Humoral immune response in mice immunized by oral route with phaseolamine extracted from common bean (*Phaseolus vulgaris*)

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ABSTRACT
The prevalence of obesity is increasing in Brazil, generating high costs for the health system. In the search for alternative therapies to control the obesity, several researches seek active compounds in natural products in order to find better pharmacological effects. Inhibitors of α-amylase such as phaseolamine extracted from common beans (*Phaseolus vulgaris*) have shown satisfactory results in weight loss. The present study aimed to verify the possibility of phaseolamine-causing allergic reactions through the humoral response in mice sensitized with phaseolamine. Swiss female mice (*n* = 10) were sensitized orally with 100 µg of lyophilized phaseolamine diluted in water for 10 consecutive days and reinforced on the 21th and 35th days after the beginning of this experiment. The production of immunoglobulins IgG, IgG1 and IgE was measured by ELISA and western blotting. The immunological response was investigated and it was found that phaseolamine induced the synthesis of IgG and did not induce the synthesis of IgG1 and IgE. The present study indicates that the product did not cause allergic reactions.

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1. Introduction

The obesity is a condition characterized by the excessive accumulation and storage of fat in the body that may harm health. Its prevalence increased substantially reaching epidemic proportions in both industrialized societies and in developing countries (Choi et al., 2016; Maffetone, Rivera-Dominguez, & Laursen, 2017; World Health Organization [WHO], 2016a).

According to the WHO (2016b), obesity has more than doubled in the last 30 years. In 2014, there were about 2 billion overweight people in the world of which 600,000 people were classified as obese. A survey conducted by the Brazilian Institute of Geography and Statistics (IBGE) in 2015 indicated that about 80 million people had a body mass index (BMI) equal or greater than 25 indicating overweight or obesity. The highest prevalence
was found in females, corresponding to 58.2% and men representing 55.6% of the reports (Brazilian Association of Endocrinology and Metabolism, 2015).

The data presented by IBGE indicate the need to develop adequate public policies for the prevention and treatment of this disease, in order to offer a better quality of life and reduce the impacts of public spending, where the estimated direct costs in Brazil in 2011 was approximately R$33.5 million (Mazzocante, Moraes, & Campbell, 2012) and spending associated with other diseases related to obesity between 2008 and 2010 was approximately $2.1 billion (Bahia et al., 2012).

Excess weight and obesity, in many cases, are associated with non-transmissible chronic diseases (NTCDs). The WHO defines chronic diseases as cardiovascular disease, cancer, chronic respiratory diseases and diabetes mellitus (WHO, 2005). Studies have shown that the decrease of 5–10% of body weight significantly reduces the risk factors for diabetes and cardiovascular diseases in overweight patients (Alberti, Zimmet, & Shaw, 2006; Dengo et al., 2010; Wing, 2010). The main recommendations for weight reduction are changes in lifestyle, increasing physical activity and healthy dietary modifications. When the patient is unable lose weight by these methods, a pharmaceutical treatment is recommended in view of the high association of this condition with comorbidities and a high mortality rate (Brazilian Association for the Study of Obesity and Metabolic Syndrome, 2010). Thus, the prevention of obesity is essential, but the treatment of obesity is also crucial.

In Brazil, the main drugs registered for the treatment of obesity are amfepramone, fenproporex, mazindol, sibutramine and orlistat. Except for orlistat, all other medications for obesity control by acting on the central nervous system by inhibiting the feeling of hunger. Orlistat acts at the intestinal level by inhibiting the absorption of lipids. The great counterpoint to the use of conventional drug therapy is because of various adverse effects associated with the use of these drugs (Brazilian Association for the Study of Obesity and Metabolic Syndrome, 2010).

In the search for alternative therapies to control obesity and its consequences, there is interest to use alternative medicine. Plants have long been used for the treatment of NTCDs, especially in developing countries where most of the population has few resources. The herbal drugs are prescribed widely because of their effectiveness, fewer adverse effects and relatively low cost (Miaffo, Poualeu, & Kamanyi, 2014).

