to carrot was used for the WB analysis. Anti-human IgE immunoglobulin, originally from goats, anti-goat IgG – biotin (rabbits), conjugate streptavidin-peroxidase and other chemicals for electrophoresis were purchased from Sigma–Aldrich. Samples were thawed carefully before testing. Further preparation of samples was done according to Lämmler [10]. SDS-PAGE protocol was used for protein separation. After electrophoresis, the gels were placed into transfer buffer (pH = 8.3); then the gels were placed onto a membrane with the patient's serum and then into a blotting chamber. A detailed description of the methodology is provided in our research report [11].

## 2.9. CD electron spectroscopy

Structural changes of rDau c1 were tested by CD electron spectroscopy. This work was performed at the Institute of Chemical Technology in Prague, using a J-810 Spectropolarimeter (Jasco, Japan). The sample was placed in crystal glass flat cell thickness of optical environment 1 mm, and carefully thermostated to 5 °C. The spectral range was studied at wavelengths between 185 and 260 nm.

## 2.10. Statistical evaluation

The data were expressed as medians and the 5th and 95th percentiles; the differences between samples were tested by the Wilcoxon non-parametric test. A *p*-value less than 0.05 was regarded as significant [12].

## 3. Results

### 3.1. BAT reaction of allergen solutions

The results of BAT for rDau c1 undiluted and 10 times diluted solutions treated in the pressure range between 400 and 550 MPa and held 3 and 10 min are presented at Tables 1 and 2. Medians and percentiles of BAT for rDau c1 HPT solutions treated at 500 MPa for 10 min at different temperatures are shown at Table 3. Results of Wilcoxon's test showed statistically insignificant difference in the confidentiality level 0.05. Neither the HPT from 400 to 550 MPa for 3 and 10 min nor the HP at 500 MPa for 10 min and temperatures 30, 40, and 50 °C had the influence on rDau c1-induced basophil activation. A statistically insignificant 15% decrease of basophile activation was found at temperatures 30 °C and 50 °C.

Table 1. Basophil activation percentage by rDau c1 high-pressure-treated undiluted solutions at different times.

Pressure–minutes (MPa–min)	Anti-IgE	000–00	400–3	400–10	450–3	450–10	500–3	550–3	550–10
Median	46	51	70	51	57	49	44	57	58
5% Percentile	23	30	34	33	35	30	29	35	40
95% Percentile	69	72	82	75	68	70	70	71	79

Note: Eighteen sensitized patients included.

Table 2. Basophil activation percentage by rDau c1 high-pressure-treated 10 timed diluted solutions at different times.

Pressure–minutes (MPa–min)	Anti-IgE	000–00	400–3	400–10	450–3	450–10	500–3	550–3	550–10
Median	46	55	53	47	50	45	42	46	50
5% Percentile	22	16	18	19	14	12	13	16	16
95% Percentile	70	69	68	67	67	68	66	69	80

Note: Eighteen sensitized patients included.

Table 3. Results of basophil activation percentage by rDau c1 high-pressure-treated undiluted solutions at different temperature (no statistical differences were found).

Pressure/time–temperature (MPa/min–°C)	Anti-IgE	000/00	500/10–30	500/10–40	500/10–50
Median	31	43	28	40	28
5% Percentile	20	6	6	12	5
95% Percentile	57	60	62	77	61

Note: Sixteen sensitized patients included.

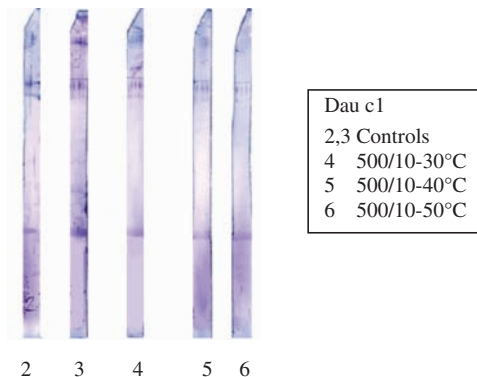


Figure 1. Influence of HPT 500 MPa for 10 min and different temperatures on allergen rDau c1 – WB method.

### 3.2. Results of WB test of allergen solutions

The IgE binding from serum samples of carrot allergic patients with rDau c1 solutions treated by HP 500 MPa for 10 min and temperature 30 °C, 40 °C, and 50 °C are presented at Figure 1. HPT of rDau c1 solutions at 500 MPa and 10 min at temperatures between 30 °C and 50 °C failed to change band characters of rDau c1.

