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Blocking IgE with L-glutamic acid analogs as an alternative approach to allergy treatment

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ABSTRACT: IgE-mediated allergic diseases have increased in the last decades. The most prevalent allergens from these seeds are Ric c1 and Ric c3, isoforms of 2S albumin. These allergenic proteins cross-react with allergens from peanut, shrimp, fish, corn, gramineous, house dust, and tobacco. The usual allergy treatment employs antihistaminic, immunotherapies and, omalizumab (Xolair)-based anti-IgE therapy. However, antihistaminics relieve symptoms, and the high cost of omalizumab limits its use for continuous treatment. We propose an alternative immunotherapeutic approach, denoted "IgE-blockage" by L-glutamic acid or modified-glutamic acid. Six compounds, D-glutamic acid, L-glutamic acid, N-methyl-L-glutamic acid, N-acetyl-L-glutamic acid, N-(4-nitrobenzoyl)-L-glutamic acid, and N-carbamyl-L-glutamic, were tested as a blocker. To evaluate motor coordination and the sedative/hypnotic activity of L-glutamic acid, a rota-rod test and a thiopental sodium-induced sleeping test were used. The compounds, L-glutamic acid and L-nitrobenzoyl glutamic acid, were the most active compounds to block the interaction of castor allergens with IgE. These compounds also prevent cross-responses with allergens from food sources and inhalants that cross-react with them. In the sleeping test, the groups that received L-glutamic acid at doses of 10 and 30 mg/kg had a sleeping time similar to the vehicle control group. No changes in the animals' behavior were observed and there was no difference between the L-glutamic acid groups and the vehicle control groups in the rota-rod test. L-glutamic acid and L-nitrobenzoyl glutamic acid can used as IgE blocker to prevent allergic diseases.

Keywords: Allergy treatment; 2S Albumin; IgE blocker.

1. INTRODUCTION

Allergy mediated by immunoglobulin E (IgE), including asthma and severe food allergy, has significantly increased in many countries in recent decades and has become a major worldwide public health issue [1,2]. IgE-mediated hypersensitivity reactions are characterized by mast cells' activation, tissue infiltration, and activation of inflammatory cells [3]. IgE antibodies generated, in response to a specific allergen,

interact with this allergen triggering a series of intracellular reactions leading to the release of histamine and other inflammatory mediators [3-6]. The histamine release causes smooth muscle contraction of the gastrointestinal tract and respiratory tract, nerve stimulation, and vasodilatation [5,7]. Specific IgE generated in response to a determined allergen may cross-react with proteins sharing structural homology in the amino acid sequence with the original immunogen [8,9]. The reserve proteins belonging to 2S albumin class are the primary allergens present in the seeds. They can cause cross-allergic reactions in previously sensitized individuals [10]. Since identifying the critical role mediator of the IgE in allergic diseases, controlling the IgE responses has become one of the main therapeutic objectives [11]. Strategies for the treatment of allergy widely studied, such as pharmacotherapy, immunotherapy, and anti-IgE therapy, have gained special attention after understanding the clinical importance of the IgE antibody's specific activity [12]. The antihistaminic drug is the most common treatment for allergy; it binds to histamine receptors and inhibits their effects, but it does not address the leading cause of allergic responses but only alleviates the symptoms [7]. In order to develop better therapeutic approaches for allergic disorders, new and safe therapies for allergy treatment are needed [13,14]. Omalizumab (Xolair)-based anti-IgE therapy binds free but not FcεRI-bound IgE, mainly used for treating severe allergic asthma. Besides, the requirement for many doses and the medicine's high cost limits the extensive use of this treatment [6]. We propose an alternative immunotherapeutic approach, denoted "IgE-blockage" [15]. This proposal is based on the interaction of free amino acid with Fab-IgE, blocking the allergen recognized by IgE. Studies developed by Deus de Oliveira et al. [16] identified amino acids, such as glutamic acid and aspartic acid, present in the IgE binding epitopes from major allergens from castor seeds, Ric c1 and Ric c3 [16]. Free Glutamic acid (Glu), when incubated with serum anti-castor allergens works as IgE binding blocker; it prevents mast cell degranulation and subsequent histamine release [15,16] of the activated mast cells. Here, we evaluated, by in vivo assays, the use of L-Glu and analog amino acids as IgE-blocker. In addition to the possible effect on the inhibition of allergy, since they are derivatives of glutamate, the effect of treatment with these compounds on neurological functions will also evaluate through behavioral tests.

