A diet enriched with cocoa prevents IgE synthesis in a rat allergy model

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Abstract

Previous studies in young rats reported the impact of cocoa intake on healthy immune status and allow suggesting it may have a role in the prevention of some immune-mediated diseases. The aim of this study was to ascertain the effect of a cocoa diet in a model of allergy in young rats. Three-week-old Brown Norway rats were immunized by i.p. injection of ovalbumin (OVA) with alum as adjuvant and *Bordetella pertussis* toxin. During the next 4 weeks rats received either a cocoa diet (containing 0.2% polyphenols, w/w) or a standard diet. Animals fed a standard diet showed high concentrations of anti-OVA IgG1, IgG2a, IgG2b and high anti-OVA IgE titres, which is the antibody involved in allergic response. In contrast, animals fed a cocoa diet showed significantly lower concentrations of anti-OVA IgG1 and IgG2a antibodies. Interestingly, the cocoa diet prevented anti-OVA IgE synthesis and decreased total serum IgE concentration. Analysis of cytokine production in lymph node cells at the end of the study revealed that, in this compartment, the cocoa diet decreased the tumor necrosis factor (TNF) -α and the interleukin (IL) -10 secretion but not IL-4 production. In conclusion, a cocoa-enriched diet in young rats produces an immunomodulatory effect that prevents anti-allergen IgE synthesis, suggesting a potential role for cocoa flavonoids in the prevention or treatment of allergic diseases.

Keywords: cocoa, flavonoid, IgE, allergy, rat, Th2 immune response

Abbreviations:

AU: arbitrary units; **BSA:** albumin from bovine serum; **CBA:** cytometric bead array; **FBS:** fetal bovine serum; **IFN:** interferon; **IL:** interleukin; **MLN:** mesenteric lymph nodes; **OPD:** o-phenylenediamine; **OVA:** ovalbumin; **PBS-Tw**: phosphate-buffered saline containing 0.05% Tween 20; **RT**: room temperature; **tBp:** *Bordetella pertussis* toxin; **TNF:** tumour necrosis factor.

1. Introduction

Allergy is a common form of hypersensitivity with an incidence that has increased dramatically in the developed world during the last fifty years and, at present, it affects more than 25% of the population in these countries [1]. Atopic individuals show a dysregulated immune response to non-pathogenic proteins, called allergens, present either in the environment (i.e., dust, pollen) or in food (i.e., eggs, milk, nuts). This response starts, like any acquired immune response, with antigenic sensitization. Allergen is taken by dendritic cells and presented to specific Th cells by means of major histocompatibility complex (MHC) class II and co-stimulatory molecules (CD80, CD86) thus performing the immunological synapse [2]. Activated allergen-specific Th cells, which usually polarize in the Th1 or Th2 effector populations, differentiate and expand into a Th2 subpopulation. Activated Th2 cells produce cytokines such as interleukin (IL) -4, IL-5, IL-10, and IL-13 which are important in switching antibody production from B cells to predominantly IgE production against the allergen, as well as stimulating mast cells and eosinophils. Specific IgE binds then to IgE receptors (FcεRI) in mast cells; when allergen crosslinks to mast cell-bound IgE, a large number of preformed and newly synthesized mediators and proteases are released and they are responsible for allergic manifestations [3]. Symptoms of allergy affect the skin, respiratory and gastrointestinal systems or even the nervous and cardiovascular systems. The most severe form of allergy is the anaphylaxis which involves two or more organic systems, sometimes producing an anaphylactic shock with hypotension that can result in death.

At present, the majority of pharmacological agents available for allergies (corticosteroids, antihistamines) are aimed at treating different allergic symptoms. Although they are highly effective, numerous factors, such as the dose and the route of administration, account for the efficacy of these treatments, and adverse effects are sometimes associated with a chronic use of these drugs [4]. Therefore, research into new anti-allergic therapy must be performed in order to make it effective from the prevention of allergic sensitization until the alleviation of allergy symptoms. Recent studies have highlighted different nutritional interventions that can impact on allergic diseases [5]. Dietary polyphenols have been found among these emerging nutraceuticals [6].

Flavonoids are products of the secondary metabolism of plants that are regularly ingested in small quantities in many edible plants. Chemically, they have a polyphenolic structure showing antioxidant

activities. These properties have aroused an increasing interest in assessing their possible beneficial role in the prevention of various diseases, as evidenced by the large number of studies focused on the effect of flavonoids on health over the last decade [7,8]. Considering the role of flavonoids in the immune system, most studies are performed *in vitro*, and those reports focusing on acquired immune response suggest that flavonoids produce an inhibitory effect on lymphocyte activation [9-11]. These studies show that flavonoids can affect both early and late phases of the immune response, including Th1 and Th2 effector responses, although a skewed effect of flavonoids favouring or inhibiting Th1 or Th2 responses has not been clearly established. Most preclinical studies on flavonoids in the acquired immune response use an antigen sensitization model followed by a challenge through different routes, searching to provoke a harmful immune response. Different types of flavonoids show the potential to suppress these damaging responses [12-14].