In this line of research, in 1986, Layer and collaborators had developed studies with a protein extracted from red kidney beans (Phaseolus vulgaris) named as phaseolamine, described and characterized in 1975 (Marshall & Lauda, 1975) as a tetrameric glycoprotein composed of two α-subunits and two β-subunits with molecular weights of 10.8 and 15.6 kDa, respectively, showing an inhibitory effect on pancreatic α-amylase enzyme animals.

In certain cases, some proteins may be identified as allergens, which may cause hypersensitivity reactions that lead to the release of chemical mediators of tissue mast cells and circulating basophiles. In this process, the immune system identifies a protein (or a substance bound to a protein) as detrimental to system integrity. Although this reaction is primarily directed to the antigen triggering the process, the production of high levels of antibodies and cellular systemic reactions can be generated from acute clinical symptoms such as a transient skin reaction even anaphylactic shock life-threatening for the individual (Abbas, Lichtman, & Pillai, 2015; Sicherer & Sampson, 2007).
In view of the benefits of the use of $\alpha$-amylase inhibitors in weight control and aid in the regulation of glucose levels in humans, and to provide further clarification about their action in the body, this study was aimed to analyze the humoral immune response in mice sensitized orally with phaseolamine extracted from common beans ($P. vulgaris$).

2. Methods

2.1. Animals

This study used female Swiss mice ($Mus musculus$), aged approximately seven to eight weeks (weighing 20.0–25.0 g). All the animals were provided by the Federal University of Ceara, Brazil. They were maintained in closed colonies at the Laboratory of Human Biochemistry at the State University of Ceara, Brazil. Water and food were given *ad libitum* to the animals. Euthanasia was performed with xylazine followed by cervical dislocation. The institutional ethics committee on the care and use of animals for experimentation approved the experimental protocols (08476492-9).

2.2. Antigen

The antigen in this study consisted of the lyophilized phaseolamine (LP) extracted from common beans. LP was provided by Biotechnological and Development Park at the Federal University of Ceara, Brazil (PADETEC).

2.3. Immunization of mice

Groups of mice ($n = 10$) were immunized by oral route with doses equivalent of 100 µg of LP diluted in distilled water and offered 10 consecutive days. The animals received boosters 21 and 35 days after the start of the immunization. The same animals before immunization (pre-immune) was used as the control group. The animals were bled by the retro-orbital plexus on days 7, 14, 21, 28, 35 and 42 after beginning of the immunization procedure. The blood samples collected were maintained at a temperature of 37°C for 15 min for clot retraction. The samples were centrifuged 3000×$g$ for 5 min and the supernatant (serum) was placed in Eppendorf tubes*, labeled and stored in a freezer (−4°C) (Guedes & Pereira, 2005).

2.4. Enzyme-linked immunosorbent assay

The method employed for the analysis of the serum of the animals was the enzyme-linked immunosorbent assay (ELISA) indirect according to Clark and Adams (1977), in order to quantify the presence of antibodies. The test was applied to serum for the determination of isotypes of immunoglobulin IgG, IgG1 and IgE. To this end, the plates (U96 – PolySorp-NUNC-IMMUNO PLATE BATCH 016181) were coated with LP (4 µg/well) diluted in phosphate-buffered saline (PBS).

LP was diluted in PBS and it was introduced into each well. After, the plates were incubated overnight at 4°C. On the next day, the plates were washed with PBS–Tween 20 (0.05%) and blocked for 2 h at 37°C with PBS containing 5% milk Molico* (PBS – Molico*). After the
incubation and washing, 100 µl PBS was added into each well. Then 100 µl of serum was diluted in PBS present on the plate serial dilutions (1:10 to 1:1.280). The plates were incubated for 2 h at 37°C. Following another round of washes, the conjugate was added immunoglobulin anti-mouse linked to peroxidase, in a dilution of 1:500 in the case of anti-IgE and 1:1000 for anti-IgG and anti-IgG1 for 2 h at a temperature of 37°C. The reaction was revealed after adding 100 µl solution containing citrate buffer and orthophenlenediamine diluted in distilled water, incubated for 15 min at 37°C. The intensity of the reaction was measured by a spectrophotometer (ELISA micro Labystems Multiskam MS®), suitable for ELISA, which measured the absorbance of the solution in the holes at a wavelength equal to 492 nm (Guedes, 1999).