2. Materials and Methods

2.1. Plant material and 2S albumin purification

Castor seeds (*R. communis* L., cultivar IAC-226) were obtained from the Instituto Agronômico of Campinas, São Paulo, Brazil. The 2S albumin fractions were isolated and characterized by SDS-PAGE and immunoblotting experiments, as described previously by Deus-de-Oliveira et al. [16].

2.2. Drugs and chemicals

The drugs and chemicals used for the experiments were diazepam 5 mg/Kg (Hipolabor Pharmaceutical, Brazil), thiopental sodium 40 mg/kg (Cristália, SP, Brazil). The amino acids D-glutamic acid, L-glutamic acid, N-methyl-L-glutamic acid, N-acetyl-L-glutamic acid, N-(4-nitrobenzoyl)-L-glutamic acid, and N-carbamyl-L-glutamic acid were from Sigma.

2.3. Experimental animals

Eight-week-old female Balb/c mice were purchased from the animal facility of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF). All experimental procedures complied with the Ethical Commission in Animal Experimentation of the UENF (Proc. CEUA-UENF/297), which fulfills the principles of ethics for animal research adopted by the National Council for Control of Animal Experimentation (CONCEA). Mice were housed in appropriate conventional animal care facilities and handled according to

international guidelines for animal experiments. The animals were divided into control and test groups containing six mice each.

2.4. Mice sensitization

Briefly, BALB/c mice were sensitized three times (at days 0, 7, 14, 21, and 28) intraperitoneally (i.p.) with 2S albumin from castor seeds. Two concentrations of allergenic protein were used, 1 and 10 µg in 200 mL per injection, at one-week intervals. The allergen was emulsified with 5 mg aluminum hydroxide gel (4 mg/mL) (Sigma, São Paulo, SP). After the third immunization (day 21), the animals received a booster with the antigen in the same concentration in the presence of 2,5 mg aluminum hydroxide gel (4 mg/mL), and this procedure was repeated on day 28 without adjuvant only using 2S albumin at concentration 50 µg in 200 mL per injection. The mice were anesthetized using Anestalcon® 5,0 mg/mL (0.5% proximethyl chloridrate) and bled. The bleeding technique was by retro-orbital plexus.

2.5. Serum immunoglobulins detection

Polystyrene 96-well plates (Nunc-Immuno Plate I F) were coated with purified 2S albumin 60 µL/well (1 µg/µL in sodium carbonate-bicarbonate buffer 50 mM pH 9.6) at 4°C overnight. After washing with PBS containing 0.05% Tween 20 (PBS-T), the plates were blocked with PBS-T containing 1% gelatin for one hour at room temperature and washed with PBS-T. Washed with PBS-T and incubated with serum samples diluted in 0.1% PBS-T gelatin at 37°C for one hour. After, the wells were washed with PBS-T and treated with peroxidase-conjugated goat anti-mouse (Southern Biotech) IgE or IgG or IgG1 antibodies (diluted according to manufacturer's instructions) in PBS-T 0.1% gelatin for one hour at 37°C and were washed. An o-phenylenediamine (OPD) peroxidase substrate (Sigma) solution was added, and the color developed by 15 min. The reaction was stopped using sulfuric acid 3M, and colorimetric intensity was measured by absorbance 492 nm using a Microplate Reader (Thermo Plate).