Cocoa is a rich source of polyphenols, particularly flavonoids such as (-)-epicatechin and (+)-catechin as well as procyanidins, the polymers derived from these monomers [15]. Previous studies demonstrated that a cocoa-enriched diet in rats was able to modify the composition and functionality of several lymphoid tissues [16-19], decreasing serum IgG, IgM and IgA concentrations [16]. In addition, a cocoa diet in rats immunized with ovalbumin (OVA) attenuated anti-OVA IgG1 (the main isotype associated with the Th2 immune response in rats), IgG2a, IgG2c and IgM concentrations but led to higher amounts of anti-OVA IgG2b (the isotype linked to the Th1 response) [18]. Similarly, a cocoa diet was able to attenuate the specific antibody response in a rat model of chronic inflammation [19].

In light of this background which demonstrates the modulatory effect of cocoa on the immune system, the aim of this study was to ascertain whether a cocoa-enriched diet could also attenuate the synthesis of IgE in a rat allergy model.

2. Materials and methods

2.1. Chemicals

The Natural Forastero cocoa (Nutrexpa, Barcelona, Spain) used in this study presented a total polyphenol content of about 22 mg/g with 2.2 mg/g (–)-epicatechin, 0.74 mg/g (+)-catechin and 1.7 mg/g procyanidin B2. Ovalbumin (OVA, grade V), albumin from bovine serum (BSA), gelatine, peroxidase-conjugated extravidin, o-phenylenediamine (OPD), and toxin from *Bordetella pertussis* (tBP) came from Sigma-Aldrich (Madrid, Spain). Imject® alum was obtained from Pierce (Rockford, IL, USA). Purified rat IgE, anti-rat IgE monoclonal antibody and biotin-conjugated anti-rat IgG1, IgG2a, IgG2b and IgE monoclonal antibodies, and rat standard, capture bead and detection reagent for interleukin (IL)-2, IL-4, IL-10, interferon (IFN) -γ and tumor necrosis factor (TNF) -α were purchased from BD Biosciences (Heidelberg, Germany). The biotin protein labelling kit used was from Roche Diagnostics (Mannheim, Germany). RPMI 1640 medium, foetal bovine serum (FBS), L-glutamine, streptomicin and penicillin were obtained from PAA (Pasching, Austria). β-mercaptoethanol was provided by Merck (Darmstadt, Germany).

2.2. Diets

The AIN-93G formulation (Harlan) was used as the standard diet. Previous studies have demonstrated that this diet does not contain detectable amounts of polyphenols [20]. A 10% cocoa diet was produced with a modification of the AIN-93G formula, consisting of subtracting the amount of carbohydrates, proteins, lipids and fibre provided by the corresponding percentage of cocoa as previously described [16]. Consequently, the resulting chow was isoenergetic with the standard diet.

2.3. Experimental design and induction of rat allergy model

Brown Norway rats were purchased from Janvier (France) and maintained in the animal facilities of the Faculty of Pharmacy under conditions of controlled temperature and humidity in a 12:12 light:dark cycle. At weaning (day 21 of life) the rats were housed in polycarbonate cages (three to four per cage) and were randomized into two dietary groups: the cocoa group, formed by animals fed chow containing 10% cocoa

starting at weaning and continuing until the end of the study (4 weeks later), and the reference group, formed by animals fed standard chow.

At weaning, the rat allergy model was induced using OVA as allergen and tBp to promote IgE synthesis [21]. Each rat was injected i.p. with 0.5 mL of an emulsion containing 0.5 mg of OVA together with 50 ng of tBp emulsified with Imject® (3:1 OVA:Alum). Blood samples were collected at the beginning of the study (before induction) and at three and four weeks after allergy induction. At week fourth, mesenteric lymph nodes (MLN) were obtained. Lymphocytes from MLN were immediately isolated in sterile conditions by passing the tissue through a mesh (100 μm, BD Biosciences). The cell suspension was left on ice for 10 min to remove tissue debris by sedimentation. Later, cells were centrifuged (600 *g*, 5 min, 4ºC) and resuspended in RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL streptomycin-penicillin, 2 mM L-glutamine, and 0.05 mM 2-mercaptoethanol. MLN cells were cultured at $5x10^6$ cells in 1 mL with OVA (50 μg/mL) for 72 h (37ºC, 5% CO_2). Supernatants were then collected to assess cytokine concentrations.

