VALIDATION OF RECIPES FOR DOUBLE-BLIND PLACEBO-CONTROLLED CHALLENGES WITH MILK, EGG WHITE AND HAZELNUT

Running title: Validation of recipes for DBPCFC

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ABSTRACT

Background: The double-blind, placebo-controlled food challenge (DBPCFC) is considered the definitive diagnostic test for food allergy. Despite this, there is a lack of validated of recipes for masking the foods, they are not standardised and differ among centres. Sensory tests are necessary to validate the recipes used for DBPCFC.

Methods: Three recipes for use in DBPCFC with milk, egg white and hazelnut were developed and the triangle test was used to validate them in a 2-phase study in which 197 volunteers participated. In each phase participants tried 3 samples (2 active-1 placebo or 2 placebo-1 active) and had to identify the odd one. In phase 1 the 3 samples were given simultaneously, whereas in phase 2 the 3 samples of foods that failed validation in phase 1 were given sequentially. A visual analogue scale (VAS) 1 to 10 was used to evaluate how much participants like the recipes.

Results: In phase 1 the egg white recipe was validated (n=89 subjects, 38.9% found the odd sample, p=0.16). Milk and hazelnut recipes were validated in phase 2 (for both foods: n= 30 subjects, 36.7% found the odd sample, p=0.36). Median VAS scores for the 3 recipes ranged from 6.6 to 9.7.

Conclusions: We have validated through sensorial testing milk, egg white and hazelnut recipes for use in DBPCFC. The validated recipes are easy to perform in a clinical setting, provide the equivalent of one serving dose, and were liked by the majority of participants.

Key words: Double-blind placebo-controlled food challenge; egg white; food allergy; hazelnut; milk; recipe; sensory test; triangle test; validation

RESUMEN

Introducción: La provocación oral doble ciego controlada con placebo (PODCCP) es la prueba diagnóstica definitiva en alergia a alimentos. A pesar de ello hay pocas recetas validadas, que además no están estandarizadas y difieren entre centros. Para poder validar recetas para PODCCP es necesario utilizar pruebas sensoriales como el test del triángulo.

Métodos: Se han desarrollado tres recetas para PODCCP con leche, clara de huevo y avellana, que se han validado mediante el test del triángulo en un estudio en dos fases con 197 voluntarios. En cada fase los participantes probaban 3 muestras (2 activo-1 placebo o 2 placebo-1 activo) y debían identificar la diferente. En la fase 1 las 3 muestras se presentaban simultáneamente, mientras que en la fase 2 las muestras de las recetas no validadas en la fase 1 eran presentadas secuencialmente a los voluntarios. Los participantes evaluaron de 1 a 10 su apreciación de las recetas en una escala visual analógica (EVA).

Resultados: En la fase 1 la receta de clara de huevo fue validada (n=90 sujetos, 38.9% identificaron la muestra diferente, p=0,16). Las recetas de leche y avellana fueron validadas en la fase 2 (cada receta fue probada por 30 sujetos, y en cada una el 36,7% identificaron la muestra diferente, p=0,36). La mediana de la puntuación en la EVA de las 3 recetas osciló entre 6,6 y 9,7.

Conclusiones: Hemos validado mediante pruebas sensoriales recetas para PODCCP con leche, clara de huevo y avellana. Las recetas validadas son fáciles de realizar en el entorno asistencial, proporcionan una cantidad equivalente a una ración, y fueron apreciadas por la mayoría de los participantes.

Palabras clave: alergia a alimentos; avellana; clara de huevo; leche; provocación oral doble ciego controlada con placebo; prueba sensorial; receta; test del triángulo; validación.