2.6. Cross-reactivity

Airborne allergens (FDA Allergenic (FDA-PRICKIT Lot 04AK00001) or food allergens (FDA-FOOD KIT lot 04AK00004 were used to investigate cross-reactivity between 2S albumin from castor seeds and allergens used for allergy diagnosis. The plates were previously sensitized with 60 µL of solution containing 10 µg/mL of each allergen. Cross-reactivity was determined by ELISA, as previously described [16]. Sensitized plates were incubated with mouse serum anti-2S albumin and peroxidase-conjugated goat anti-mouse (Southern Biotech) IgE antibodies conjugated to enzyme HRP.

2.7. Effect of glutamic acid and analogs as an IgE blocker

The binding of specific IgE to allergens ability of L-glutamic acid and analogs such as D-glutamic acid, N-methyl-L-glutamic acid, N-acetyl-L-glutamic acid, N-(4-nitrobenzoyl)-L-glutamic acid, and N-carbamyl-L-glutamic acid was assessed by ELISA inhibition. These amino acids were prepared in saline solution at concentrations of 0,5 mM. Briefly, the serum pool from animals sensitized against 2S albumin from *R. communis* (1:5 diluted) was pre-incubated for 20 minutes with glutamic acid and their analogs at a concentration of 10 µg/mL. The ELISA assays were performed as previously described. The percentage of IgE binding inhibition achieved by the pre-incubation treatment was calculated as follows: percentage of IgE binding = 100 - (ODI/ODT x 100); ODI represents the absorbance after incubation of glutamic acid-treated human serum, and ODT represents the absorbance of untreated animal serum. Also, the serum pool of specific IgE 2S anti-

albumins was pre-incubated with increasing volumes (10 µL, 15 µL, and 20 µL) of the solution of each amino acid.

2.8. Thiopental sodium-induced sleeping time test

The method employed in this study was described by Sharmen et al. [17]. The animals were randomly divided into five groups consisting of six mice each. The test groups received L-glutamic acid at the doses of 10, 30, and 50 mg/kg (i.p.), while the positive control group was treated with diazepam (5 mg/kg; i.p.) and the negative control group with vehicle (0.9% physiological saline; i.p.). Thirty minutes later, thiopental sodium (40 mg/kg; i.p.) was administered to each mouse to induce sleep. Sleeping time was calculated as the interval between the loss and the recovery of the righting reflex.

2.9. Motor test

The motor coordination effect was assessed using a Rotarod apparatus. Experimental animals were subjected to a pretest after training on the apparatus for two days before the experiment. Only those animals, which demonstrated their ability to remain on the revolving rod, were used. On the day of the experiment, the test groups received L-glutamic acid at 10, 30, and 50 mg/kg (i.p.), whereas the negative control group received vehicle (0.9% physiological saline; i.p.). In the positive control group, animals received diazepam (5 mg/ kg; i.p.). Thirty minutes after administering drugs, each mouse was placed on the rotating rod (rotational speed of 20 RPM) for five minutes (300s). Time spent in the apparatus was observed for 5 min duration (300 s).

2.10. Statistical analysis

Experimental values were expressed as means ±SD. Data were analyzed using one-way analysis of variance, or where applicable, ANOVA followed by post hoc analysis with Duncan comparison test. The *P* values were considered significant if $p < 0.05$ or $p > 0.05$. The analysis was performed using GraphPad 5 software.

3. RESULTS

3.1. Evaluation of immunoglobulin profile (IgE, IgG, and IgG1)

The immunoglobulins profile obtained after 2S albumin (Ric c1 + Ric c3) immunization is presented in Figure 1.