### 3.3. Structural changes of allergen solutions

The CD and absorption spectra and structural changes were evaluated for rDau c1 solution treated with HP in the range 400–550 MPa for 3 and 10 min and temperature below 25 °C. Changes only at the borderline pattern were found between treated and untreated samples. Solutions of rDau c1 treated by HP 500 MPa for 10 min at temperature between 30 °C and 50 °C (Figure 2) did not show statistically significant changes compared to the untreated control. A small increase of beta-sheet structure compensated with a decrease of alpha-helical structure was found in HPT sample performed at highest (50 °C) temperature used (Figure 3(a) and (b)).

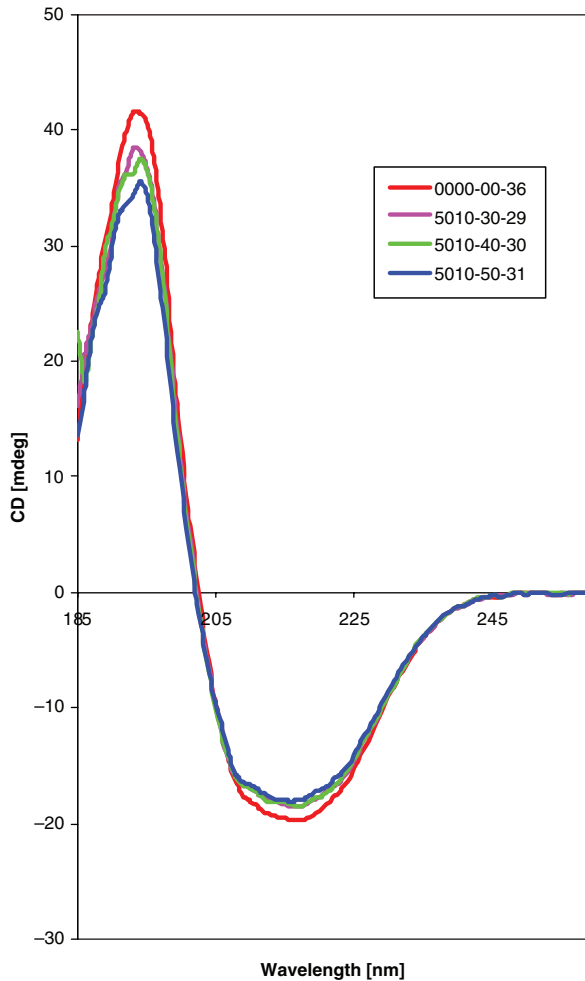


Figure 2. ECD spectra of allergen rDau c1 (0000-00 untreated sample, 5010-30 sample treated at 500 MPa for 10 min at 30 °C, 5010-40 sample treated at 500 MPa for 10 min. at 40 °C, 5010-50 sample treated at 500 MPa for 10 min. at 50 °C).

### 3.4. *Dau c1* detection in processed carrot juices

The highest content of Dau c1 protein was found in untreated carrot juice (22.6 mg Dau c1/100 mL of juice). HPT lead to 58% decrease of Dau c1 protein content (9.5 mg Dau c1/100 ml of juice) in carrot juice. Content of Dau c1 in cooked carrot juice decreased even for 66% (7.7 mg of Dau c1/100 mL of juice).

### 3.5. *SPT and BAT test reaction of carrot juice*

Fresh carrot (positive control) and untreated carrot juice elicit the same reaction in SPT (Table 4). The same reaction evoked also samples of HP-treated carrot juice. This gives evidence of the great stability of Dau c1 allergen in the natural pH of carrot juice. Results of the BAT test with carrot juice treated with HP in range 450–550 MPa for 3 and 10 min are shown in Table 5. Results of Wilcoxon's test showed a statistically insignificant difference in the confidentiality level 0.05. There was no HPT influence on the allergenicity of Dau c1 in carrot juice shown in SPT and BAT tests.