Experimental procedures were reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Barcelona.

2.4. Quantification of anti-OVA IgG1, IgGa and IgG2b antibodies

Serum anti-OVA IgG1, IgG2a and IgG2b concentrations were quantified by ELISA as previously described [18]. In brief, 96-well polystyrene plates (Nunc MaxiSorp, Wiesbaden, Germany) were coated with OVA (10 μg/mL in PBS) and, after blocking with 0.5% gelatine, appropriate diluted samples (ranging from 1/50 to 1/500000) were added. After washing, biotin-conjugated anti-rat IgG1, IgG2a or IgG2b antibodies and subsequently, peroxidase-conjugated extravidin were added. An OPD-H_2O_2 solution was used for the detection of bound peroxidase. OD was measured on a microtiter plate photometer (Labsystems Multiskan, Helsinki, Finland) at 492 nm. Data were interpolated by means of Ascent v.2.6 software (Thermo Fisher Scientific, S.I.U., Barcelona, Spain). The relative concentration of each anti-OVA isotype was calculated by comparison with a pool of OVA-immunized rat sera to which

arbitrary units (AU) were assigned according to the dilution of the serum samples used for each isotype determination (100000 AU/mL for IgG1 and IgG2a; 10000 AU/mL for IgG2b).

2.5. Quantification of anti-OVA IgE antibodies

To detect OVA-specific IgE, an ELISA based on that described by Knipples *et al.* [22] was applied. In brief, 96-well polystyrene plates (Nunc MaxiSorp) were coated with mouse anti-rat IgE antibody (0.5 mg/mL overnight, 4 ºC) and blocked with 0.5% gelatine in PBS (1 h). After washing, appropriately diluted serum samples (1/2 – 1/100) were added for 3 h at room temperature (RT). Then, an OVA-biotin complex obtained after conjugation of OVA with biotin was incubated (2 h, RT). Thereafter, peroxidase-conjugated extravidin and OPD solution were subsequently added as detailed above. The relative concentration of anti-OVA IgE was calculated by comparison with a pool of OVA-immunized rat sera to which 10 AU/mL were assigned.

2.6. Quantification of total IgE

Serum IgE concentration was quantified by a sandwich ELISA using anti-rat IgE antibody as capture reagent and biotin-conjugated anti-rat IgE antibody as detection reagent. Thereafter, peroxidase-conjugated extravidin and OPD-H_2O_2 were added as detailed above. IgE standard was applied in a concentration range of 0.15- 20 ng/mL and samples were diluted to between 1/20 and 1/200.

2.7. Determination of cytokines by cytometric bead array immunoassay

IL-2, IL-4, IL-10, IFN-γ and TNF-α cytokine concentrations were measured using the BD™ CBA Rat Soluble Protein Flex Set (Madrid, Spain). Briefly, samples or standards were incubated with a mix of specific fluorescent beads for each analyte (1 h, RT, darkness). Later, a mix with the detection antibodies conjugated with phycoerithrin was added and incubated for 2 h under the same conditions. Samples were washed by centrifugation at 200 *g* for 5 min and analyzed using a BD FACSAria™ (BD Biosciences) cytometer and the FCAP Array™ Software (BD Biosciences). The minimum detectable concentrations

were 0.46 pg/mL for IL-2, 3.4 pg/mL for IL-4, 19.4 pg/mL for IL-10, 6.8 pg/mL for IFN-γ and 27.7 pg/mL for TNF-α.

2.8. Statistical Analysis

The software package SPSS 18.0 (PASW Statistics, SPSS, Chicago, IL, USA) was used for statistical analysis. The Levene's and Kolmogorov-Smirnov's tests were applied to assess variance equality and normal distribution, respectively. The parametric t-Student test was used to assess significance. Differences were considered statistically significant for p values <0.05.

3. Results

3.1. Body weight

At the beginning of the study rats weighed 44.75 ± 6.79 g (mean ± S.E.M.). Body weight was monitored throughout the study and its time course was similar between both groups. At the end of the study, body weight was 99.31 ± 24.17 g [64.3-123.8 g] and 107.91± 15.19 g [90.4-135.3 g] in the reference and cocoa groups, respectively.

3.2. Serum anti-OVA IgG1, IgG2a and IgG2b antibodies

The serum concentrations of specific anti-OVA antibodies belonging to the IgG1, IgG2a and IgG2b isotypes are summarized in Figure 1. Three weeks after OVA injection, the reference group showed high concentrations of anti-OVA IgG1 and IgG2a, which increased about 6-fold one week later (Fig. 1A and 1B). The cocoa group showed much lower anti-OVA IgG1 and IgG2a concentrations than the reference group both at three weeks (80- and 30-fold for anti-OVA IgG1 and IgG2a, respectively) and four weeks (500- and 200-fold for anti-OVA IgG1 and IgG2a, respectively) from allergy induction ($p<0.001$). The anti-OVA IgG2b concentrations in both groups were much lower than the other IgG isotypes and there were no significant differences between the diets (Fig. 1C).