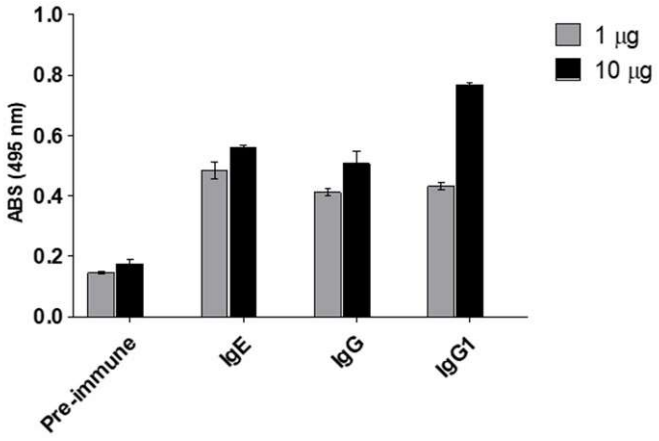

Figure 1. Detection of immunoglobulins in the serum of mice sensitized with 2S albumin, 1 and 10 µg/200 µL. Primary antibody: 1:5 (IgE); 1:500 (IgG and IgG1). Anti-IgE: 1:2000 (IgE); The means for six mice per group are shown.

The presence of IgG1 and IgE characterize the humoral response pattern induced by Th2 helper T lymphocytes. The levels of each immunoglobulin were slightly higher when immunization was performed with 10 µg protein than immunizations with 1 IgE blocking assays using modified glutamic acids.

Figure 2 shows that, among the amino acids evaluated as possible blockers, L-glutamic acids (60% of blockage) and N-(4-nitrobenzoyl)-L-glutamic acid (93% of blockage) are the best compounds for protection of the interaction between IgE and allergenic proteins (Ric c1 + Ric c3). N-carbamyl-L-glutamic acid (42%) and N-acetyl-L-glutamic acid (36%) could be used as IgE blockage; however, N-methyl-L-glutamic acid blocker (E2) (25%) and D-glutamic (14%) presented a low percentage of IgE blockade.

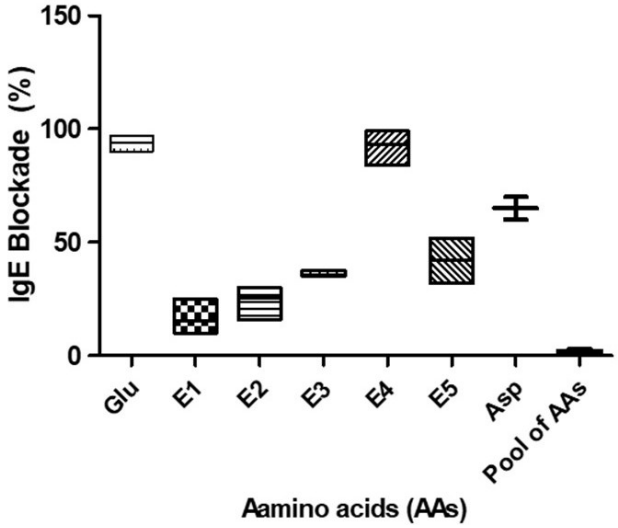

Figure 2. Effects of blocking modified glutamic acids on IgE binding to 2S albumin by ELISA. A serum without treatment was used as control (100% of mast cell degranulation. L-glutamic acid (Glu), D-Glu (E1), N-methyl-L-glutamic acid (E2), N-acetyl-L-glutamic acid (E3), N-(4-nitrobenzoyl)-L-glutamic acid (E4), and N-carbamyl-L-glutamic acid (E5) were each mixed with a diluted pooled plasma at a ratio of 1:10 (v/v), and incubated in a microplate coated with 2S albumin. IgE binding was detected using a Goat anti-mouse IgE HRP conjugate (1:2000) and an OPA substrate. Statistical analysis using one-way ANOVA followed by Tukey's post-test. * P<0,01 e * * P<0,05.

3.2. The dose-response curve of blocking agents

Figure 3 shows the influence of modified glutamic amino acid concentration to block IgE-2S albumin interaction, investigated by ELISA. This result shows each of the modified glutamic acids' blocking action, comparing the relationship between the IgE blockade and the concentration of these amino acids. The best results were observed when L-glutamic acid and N-(4-nitrobenzoyl)-L-glutamic acid (E4) were used. These amino acids promoted ~100% IgE blockade in the more significant tested concentration. D-glutamic acid (E1) and N-carbamyl-L-glutamic acid (E5) blocked ~40%. However, when N-acetyl-L-glutamic acid (E3), and N-methyl-L-glutamic acid (E2), were used, lower levels of blockage (~20%) were observed.