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INTRODUCTION

The oral challenge is the only test that confirms the diagnosis of food allergy. Its aim is to confirm or exclude the patient's reactivity to a food. There are three types of oral food challenges: open challenge, single-blind placebo controlled and double-blind placebo controlled (DBPCFC). In all of them, increasing doses of the food under investigation are given progressively at time intervals. A blinded oral challenge consists on the administration of the food masked in order to hide its consistency, odour, colour and flavour. When the challenge is placebo-controlled, some doses contain only food and other placebo, and their administration is determined randomly in two separate days. Fresh food is usually used, hiding its features in carrier liquids (juices, smoothies) or semisolids. They are very useful to assess symptoms, especially chronic or subjective ones, since the patient does not know when s/he is receiving active or placebo. The DBPCFC is considered the "gold standard" in diagnosing food adverse reactions [1, 2, 3] and it is the only accepted test in research. Despite this, it is a non-standardised test, and each clinical group develops its own masking recipes and dosing procedures or protocols. To perform a DBPCFC, a true blinding of the food is necessary. Sensory testing for difference has been used for validating food recipes for DBPCFCs [4-11]. Discrimination tests are some of the most common methods employed in sensory science for application in market research and food industry. They are used to discriminate if a difference (or similarity) exists between two or more samples. The test most frequently used is the triangle test, whose objective is to establish if a difference exists between two samples (i.e. placebo or active food), no matter which attribute differs between samples [12-14].

The Committee of Food Allergy of the Spanish Society of Allergy and Clinical Immunology (SEAIC) set a target of developing recipes for performing blinded oral challenges with the foods most often implicated in allergic reactions. The recipes had to be easy to perform to facilitate its implementation in the clinical setting, applicable to children and adults, and able to deliver a full serving dose. Furthermore, they had to be validated by sensory methods. In this manuscript we present the validation by applying the triangle test of recipes for masking milk, egg white and hazelnut for DBPCFC.

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METHODS

Recipes

The foods masked were milk, egg white and hazelnut. Ingredients used to prepare the recipes are summarized in Table 1. The same ingredients and procedures were used to prepare the active and placebo recipes. Additional requirements were an acceptable taste, enough challenge dose (total amount of allergenic food equivalent to one serving dose) in a suitable volume for children and adults, and a good correlation between sensory properties of placebo and active test.

Study design

The validation of the recipes was performed by sensory testing for difference using the triangle test. The volunteers tasted three samples of each food, distributed between active (A) and placebo (P) ones, in a randomized way. Each sample was labelled with a three-digit code. Six possible combinations of A and P (AAP, APA, PAA, PPA, PAP, APP) were offered with equal frequency in random order to the subjects. [They were told that two samples were similar and one was different, were asked to assess the samples in the order provided, and were not allowed to retest the samples [13]. Crackers and water were used as cleaners after each sample. The participants were told to fill out a questionnaire where they were required to identify the odd one (they were forced to choose one option even if they could not find differences among them). In addition, the participants had to select the characteristics that made the sample different, in terms of colour, taste, texture and smell. Finally, they also evaluated in a visual analogue scale (VAS) from 0 to 10 how much they like the recipe.

The study was performed in 2 phases. In phase 1 participants tested two different foods and received the 3 samples of each tested food simultaneously (all together in a tray). In phase 2 participants tested only one food and received the 3 samples sequentially (one after the other) which is another way of applying the triangle test [12-14]. Phase 2 was planned only for those foods that (if ever) failed validation in phase 1. These two variations of the test are methodologically sound and can be used indifferently in the validation of recipes [12-14].

Subjects

Within the framework of the 2010 annual Congress of the SEAIC the phase 1 of the study was performed. Individuals attending this meeting were invited to participate in the sensorial evaluation of these recipes in a controlled environment. All of them had received information in the congress bag and approached voluntarily the area were the sensory testing was carried out.

Inclusion criteria were: adult (≥ 18 years); non-smoker; with no food allergy; with no active respiratory disease, rhinitis, anosmia or impaired olfaction; with no active respiratory allergy; with no oropharyngeal disease; fasting in the previous two hours. Subjects with lactose intolerance and celiac disease were excluded.

Phase 2 of the study was performed in the Allergy Units of Hospital Universitario de Fuenlabrada and Hospital Universitario Fundación Alcorcon, applying the same inclusion and exclusion criteria.

Statistics

For phase 1 a probability of 33.3% (p=1/3, the probability of identifying the odd sample by chance) with a 10% accuracy and a 95% confidence, a sample size of 85 subjects was estimated. For phase 2, with the same conditions, a sample size of 30 subjects for each food was estimated (60 subjects in total).