3.3. Serum anti-OVA IgE and total IgE antibodies

The serum concentrations of anti-OVA IgE antibodies and total IgE are summarized in Figure 2. The reference animals produced high titres of specific anti-OVA IgE after three weeks of allergy induction, which increased 6-fold one week later (Fig. 2A). The animals fed the cocoa diet did not produce such high anti-OVA IgE titres either at three weeks (40-fold lower) or four weeks (60-fold lower) after allergy induction ($p<0.001$).

The effect of cocoa diet on IgE was also apparent in the total serum IgE concentration (Fig. 2B). Values of IgE before allergy induction were 75.55 ± 15.45 ng/mL and 74.57 ± 16.78 ng/mL (mean ± standard

error) in the reference and cocoa groups, respectively. In the reference animals, serum IgE concentration increased up to about 3300 ng/mL after four weeks of allergy induction (Fig. 2). The cocoa diet prevented total IgE increase at three and four weeks from allergy induction ($p<0.01$).

3.4. Cytokine production

At the end of the study, cells from MLN were incubated to establish a cytokine pattern (Table 1). After OVA stimulation, cells obtained from the cocoa group secreted lower concentrations of TNF-α and IL-10 but higher IL-4 than those from the reference group. No difference was observed in IL-2 values and IFN-γ production was non-detectable ($p<0.05$).

The same cytokines were tested in serum in the third week of the study. Non-detectable values were obtained in these samples from both the reference and cocoa groups.

4. Discussion

This study demonstrates that a diet containing 10% cocoa is able, in young rats, to prevent the synthesis of antibodies involved in allergic reaction. This result suggests that cocoa could be used as a nutraceutical in allergic diseases.

Previous studies demonstrated that cocoa diets attenuated antibody synthesis in Wistar rats after immunization [18, 19]. These results prompted us to ascertain what happens in an allergy model. In this study we induced the IgE synthesis by injecting *Bordetella pertussis* toxin together with ovalbumin (OVA) and alum adjuvant into Brown Norway allergy-prone rats. A similar model in Brown Norway rats was applied by Dong *et al.* [21] and that involved high titres of anti-OVA IgE, 3 weeks after injection. In this study, we demonstrate that young rats that were fed a diet containing 10% cocoa produced drastically less anti-OVA IgE and, moreover, showed lower concentrations of anti-OVA IgG1 and IgG2a, Th2-related antibodies in rats. From these results, lower mast cell sensitization and a reduction of mediators released from these cells after allergen challenge would be expected. Therefore, it possible to suggest that cocoa has the potential to prevent allergic manifestations.

The cocoa compounds responsible for these effects are still to be ascertained, but flavonoids are good candidates. Cocoa has a relatively high content of flavanols such as catechin and epicatechin and unique long procyanidins which, while most of them cannot be directly absorbed, are metabolized by microbiota to phenolic metabolites that are eventually absorbed [23]. Cocoa also contains lower amounts of flavonols such as quercetin, flavones such as luteolin, and flavanones such as naringenin [15,24]. Some preclinical studies show the effect of dietary flavonoids on specific IgE production and allergic manifestations. In a model of food allergy induced in mice with OVA, the intake of apple condensed tannins, which are rich oligomeric flavanols, decreased serum OVA-specific IgE and IgG1 synthesis and inhibited the development of oral OVA sensitization [25]. Single flavonoids such as quercetin and silibinin administered p.o. have also demonstrated attenuating activity on specific IgE synthesis [26,27]. In addition, in an allergy model, mice treated daily p.o. with an extract of *Kalanchoe pinnata* (Crassulaceae), which contains quercetin derivatives among other flavonoids, reduced the production of specific IgE and were protected against fatal allergenic challenge [28].

The cocoa diet partially prevented the increase of total IgE in serum that occurs after allergy induction. These results are in line with some studies using flavonol- or flavone-enriched diets. In first case, a leaf extract rich in the flavonol myricitrin decreased total IgE in an allergy model in transgenic mice [29]. The flavones chrysin and apigenin suppressed total IgE concentrations whereas IgG, IgM and IgA concentrations were not affected [30] but later studies show that apigenin in a mice model of atopic dermatitis decreased serum total IgG1 and IgE concentrations, without modifying significantly specific antibodies**,** and ameliorated the development of skin lesions [12].