3.3. Glutamic acid protects against cross-reactivity

The pre-incubation of serum with glutamic acid promoted inhibition of IgE reactivity to both airborne and food allergens, indicating that the carboxyl group of these amino acids could be significant in IgE-epitope interactions. Lower blockages were observed for gramineous and peanut (Figure 4).

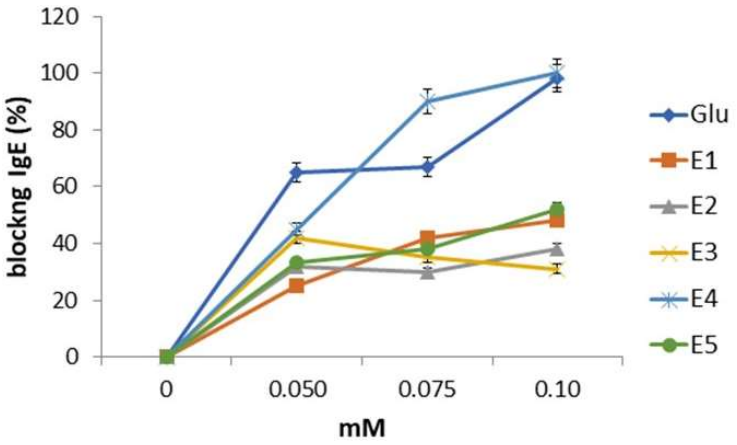

Figure 3. Blocking profile of IgE binding to 2S albumin protein by ELISA. Total binding determined by binding of immune serum anti-2S albumin incubated with Glu, L-glutamic acid; E1, D-glutamic acid; E2: acid-N-methyl-L-glutamic acid; E3 acid - N-acetyl-L-glutamic acid; E4, acid - N-(4-nitrobenzoyl)-L-glutamic acid; E5, N-carbamyl-L-glutamic acid. All amino acid derivatives at a concentration of 0.5 µM diluted 1:5 in immune serum. Microplates were incubating with albumin 2S protein (20 µg). The IgE binding was detected using a Goat anti-mouse IgE HRP conjugate (1: 2000) and a colored substrate. Absorbance at 492 nm. Values represent mean from 3 assays.

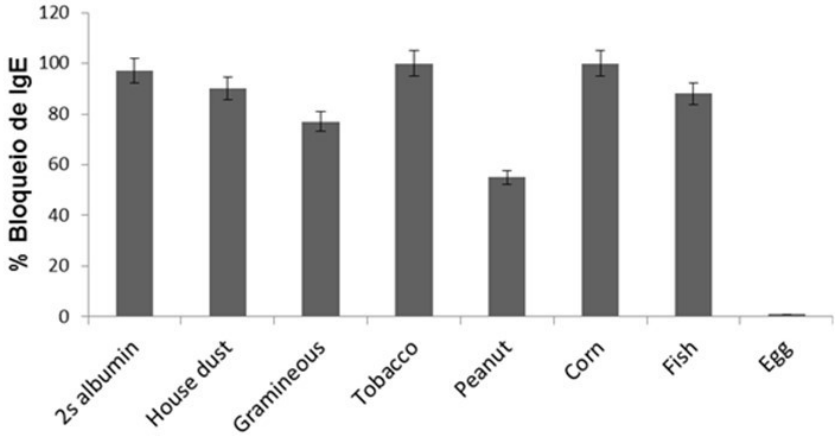

Figure 4. Blocking profile of IgE binding to castor-2S albumin and allergens from house dust, gramineous, tobacco, peanut, corn, and fish quantified by ELISA. Immune 2S anti-albumin serum diluted (1:5) was treated with L-glutamic acid before immune assay. Micro plates were coated with 10 µL of allergens solutions (0.5 µM). The IgE binding was detected using a Goat anti-mouse IgE HRP conjugate (1: 2000) and a colored substrate. The Mean values from 3 assays ±SD are presented.