2.5. Electrophoresis (SDS-PAGE)

LP was submitted to gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (1970) by using a 12% separation gel and a 4% stacking gel. The protein concentration of the sample was 5 µg per lane.

2.6. Western blotting

LP was used in the western blotting test. The antigen was subjected to polyacrylamide gel electrophoresis in 4–12% (SDS-PAGE) as described previously, and transferred to a nitrocellulose membrane, (SIGMA*) with pores of 0.2 µM in an electro vessel transfer semi-dry with a transfer buffer composed of 39 mM glycine, 0.0375% SDS (w/v) and 20% methanol (v/v) in 48 mM Tris buffer pH 7.2, under conditions of constant amperage of 150 mA. After the transfer, the membrane was immersed in a dye solution Ponceau 0.5% (0.5 g colorant Ponceau, 1.0 ml of glacial acetic acid and 100 ml of Milli-Q water qs) for 10 min, followed by washing with distilled water to remove excess, when it was possible to visualize the protein in the membrane. Then the membrane was incubated with buffer PBS-Molico® and kept under refrigeration for 18 h to block the free active sites of the membrane. After blocking, the membrane was divided into longitudinal cuts into three strips. Each membrane strip was incubated with serum containing antibodies of immunized mice with LP at a dilution of 1:100 in PBS–Tween and incubated for 2 h. Then, each membrane strip was subjected to three washes of 15 min in PBS–Tween and transferred to a plate of petri containing secondary antibodies conjugated to peroxidase anti-mouse (Thermo Fisher Scientific*), diluted 1:1000, for 2 h under slow agitation at room temperature to detect the immunoglobulins (anti-IgG, anti-IgG1 and anti-IgE). The membranes were again subjected to four washes of 15 min in PBS–Tween, and placed in a revealing solution with substrate for peroxidase comprising diaminobenzidine (DAB) 0.1 g/ml and nickel chloride (NiCl₂) 0.4 g/ml in 100 mM Tris-HCl, pH 7.2 buffer with the addition of 12.5 µl of hydrogen peroxide (H₂O₂).

2.7. Definition of the cut-off and statistical analysis

In order to establish the cut-off (positivity limit), pre-immune serum was used as a control. The cut-off value was determined by the arithmetic mean of the optical density values of the negative control sera plus three times the standard deviation. Values above the cut-off
indicate the synthesis of the antibodies against the LP proteins. The statistical analysis of the data was achieved by means of Microsoft Office Excel.

3. Results

3.1. Electrophoresis

The electrophoretic profile extract used in SDS-PAGE revealed the migration of 10 protein fractions, 5 were most evident, with molecular masses estimated at approximately 35, 34, 28, 26 and 19 kDa (Figure 1).

3.2. ELISA

The immune responses of mice sensitized by the oral route with 100 µl of LP were evaluated by the ELISA method and western blotting using the serum of animals immunized without adjuvant use, in order to assess the detection of immunoglobulin class IgG, IgG1 and IgE.

The results of the ELISA tests showed that LP induced the synthesis of specific IgG class immunoglobulins (Figure 2(A)). On the other hand, this protein was not able to induce specific IgE and IgG1 synthesis from protein fraction present in LP (Figure 2(B, C)).

3.3. Western blotting

In the Western blotting test, using anti-IgG as the secondary antibody, anti-IgG1 and antimeouse IgE (bound to peroxidase) were verified that the LP induced the formation of IgG immunoglobulin which specifically recognized some protein fractions of the LP. Moreover, there was a strong reaction to the protein fractions ranging between 30 and 45 kDa, suggesting an increased immunogenicity of these fractions to such immunoglobulins. In addition, specific bands were not detected in the western blotting test for IgE and IgG1 immunoglobulins (Figure 3).

Figure 1. Electrophoretic profile of phaseolamine in SDS-PAGE 12%. Column 1: molecular weight marker and Column 2: phaseolamine.
Figure 2. Production of immunoglobulins in mice sensitized orally with phaseolamine extracted from common beans during the experiment. In A (IgG); B (IgE) and C (IgG1).