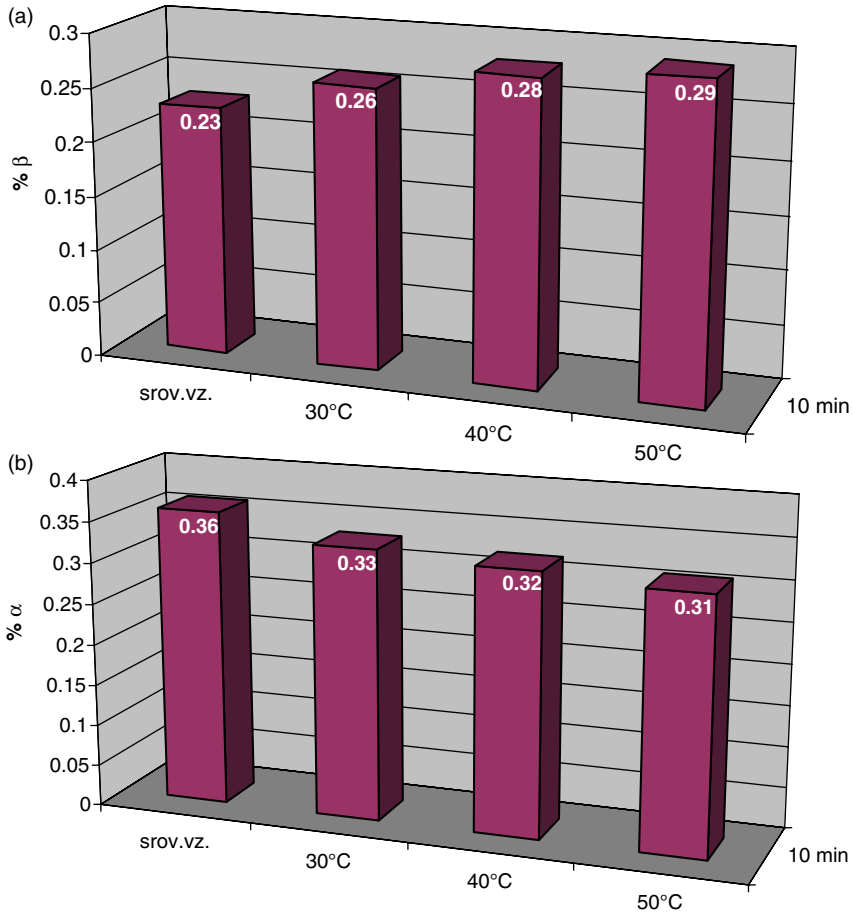


Figure 3. (a) Presence of beta-structure in Dau c1 allergen (500 MPa, 10 min, temperature 30 °C, 40 °C, and 50 °C) (“srov. vz.” represents untreated control). (b) Presence of alpha-structure in Dau c1 allergen (500 MPa, 10 min, temperature 30 °C, 40 °C, and 50 °C) (“srov. vz.” represents untreated control).

Table 4. Results of SPT with high-pressure-treated carrot juice.

Pressure/time (MPa/min)	Wheal diameter (mm)								
	Fresh carrot	Positive ctrl	000/00	450/3	450/10	500/3	500/10	550/3	550/10
Median	6.0	7.0	6.0	6.0	6.0	5.0	5.0	6.0	5.0
5% Percentile	3.3	4.3	4.0	3.3	4.0	4.0	3.3	4.0	4.0
95% Percentile	11.4	8.0	10.0	10.0	8.0	10.0	8.0	11.4	10.0

Note: Twenty-four sensitized patients included.

Table 5. Results of basophil activation percentage by carrot juice high-pressure-treated at different holding times.

Pressure/time (MPa/min)	Anti-IgE	000/00	450/3	450/10	500/3	500/10	550/3	550/10
Median	37	64	73	65	66	66	66	66
5% Percentile	18	32	37	35	36	34	32	29
95% Percentile	70	93	91	92	90	91	91	92

Note: Twenty-six sensitized patients included.