3.4. Behavioral evaluation of animals after treatment

3.4.1. Motor test

The residence time in the Rota-Rod apparatus after treatment with glutamic acid, at the tested concentration (10, 30, or 50 mg/kg) presented a higher time of permanence in the apparatus than the group of diazepam (Figure 5), similar to the control (animal treated with vehicle).

3.4.2. Sleeping time test

The effect of glutamic acid (10 mg/kg or 30 m/kg) on sleep onset was comparable to that of standard (vehicle). A significant reduction in the time of onset of sleep in a dose-dependent manner was observed with glutamic acid at 50 mg/kg (Figure 6).

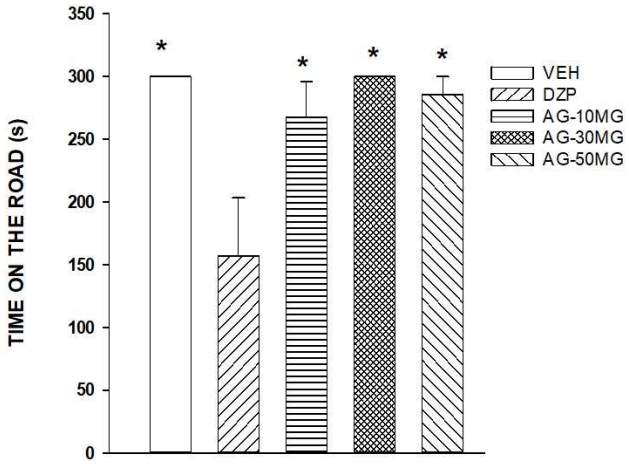

Figure 5. Evaluation of the permanence time of mice treated with glutamic acid in the Rota-Rod apparatus after treatment with glutamic acid 1. Vehicle (saline solution); 2. Diazepam, 3. Glutamic acid (10 mg / kg); 4. glutamic acid (30 mg / kg); 5. glutamic acid (50 mg / kg). Values represent means for six mice per group. In comparison with diazepam, by one way ANOVA * P <0.01 and * * P <0,05.

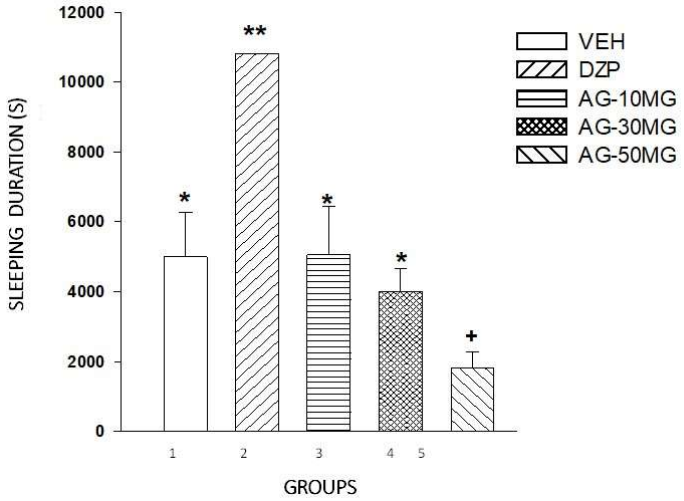

Figure 6. Evaluation of the potentiation effect of sleep time by sodium thiopental (TPT) on the induction of sleep time in mice after treatment with glutamic acid. 1. Vehicle (saline solution); 2. Diazepam, 3. Glutamic acid (10 mg/kg); 4. glutamic acid (30 mg/kg); 5. glutamic acid (50 mg/kg). Statistical analysis was performed using one-way ANOVA followed by Duncan post-test. * P<0,01 e * * P<0,05.