The mechanism involved in the regulation of IgE synthesis by cocoa flavonoids is still to be clarified. As IgE antibodies are associated with Th2-effector cells, a lower production of cytokines by these cells was expected. However, results in supernatants of mesenteric lymph node (MLN) cells did not totally confirm this hypothesis. The production of Th2-cytokines IL-4 and IL-10 by MLN cells increased and decreased, respectively, in animals fed cocoa. As IL-4 is the major inducer for class-switching to IgE biosynthesis in B lymphocytes, the decrease in serum IgE in cocoa-fed rats should be associated with lower IL-4 production. It is noteworthy that although some authors reported low levels of IL-4 after flavonoid treatment [30,31] others did not find any difference [12]. On the other hand, previous studies with a similar cocoa diet in rats noted a decrease of IL-4 production in spleen [16,18] but not in MLN cells [18]. Therefore, a systemic compartment, such as the spleen, could perhaps better reflect the Th2-specific effect of cocoa than cells from the intestinal immune system, such as MLN lymphocytes. Moreover, it would be interesting to investigate the production of cytokines earlier in the process, when IgE production is not yet decreased. In any case, the effect of the cocoa diet on IL-10 and TNF-α production, which were the most abundant cytokines among those considered, should be noted. The cocoa diet decreased IL-10 secretion by about 60% and TNF-α production about 85%. Inhibition of IL-10 production can reflect the downregulation of Th2-cell function. With regard to TNF-α, this can be secreted by mast cells and could be implicated in Th2-mediated allergic inflammation promoting the homing of Th2 cells to the site of allergic inflammation [32]. Similar effects on IL-10 and TNFα mediated by flavonoids given in allergic conditions have been reported [28,33,34]. In addition, the inhibitory effect of a cocoa diet on TNF-α synthesis has been described [16,35].

Apart from the influence of flavonoids on allergen-specific IgE and cytokine production, it is well established that some flavonoids are able to act directly on mast cells and inhibit their degranulation. In this regard, Maeda-Yamamoto et al. [36] demonstrated the inhibitory effect of some tea catechins (very similar to those in cocoa) on mast cell activation, and established the intracellular mechanisms involved. More recently, Tamura et al. [37] reported the downregulatory effect of epigallocatechins on the surface expression of FcεRI in mast cells, and Venkatesh et al. [38] demonstrated with *ex vivo* studies the inhibitory effect of extracts rich in catechin on mast cell activation. In this study, *ex vivo* mast cell function was not assessed, but the direct effect of cocoa flavonoids on these cells cannot be disregarded. In any case, it seems that some flavonoids, including those present in cocoa, could be an adjuvant or an alternative to anti-allergic treatment, and this suggestion has driven some human trials. Flavonoids, such as apple polyphenols, Pycnogenol® and isoquercitrin, have been applied in the prevention of seasonal allergy [39-41]. Although the cohort of the studies was small, flavonoid intake over a long period seems to be effective for the relief of allergic symptoms [39-41]. In the case of cocoa, further studies need to be performed to establish its anti-allergic dose in humans, although following the conversion of animal doses into human equivalent doses [42], the 10% cocoa diet was equivalent to 0.908 g cocoa/kg human per day. Moreover, the polyphenolic composition of cocoa also needs to be also considered as this depends on cocoa's origin and method of processing and can impact in its nutraceutical potential [43].

5. Conclusion

In conclusion, a cocoa-enriched diet in young rats produces an immunomodulatory effect that prevents anti-allergen IgE synthesis and other Th2-associated antibodies. This diet also reduces TNF-α secretion which can play a role in allergic inflammation. All these results suggest a potential role for cocoa flavonoids in the prevention or treatment of allergic diseases.

Acknowledgements

This study was supported by a grant from the Spanish Ministry of Science and Innovation (AGL2008-02790). The authors declare they have no conflicting interests. The authors wish to thank Nutrexpa S.L. for providing cocoa. The authors would also like to thank Alba Garcia-Just for her technical support.

5. References

[1] Larché M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. Nat Rev Immunol 2006;6:761-71.

[2] Valenta R, Ball T, Focke M, Linhart B, Mothes N, Niederberger V, Spitzauer S, Swoboda I, Vrtala S, Westritschnig K, Kraft D. Immunotherapy of allergic disease. Adv Immunol. 2004;82:105–53.

[3] Amim K. The role of mast cells in allergic inflammation. Resp Med 2012;106:9-14.

[4] Coureau B, Bussieres JF, Tremblay S. Cushing's syndrome induced by misuse of moderate-to high-potency topical corticosteroids. Ann Pharmacother 2008;42:1903–7.