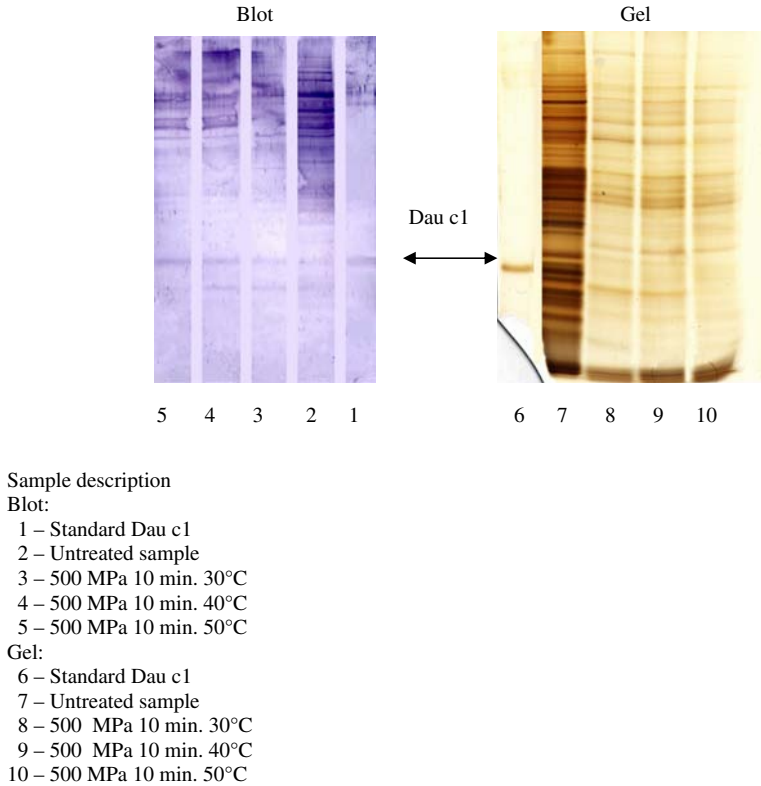


Figure 4. Influence of HPT 500 MPa for 10 min and different temperatures on Dau c1 allergen in carrot juice – WB method.

**3.6. WB analysis of carrot juice**

Serum of a patient sensitive to carrot reacted with carrot juice HP treated at 500 MPa for 10 min and at temperatures 30 °C, 40 °C, and 50 °C. High temperature (50 °C) influenced protein in gel, but no changes were seen at the Dau c1 band in the blot (Figure 4). Other samples of carrot juice were pressure treated in the range between 450 and 550 MPa for 3 and 10 min at a temperature of 30 °C. We can conclude that longer HPT (for 10 min) was more effective and again had influence on protein in gel, but no difference between treated and untreated samples was seen in the blot.

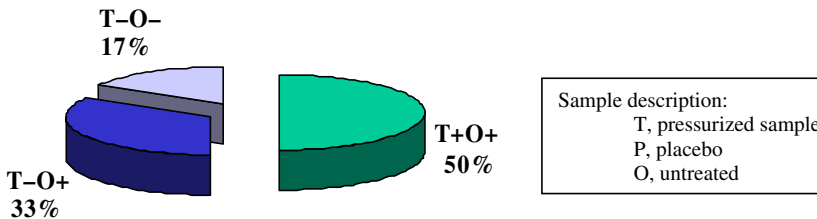


Figure 5. Results of DBPCFC with carrot homogenate. Total number of patients was 19. Thirteen patients were excluded for placebo reaction, one patient did not react to any material (placebo, HPT material and fresh frozen carrot juice) and the test was completely negative, three patients had a positive test (reacted to the HPT material and untreated fresh frozen carrot juice) and two patients had a negative reaction (reacted only to the fresh frozen material).

### 3.7. DBPCFC with carrot juice

Nineteen patients underwent DBPCFC. Thirteen of them reacted to the placebo and were excluded, one patient did not react at any material (placebo, HPT material or fresh frozen carrot juice) and the test was completely negative, three patients had a positive test (reacted to HPT material and untreated fresh frozen carrot juice), and two patients had a negative reaction (reacted only to fresh frozen material) (Figure 5).

## 4. Discussion

In food allergic patients (pollen/food cross-reactive allergy syndrome), the processing of some fruits, such as apple, results in the loss of the eliciting potential of Bet v1 homologues to trigger allergy, which has led to the premise that all Bet v1 homologues are thermolabile and may be susceptible to other procedures. A recent study showed celery root retains its eliciting potential after cooking; also, Bet v1 itself is relatively thermostable. No exact data about the potential of carrot protein unfolding due to the influence of HPT had been published. Our data supports the hypothesis that 16 kD PR10 carrot protein, Dau c1, belongs to a group of highly stable allergens. In addition, the second allergen family responsible for food allergies, called lipid transfer proteins, are probably present in carrot [13]. This protein appears to be highly resistant to processing and this can also explain the failure of HPT to influence the reactivity of carrot juice. The high rate of reactivity to placebo material can be explained by some persistence of active allergens in the twice-boiled carrot juice in the placebo material, or by non-specific subjective reactivity of the patients tested.

## 5. Conclusion

In our study, we did not confirm the influence of HPT on allergenicity of rDau c1 and carrot juice using *in vitro* and *in vivo* tests. These proteins seem to be very resistant to the variety of conditions related to the HPT procedure.

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