4. DISCUSSION

Understanding the clinical importance of the IgE antibody's specific activity is crucial in developing new therapeutic strategies for treating allergies. This immunoglobulin has a crucial role in the immune response to allergic diseases. Moreover, the frequency of IgE-mediated allergic diseases has significantly increased in the last decades, amplifying the concern on developing preventive and alternative approaches for implementing novel treatment strategies to control allergic disorders [18].

The presented study's objective was to evaluate the use of free glutamic acid and analogs as IgE blocking agents to develop a new allergy treatment strategy based on blocking the interaction of allergens with IgE antibodies. The importance of glutamic acids in forming IgE-binding epitopes was demonstrated for two allergenic isoforms, Ric c 1 and Ric c 3, from castor 2S albumin [15,16]. Castor 2S albumin can cross-react with allergens from shrimp, fish, corn, wheat, soybean, peanut, house dust, tobacco, and airborne fungi. Thus,

exposure to the allergens present in pollen or castor seeds can become an individual sensitized and trigger an allergic response when exposed to other allergenic sources. It was found that the L-Glu could be blocking the cross-reaction between castor 2S albumin and peanut, shrimp, fish, corn, gramineous, house dust, and tobacco (Figure 4).

Glutamic acid is the major excitatory neurotransmitter of the Central Nervous System. Therefore, the excessive stimulation of glutamate receptors could cause neurotoxic effects, also known as neurotoxic excitation [19]. Additionally, because Monosodium glutamate (MSG) is widely available as a chemical in natural foods and as an additive in many prepared foods, the need to evaluate if L-Glu is used for a long time could be a dangerous allergy treatment in humans. Moreover, exposure to MSG in asthmatic patients may potentiate asthma symptoms [20]. Also, some studies associate MSG ingestion may cause headaches [21]. As proposed by Aswar et al. [22], we investigated if the possible therapeutic doses could induce behavioral alterations in motor tests and sleeping time. A decrease in locomotor activity indicates a sedative effect [23] as observed for diazepam use [24]. In all doses tested, 10, 30, and 50 mg/kg of body weight, L-Glu showed performance similar to a positive control (vehicle) in a locomotor score (Figure 5). Doses of 10 mg/kg or 30 mg/kg of this amino acid do not cause sedative activity; however, a decrease in the potentiation of the hypnotic effect induced by the thiopental sodium was observed in doses of 50 mg/kg of L-Glu [22]. Alternative compounds, derived from glutamic acid, were also capable of preventing allergen's binding, castor albumin 2S, as N-(4-nitrobenzoyl) L-glutamic acid that blocked 100% the binding of allergen to IgE (Figure 2). The binding between IgE and L-Glu or derivatives occurred at pH 7.0, a physiological pH, probably due to electrostatic interaction. At this pH, the amino acid Glutamic carries negative charges from the carboxyl groups and thus can form ion pairs with the positively charged amino acid residues on the IgE molecules. Hubbard et al. [25] demonstrated that molecular recognition between antibody and antigen also involves interactions between surfaces continuously in movement, and binding reactions may involve some conformational changes by the antigen or antibody before the formation of the antigen-antibody complex [25].

5. CONCLUSION

We observed that D-glutamic does not block the interaction between allergen and IgE, suggesting a stereo-selectivity for this interaction. Other L-modified glutamic acids such as N-(4-nitrobenzoyl)-L-glutamic acid and N-carbamyl-L-glutamic acid could also be an IgE blockers; However, animal behavior studies are required for the indication of these compounds for begging purposes. Blocking IgE by glutamic acid-free may be an approach for allergy treatment.

Authors' contributions: Study conception and design: OLTM, MP and AG-G; Acquisition of data: DMC-M, GSS; Analysis and interpretation of data: All authors; Drafting of manuscript: MOLT; Critical revision: MOLT, MP and AG-G. All authors discussed the results and contributed to the final manuscript.

Conflict of interest: The authors declare no potential conflict of interest.

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