[5] Kalliomäki M, Antoine JM, Herz U, Rijkers GT, Wells, JM, Mercenier A. Guidance for substantiating the evidence for beneficial effects of probiotics: prevention and management of allergic diseases by probiotic. J Nutr 2010;140:S713–21.

[6] Singh A, Holvoet S, Mercenier A. Dietary polyphenols in the prevention and treatment of allergic diseases. Clin Exp Allergy 2011; 41:1346–59.

[7] Masuoka N, Matsuda M, Kubo I. Characterisation of the antioxidant activity of flavonoids. Food Chem 2012;131:541–5.

[8] Han X, Shen T, Lou H. Dietary polyphenols and their biological significance. Int J Mol Sci 2007;8:950-88.

[9] Miles EA, Zoubouli P, Calder PC. Effects of polyphenols on human Th1 and Th2 cytokine production. Clin Nutr 2005;24:780–4.

[10] Yu ES, Min HJ, An SY, Won HY, Hong JH, Hwang ES. Regulatory mechanisms of IL-2 and IFNγ suppression by quercetin in T helper cells. Biochem Pharmacol 2008;76:70-8.

[11] Ramiro-Puig E, Castell M. Cocoa: antioxidant and immunomodulator. Brit J Nutr 2009;101:931-40.

[12] Yano S, Umeda D, Yamashita S, Yamada K, Tachibana H. Dietary apigenin attenuates the development of atopic dermatitis-like skin lesions in NC/Nga mice. J Nutr Biochem 2009;20:876–81.

[13] Iwamura C, Shinoda K, Yoshimura M, Watanabe Y, Obata A, Nakayama T. Naringenin chalcone suppresses allergic asthma by inhibiting the type-2 function of CD4 T cells. Allergol Int 2010;59:67-73.

[14] Sakai T, Furoku S, Nakamoto M, Shuto E, Hosaka T, Nishioka Y, Sone S. The soy isoflavone equol enhances antigen-specific IgE production in ovalbumin-immunized BALB/c mice. J Nutr Sci Vitaminol 2010;56:72-6.

[15] Borchers A, Keen C, Hannum S, Gershwin, E. Cocoa and chocolate: composition, bioavailability, and health implications. J Medicinal Food 2000;3:77-105.

[16] Ramiro-Puig E, Pérez-Cano FJ, Ramírez-Santana C, Castellote C, Izquierdo-Pulido M, Permanyer J, Franch A, Castell M. Spleen lymphocyte function modulated by a cocoa-enriched diet. Clin Exp Immunol 2007;149:535–42.

[17] Ramiro-Puig E, Pérez-Cano FJ, Ramos-Romero S, Pérez-Berezo T, Castellote C, Permanyer J, Franch A, Izquierdo-Pulido M, Castell M. Intestinal immune system of young rats influenced by cocoa-enriched diet. J Nutr Biochem 2008;19:555–65.

[18] Pérez-Berezo T, Ramiro-Puig E, Pérez-Cano FJ, Castellote C, Permanyer J, Franch A, Castell M. Influence of a cocoa-enriched diet on specific immune response in ovalbumin-sensitized rats. Mol Nutr Food Res 2009;53:389-97.

[19] Ramos-Romero S, Pérez-Cano FJ, Castellote C, Castell M, Franch A. Effect of cocoa-enriched diets on lymphocytes involved in adjuvant arthritis in rats. Br J Nutr 2012; 107:378–87.

[20] Ramiro-Puig E, Urpí-Sardà M, Pérez-Cano FJ, Franch A, Castellote C, Andrés-Lacueva C, Izquierdo-Pulido, M, Castell M. Cocoa-enriched diet enhances antioxidant enzyme activity and modulates lymphocyte composition in thymus from young rats. J Agric Food Chem 2007;55:6431–8.

[21] Dong W, Selgrade MJK, Gilmour MI. Systemic administration of *Bordetella pertussis* enhances pulmonary sensitization to house dust mite in juvenile rats. Toxicol Sci 2003;72:113-21.

[22] Knipples LMJ, Penninks AH, Spanhaak S, Houben GF. Oral sensitization to food proteins: a Brown Norway rat model. Clin Exp Allergy 1998;28:368-75.

[23] Monagas M, Urpi-Sarda M, Sánchez-Patán F, Llorach R, Garrido I, Gómez-Cordovés C, Andres-Lacueva C, Bartolomé B. Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. Food Funct 2010 1:233-53.

[24] Sánchez-Rabaneda F, Jauregui O, Casals I, Andrés-Lacueva C, Izquierdo-Pulido M, Lamuela-Raventós RM. Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa *(Theobroma cacao)*. J Mass Spectrom 2003;38:35-42.