In phase 1 each participant was randomly assigned to a pair of foods (egg-milk, egg-hazelnut, milk-egg, milk-hazelnut, hazelnut-egg, hazelnut-milk) and to one of the 6 possible combinations of A and P (AAP, APA, PAA, PPA, PAP, APP) using a uniform random distribution. In phase 2 subjects tasted only one food with the same 6 possible combinations of A and P using also a uniform random distribution.

For each food the proportion of subjects who identified the odd sample was calculated, as well as the right-tail probability of observing k correct answers (or more) in a binomial distribution with parameters n=number of participants who tasted each food, and p=1/3 probability of getting it right by chance. The right-tail probability is chosen because we were interested in detecting high frequencies of correct answers. In case A and P were similar (food well masked) the right-tail probability p should not be significantly ($p \geq 0.05$) different from p=1/3. In case A and P are different (food not adequately masked), the right-tail probability p should be significantly ($p < 0.05$) different.

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The percentage of subjects who found differences in colour, taste, texture and smell were compared between those who identified the odd sample and those who did not by a chi square test. A p value <0.05 was considered significant.

RESULTS

A total of 137 volunteers (conference attendants) participated in the phase 1 evaluation of the recipes. Each subject was given randomly two of the three foods to taste. Hazelnut and milk were evaluated by 92 individuals, and egg white by 90 subjects. The mean age of the group was 42.6 years (SD: 9.5), with a gender distribution of 86 females (62.8%) and 51 males (37.2%). Eighty-one participants (59.1%) were medical doctors (allergists or residents in Allergy), 26 (19.0%) were allergy nurses and 30 (21.9%) had other professions. Analyzing how many people found out the odd sample, 56 out of 92 (60.9%) individuals identified the different one in the milk recipe (p<0.001), 35 out of 09 (38.9%) in the egg white recipe (p=0.16) and 41 of 92 (44.6%) in the hazelnut recipe (p=0.02). Table 2 depicts the differences between samples in terms of colour, taste, texture and smell, reported by the participants who found out the different sample and those who did not.

According to profession and sex, no significant differences in the rate of correct answers were observed for any of the three recipes. Among those who identified the odd sample, a similar rate of males and females observed differences in colour, smell, taste and texture between the samples. From all who selected the option "all the samples look the same" (42 subjects) but were forced to choose one as the odd, 21.4% chose the first sample, 38.1% the second one and 40.5% the third one (p=0.131). In the VAS evaluation of how much the participants liked each recipe [ranging from 0 ("I don't like it") to 10 ("I like it very much")], a median of 6.6 [IQR: 4.4-7.7] was found for the milk recipe, 6.6 [IQR: 4.9-7.6] for the egg white recipe, and 7.3 [IQR: 5.6-8.4] for the hazelnut recipe.

Phase 2 evaluation was carried out two months later for milk and hazelnut, since 60.9% and 44.6% (p<0.05) of the participants in phase 1 identified the different sample for these foods respectively. Phase 2 was performed at the Allergy Unit of Hospital Universitario de Fuenlabrada where the milk recipe was tested, and at the Allergy Unit of Hospital Universitario Fundación Alcorcón, where the hazelnut recipe was evaluated.

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A total of 30 volunteers (doctors, nurses and other hospital staff) in each center, participated in the evaluation of the recipes (60 subjects in total). Each subject evaluated only one food. The mean age of the group was 42.6 years (SD: 8.5), with a gender distribution of 43 females (71.7%) and 17 males (28.3%). Nineteen participants (31.7%) were medical doctors, 15 (25.0%) were nurses and 26 (43.3%) had other professions.

Analyzing how many people found out the odd sample, 11 out of 30 (36.7%) individuals identified the different one in the milk recipe (p=0.36), and 11 of 30 (36.7%) in the hazelnut recipe (p=0.36). No differences between samples, in terms of colour, taste, texture and smell were found between participants who found out the different sample and those who did not (Table 3). According to profession and sex, no significant differences in the rate of correct answers were observed for any of the two recipes on the VAS evaluation of how much the participants liked each recipe [ranging from 0 ("I don't like it") to 10 ("I like it very much")], a median of 7.2 [IQR: 6.0-8.0] was found for the milk recipe, and 9.7 [IQR: 7.8-10] for the hazelnut recipe.