Figure 3. Western blotting for detection of IgG, IgE and IgG1 produced in response to sensitization with lyophilized phaseolamine extracted from common beans. Column 1: molecular weight marker; Column 2: phaseolamine; Column 3: confirmation of the presence of IgG immunoglobulins; Column 4: absence of IgG1 immunoglobulin; Column 5: absence of IgE immunoglobulin and Column 6: negative control (pre-immune).
4. Discussion

The main allergenic foods identified are proteic structure. The results found in this research are relevant because they show which immunoglobulins are produced through the oral use of LP from common bean for therapeutic purposes.

The electrophoretic profile of the sample used in the present study corroborates with the results reported by Wang, Chen, Jeng, and Sung (2011) who found a similar profile migration for protein extract obtained from a commercial variety (PI40) of common bean (P. vulgaris L.). In that case, the authors verified that the α-amylase inhibitor found in the bean sample was a commercial tetrameric glycoprotein (α2β2) molecular weight in the range 36–56 kDa, which during electrophoresis dissociated into two peptides of molecular weight in the range of 30–35 kDa and two small peptides of molecular weight varying in the range of 14.4–25.5 kDa.

The ELISA test of the sample used in the present study corroborates with the results reported by Santos et al. (2012) who found similar results evaluating the immunological response of mice immunized with a protein present in a functional food, such as phaseolamine. They used the serum samples obtained from the mice sensitized, it was observed that the protein was able to induce the synthesis of IgG immunoglobulins by subcutaneous and oral routes and it did not induce the synthesis of IgE and IgG1 immunoglobulins.

The results present in this study are in contrast to the data found by Weiner et al. (1994) who used isosoy proteins and the ELISA test showed a primary and secondary responses in the production of IgE and IgG1 immunoglobulins by oral immunization.

Analyzing IgE and IgG1, positive results were observed in the western blotting test showing that LP used was not able to generate the production of immunoglobulins. This result is relevant because allergic reactions from foods are characterized by specific IgE and IgG1 levels in serum and by activating gut mast cells (Guedes & Pereira, 2005; Oliveira et al., 2013). The western blotting test of the sample used in the present study is not similar to the results reported by Sandeep, Alok, Amita, Anurag, and Bhushan (2011) who evaluated the allergic responses of BALB/c mice immunized with red kidney beans (P. vulgaris cv. chitra) producing high production of IgE compared to the control group; therefore, this protein can cause allergic reactions with unsafe use.

According to Santos et al. (2012) in the western blotting test, using the polyclonal antibodies obtained by the subcutaneous route, it was demonstrated that the isosoy* proteins, present in a functional food similar to phaseolamine, only induced the formation of type IgG immunoglobulins and IgG1 and IgE were not detected, corroborating with the results found in our study, suggesting that the product is immunologically safe for use. Although positive results were found in this research, there are still few studies related to phaseolamine and its use as a pharmacological tool.

The findings of this study are encouraging, since many authors report the effectiveness of the use of α-amylase inhibitors present in many types of beans as a tool in weight management, with significant reduction in BMI; thus, forming more possibility of treatment of overweight and obesity since LP does not present allergenic properties (Celleno, Tolaini, D’amore, Perricone, & Preuss, 2007; Helmstadter, 2010; Koike, Koizumi, Tang, Takahara, & Saitou, 2005).

In addition to this favorable results several authors report on studies conducted in animal models, positive effects of α-amylase inhibitors found in beans in diabetes
management. This fact is observed both by reducing the weight and postprandial glucose levels (Gouveia et al., 2014). Other workers demonstrated in an animal model that using aqueous extract of *P. vulgaris* administered orally (200 mg/kg) for over 45 days showed improvement of lipid profile and decreased blood oxidative markers (Venkateswaran, Pari, & Saravanan, 2002).

5. Conclusion

In accordance with the above findings, there are some proteins immunogenic in LP which induced the synthesis of IgG and did not induce the production of IgE and IgG1. However, as IgG1 and IgE were not detected, which are classes of immunoglobulins that indicate risk of allergic reactions, it can be suggested that the product did not cause allergic reactions in sensitized mice.

Although the results of this study demonstrate a level of safety in using the LP, much needs to be explored in order to verify issues related to effectiveness, cytotoxicity and interaction with drugs, requiring further studies in this area.

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