[25] Akiyama H, Sato Y, Watanabe T, Nagaoka MH, Yoshioka Y, Shoji T, Kanda T, Yamada K, Totsuka M, Teshima R, Sawada J, Goda Y, Maitania T. Dietary unripe apple polyphenol inhibits the development of food allergies in murine models. FEBS Lett 2005;579:4485-91.

[26] Shishehbor F, Behroo L, Broujerdnia MG, Namjoyan F, Latifi, SM. Quercetin effectively quells peanut-induced anaphylactic reactions in the peanut sensitized rats. Iran J Allergy Asthma Immunol 2010;9:27-34.

[27] Kuo FH, Jan TR. Silibinin attenuates antigen-specific IgE production through the modulation of Th1/Th2 balance in ovalbumin-sensitized BALB/c mice. Phytomedicine 2009;16:271-6.

[28] Cruz EA, Da-Silva SA, Muzitano MF, Silva PM, Costa SS, Rossi-Bergmann B. Immunomodulatory pretreatment with *Kalanchoe pinnata* extract and its quercitrin flavonoid effectively protects mice against fatal anaphylactic shock. Int Immunopharmacol 2008;8:1616-21.

[29] Shimosaki S, Tsurunaga Y, Itamura H, Nakamura M. Anti-allergic effect of the flavonoid myricitrin from *Myrica rubra* leaf extracts *in vitro* and *in vivo*. Nat Prod Res 2011;25:374-80.

[30] Yano S, Umeda D, Yamashita T, Ninomiya Y, Sumida M, Fujimura Y, Yamada K, Tachibana H. Dietary flavones suppresses IgE and Th2 cytokines in OVA-immunized BALB/c mice. Eur J Nutr 2007;46:257-63.

[31] Hazlewood LC, Wood LG, Hansbro PM, Foster PS. Dietary lycopene supplementation suppresses Th2 responses and lung eosinophilia in a mouse model of allergic asthma. J Nutr Biochem 2011;22:95-100.

[32] Iwasaki M, Saito K, Takemura K, Sekikawa H, Fujii H, Yamada Y, Wada H, Mizuta K, Seishima M, Ito Y. TNF-alpha contributes to the development of allergic rhinitis in mice. J Allergy Clin Immunol 2003;112:134–40.

[33] Jung WK, Choi I, Oh S, Park SG, Seo SK, Lee SW, Lee DS, Heo SJ, Jeon YJ, Je JY, Ahn CB, Kim JS, Oh KS, Kim YM, Moon C, Choi IW Anti-asthmatic effect of marine red alga *(Laurencia undulata)* polyphenolic extracts in a murine model of asthma. Food Chem Toxicol 2009;47:293-7.

[34] Cruz EA, Reuter S, Martin H, Dehzad N, Muzitano MF, Costa SS, Rossi-Bergmann B, Buhl R, Stassen M, Taube C. *Kalanchoe pinnata* inhibits mast cell activation and prevents allergic airway disease. Phytomedicine 2012;19:115-21.

[35] Ramos-Romero S, Pérez-Cano FJ, Pérez-Berezo T, Castellote C, Franch A, Castell M. Effect of a cocoa flavonoid-enriched diet on experimental autoimmune arthritis. Br J Nutr 2012; in press.

[36] Maeda-Yamamoto M, Inagaki N, Kitaura J, Chikumoto T, Kawahara H, Kawakami Y, Sano M, Miyase T, Tachibana H, Nagai H, Kawakami T. O-methylated catechins from tea leaves inhibit multiple protein kinases in mast cells. J Immunol 2004;172:4486-92.

[37] Tamura S, Yoshihira K, Fujiwara K, Murakami N. New inhibitors for expression of IgE receptor on human mast cell. Bioorg Med Chem Lett 2010;20:2299-302.

[38] Venkatesh P, Mukherjee PK, Kumar NS, Bandyopadhyay A, Fukui H, Mizuguchi H, Islam N. Anti-allergic activity of standardized extract of *Albizia lebbeck* with reference to catechin as a phytomarker. Immunopharmacol Immunotoxicol 2010;32:272-6.

[39] Enomoto T, Nagasako-Akazome Y, Kanda T, Ikeda M, Dake Y. Clinical effects of apple polyphenols on persistent allergic rhinitis: A randomized double-blind placebo-controlled parallel arm study. J Investig Allergol Clin Immunol 2006;16:283-9.

[40] Wilson D, Evans M, Guthrie N, Sharma P, Baisley J, Schonlau F, Burki. C. A randomized, double-blind, placebo-controlled exploratory study to evaluate the potential of Pycnogenol® for improving allergic rhinitis symptoms. Phytother Res 2010;24:1115-9.