DISCUSSION

In this study, we have validated some easy to perform recipes to be used in DBPCFC with milk, egg white and hazelnut, foods commonly involved in allergic reactions.

Following a strict and rigorous methodology, the validation of recipes to be used in blinded challenges should be done by a panel of professional and well-selected panelists [12-14]. However, this is a very expensive and time consuming procedure that requires special facilities, and is only affordable to few groups [4,8,9]. In this study we have included 197 volunteers related to the Allergy field, 141 of whom (71.6%) were allergists, residents in Allergy or allergy nurses who understood the aim and relevance of the study. With the evaluation of active and placebo foods by these volunteers separated by a few minutes we consider it is easier to detect differences between samples than in routine clinical practice, where active and placebo foods are given on two different days. So, although not done by professional panelists, which is a limitation of the study, it could be an appropriate approach for the validation of our recipes.

When masking a food for DBPCFCs there are two fundamental requirements. The first one is that the allergenic food must be properly dressed so as not to distinguish between active and placebo, thus minimizing the possible psychological effects. The objective of

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the triangle test for validation of recipes for DBPCFC is to determine if two samples (active and placebo) are similar enough to be used exchanged. As the volunteers were testing three samples of each recipe, the probability of hitting by chance is 1/3. A success rate greater than 33% means that probably one or more samples are different. When comparing the rate of people observing differences or not between the samples, a statistically non-significant result means that the observed differences may be attributed to chance, and implies that both samples (active/placebo) are sufficiently similar.

The second fundamental requirement when masking a food for DBPCFCs is that a sufficient amount of the allergenic food in a total volume appropriate for the patient must be masked. The recipes tested in this study contained the equivalent of a food serving of the allergenic food. Additionally, the recipes are easy to prepare and use easily available ingredients. All this facilitates the implementation of these recipes in daily clinical practice.

In the phase 1 evaluation, close to 39% of the patients found the odd sample in the egg white recipe ($p=0.16$). This figure is close to the percentage of hits that can be attributed to chance (33.3%), so we could conclude that there were no differences between samples in the egg white recipe. Furthermore, participants did not find differences in colour, taste, smell, or texture of the samples (Table 2).

Cow's milk and hazelnut recipes did not reach the validation in the phase 1 study, because 60.9% and 44.6% respectively of the subjects identified the different sample ($p<0.05$), suggesting that differences between active and placebo did exist. Volunteers appreciated differences in the colour of the milk samples, and in the colour and texture of the hazelnut ones (Table 2).

In the phase 1 evaluation the 3 samples were presented together to the participants, and curiously colour was one of the main characteristics that allowed the identification of the odd sample in milk and hazelnut. However, in the clinical practice, patients or their relatives, as well as the blind evaluators, cannot observe (or recall) small differences in colour or in other characteristics, since active and placebo meals are given on different days. For this reason, we conducted a second evaluation of the milk and hazelnut recipes following the same methodology with just one modification: volunteers were offered the different samples to test in a row (one after the other), and they could not compare their colour. This posed a situation closer to that of DBPCFC in clinical practice, where patients receive placebo or active on different days. Furthermore, this is

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also a correct way to apply the triangle test: when samples show slight differences in appearance they may be offered sequentially without invalidating the test [12-14].
In the assessment of overall acceptance of the recipes (how much the participants liked them in a VAS 1 to 10), very good results were obtained. Median VAS scores for the 3 recipes ranged from 6.6 to 9.7.

In summary, we have developed easy to perform recipes to blind full serving doses of egg white, milk and hazelnut for DBPCFC, and we have validated them through the triangle test. These recipes can be used in DBPCFCs in both children and adults. The hazelnut recipe can be adapted to blind other tree nuts or peanut, although sensorial testing would be needed.
Validated challenge recipes facilitate the implementation of DBPCFC in clinical practice ensuring a reliable diagnosis by minimizing the bias generated by the subjectivity of patients and clinical evaluators.

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Table 1. Milk, egg white and hazelnut recipes

Recipes	Active sample ingredients	Placebo sample ingredients
Cow's milk	250 ml of whole UHT cow's milk	250 ml of UHT soy milk
	2 scoops of soy milk powder (9 g)	2 scoops of soy milk powder (9 g)
	2 scoops of milk hydrolysate powder (9 g)	2 scoops of milk hydrolysate powder (9 g)
	4 scoops of soluble cacao (18 g)	4 scoops of soluble cacao (18 g)
	4 scoops of vanilla sugar (18 g)	4 scoops of vanilla sugar (18 g)
	red food coloring	red food coloring
Egg white	34 ml of pasteurized liquid egg white [1]	240 ml of UHT chocolate soy milk
	206 ml of chocolate soymilk	2 scoops of milk hydrolysate (9 g)
	2 scoops of milk hydrolysate (9 g)	2 scoops of vanilla sugar (9 g)
	2 scoops of vanilla sugar (9 g)	
Hazelnut[3]	8 hazelnuts (10 g)	1 can of pickled tuna paté [2]
	1 can of pickled tuna paté [2] (82 g)	5 ml vinegar
	10 ml vinegar	10 g wheat bran
	10 g wheat bran	2 scoops of brown sugar (9 g)
	1 scoop of brown sugar (4.5 g)	

[1] 34 ml of pasteurized liquid egg white equals one whole egg

[2] Ingredients: Tuna, tapioca, potato, milk, sunflower oil, salt and vinegar

[3] The paste is spread over a bread toast and eaten with the toast

Table 2. Differences between samples reported by the volunteers who identified the active food sample (correct answer) or not (uncorrect answer) in Phase 1 evaluation.

	Colour difference			Smell difference			Taste difference			Texture difference		
	Correct answer	**Uncorrect answer**	**p**	**Correct answer**	**Uncorrect answer**	**p**	**Correct answer**	**Uncorrect answer**	**p**	**Correct answer**	**Uncorrect answer**	**p**
Milk recipe	15/56 (26.8%)	3/36 (8.3%)	<0.05	6/56 (10.7%)	6/36 (16.7%	n.s.	48/56 (85.7%)	28/36 (77.8%)	n.s.	7/56 (12.5%)	7/36 (19.4%)	n.s.
Egg white recipe	2/34* (5.8%)	1/55 (1.8%)	n.s.	3/34 (8.8%)	9/55 (16.4%)	n.s.	23/34 (67.6%)	36/55 (65.4%)	n.s.	7/34 (20.5%)	5/55 (9%)	n.s.
Hazelnut recipe	8/41 (19.5%)	2/51 (3.9%)	<0.05	6/41 (14.6%)	4/51 (7.8%)	n.s.	35/41 (85.4%)	39/51 (76.4%)	n.s.	13/41 (31.7%)	7/51 (13.7%)	<0.05

n.s.: non significant ($p \geq 0.05$)

* n= 34 because one of the 35 subjects who identified the odd sample in egg white recipe did not provide answers to the differences in colour, smell, taste and texture.

Table 3. Differences between samples reported by the volunteers who identified the active food sample (correct answer) or not (wrong answer) in Phase 2 evaluation.

	Colour difference			Smell difference			Taste difference			Texture difference		
	Correct answer	**Uncorrect answer**	**p**	**Correct answer**	**Uncorrect answer**	**p**	**Correct answer**	**Uncorrect answer**	**p**	**Correct answer**	**Uncorrect answer**	**p**
Milk recipe	0/11 (0%)	1/19 (5.2%)	n.s.	0/11 (0%)	4/19 (21%)	n.s.	11/11 (100%)	14/19 (73.6%)	n.s.	1/11 (9%)	3/19 (15.7%)	n.s.
Hazelnut recipe	0/11 (0%)	3/19 (15.7%)	n.s.	0/11 (0%)	2/19 (10.5%)	n.s.	11/11 (100%)	15/19 (78.9%)	n.s.	0/11 (0%)	4/19 (21%)	n.s.

n.s.: non significant ($p>0.05$)