[41] Hirano T, Kawai M, Arimitsu J, Ogawa M, Kuwahara Y, Hagihara K, Shima Y, Narazaki M, Ogata A, Koyanagi M, Kai T, Shimizu R, Moriwaki M, Suzuki Y, Ogino S, Kawase I, Tanaka T. Preventative effect of a flavonoid, enzymatically modified isoquercitrin on ocular symptoms of Japanese cedar pollinosis. Allergol Int. 2009;58:373-82.

[42] Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J 2008;22:659–61.

[43] Rusconi M, Conti A. *Theobroma cacao* L., the food of the gods: a scientific approach beyond myths and claims. Pharmacol Res 2010;61:5-13.

FIGURE LEGENDS

Figure 1: Concentration of serum anti-OVA IgG1 (**A**), IgG2a (**B**) and IgG2b (**C**) antibodies in reference (white bars) and cocoa groups (striped bars) at three and four weeks from allergy induction. Results are expressed as mean plus standard error of AU/mL logarithm. * $p<0.001$ between reference and cocoa groups.

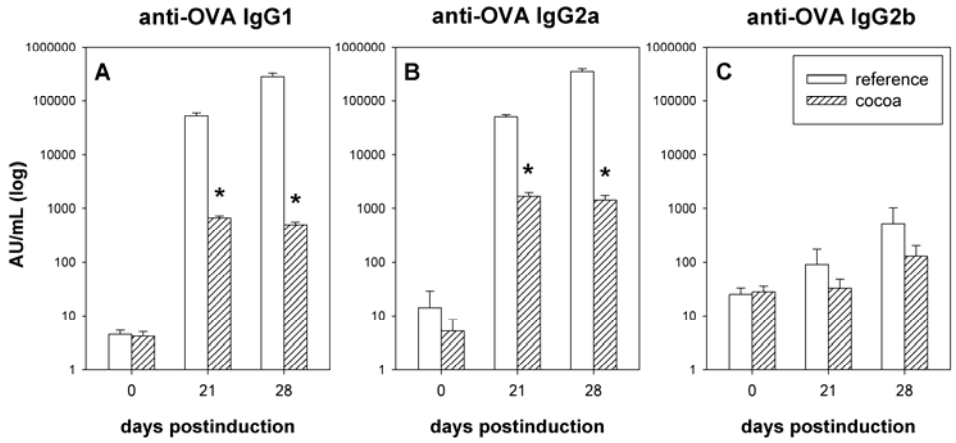

Figure 2: Concentration of serum anti-OVA IgE (**A**) and total IgE (**B**) in reference (white bars) and cocoa groups (striped bars) at three and four weeks from allergy induction. Results are expressed as mean plus standard error of AU/mL logarithm in anti-OVA IgE and as mean plus standard error of total IgE concentration (ng/mL). * $p<0.01$ between reference and cocoa groups.

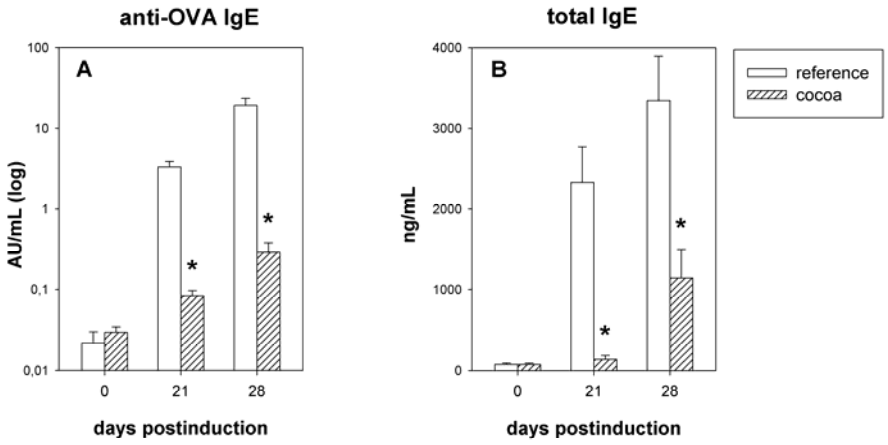

Table 1: Cytokine concentration in MLN cell supernatants after stimulation with OVA. Results are expressed as mean ± standard error of the mean.

groups	IL-2 (pg/mL)	IFN-γ (pg/mL)	TNF-α (pg/mL)	IL-4 (pg/mL)	IL-10 (pg/mL)
reference	6.72 ± 0.61	n.d.[a]	1677.34 ± 234.08	5.74 ± 1.39	226.88 ± 38.61
cocoa	6.72 ± 0.86	n.d.	266.65 ± 45.12	11.69 ± 2.05	94.22 ± 15.11
P value	NS	-	< 0.005	<0.005	<0.05

[a]Non-detectable values.