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Immunomodulatory effect of *Polypodium leucotomos* (Anapsos) in child palatine tonsil model

Carolina Sánchez-Rodríguez[a,*], Keyliz R. Peraza Cruces[b], Laura Rodrigáñez Riesco[c], José Antonio García-Vela[d], Ricardo Sanz-Fernández[e]

[a] European University of Madrid, Calle del Tajo S/N 28670 Villaviciosa de Odón, Madrid, Spain
[b] Department of Biochemistry, University Hospital of Getafe, Carretera de Toledo, km 12, 5, 28905, Getafe, Madrid, Spain
[c] Department of Otolaryngology, University Hospital of La Paz, Paseo de la Castellana, 261, 28046, Madrid, Spain
[d] Department of Hematology, University Hospital of Getafe, Carretera de Toledo, km 12, 5, 28905, Getafe, Madrid, Spain
[e] Department of Otolaryngology, University Hospital of Getafe, Carretera de Toledo, km 12, 5, 28905, Getafe, Madrid, Spain

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ABSTRACT

Background: Recurrent tonsillitis might reduce the immunological capability of fighting against the infection of tonsil tissue. *Polypodium leucotomos* (Anapsos) immunomodulating effect has been subject of research in the last years. The aim of this research is to test the in vitro immunomodulating capacity of Anapsos in a child palatine tonsil explants model.

Methods: Palatine tonsils explants of children undergoing amigdalectomy were stimulated with mononuclear cells obtained from their own blood by density gradient centrifugation. Some were then treated with Anapsos while others rest untreated. Cytokines were measured by ELISA, immune cells activation was measured by flow cytometry and activation of immunoglobulins was appreciated by indirect immunofluorescence in tonsils tissue.

Results: Anapsos activates Natural Killers cells. It increases IL-2 and IFN-γ levels by the activation of Th2 lymphocytes, and IL-10, by the Th1 lymphocytes. Anapsos also increases immunoglobulins IgM, IgD and IgG4 by B-lymphocyte activation in tonsils tissue.

Conclusion: Anapsos has an immunomodulating effect, both in humoral and cellular responses, which might benefit children suffering of recurrent tonsillitis as it could enhance their immune system. This effect might reduce the number of episodes suffered and therefore the number of children undergoing surgery.

1. Introduction

Infectious tonsillitis is common cause for consulting the otorhinolaryngologist in the pediatric population [1,2]. Recurrent tonsillitis episodes may be due to antibiotic resistances or a lack of infection eradication, which might reduce the immunological capability of fighting against the infection of tonsil tissue [1,2]. This process usually ends with the surgical removal of the palatine tonsils of Waldeyer's ring with the consequent decrease of quality and quantity of the local host response to infection as they content a cluster of B and T cells, interdigitating cells (IDC), macrophages, antibody-forming cells (AFC) and follicular dendritic cells (FDC) [2]. One major function of cytokines produced by T cells in lymphoid tissues is to provide necessary signals for activation, proliferation and differentiation of B cells that have been exposed to antigens [3].

The fern *Polypodium leucotomos* grows in the rainforest of Central and South America.

Early studies showed evidence of antitumor effects [4–6]. Subsequent studies demonstrated that it has antioxidant, anti-inflammatory, and photoprotective properties: inhibit oxidative stress, lipid peroxidation, dermal mast cell infiltration, inflammatory cytokines, DNA damage and UV skin damage [7–9]. *In vitro* and in vivo studies performed with a phytoextract called Anapsos, obtained from the rhizomes of this fern, have already shown changes in certain immune cell subsets and cytokines [4,10–12]. It has been the subject of research in the last years, in order to analyse its possible immunomodulating effect [6,13] and its application in some autoimmune diseases [14–16].

Anapsos oral administration increases suppression rate, lymphoblast response to mitogens, serum immunoglobulin levels, and the proportion of $CD8^+$ cells (cytotoxic/suppressor) [17]. Only minor side effects have been reported, such as abdominal pain. Therefore it modulates IL-1β,

* Corresponding author.
E-mail addresses: carolina.sanchez2@universidadeuropea.es (C. Sánchez-Rodríguez), keylizmd@gmail.com (K.R. Peraza Cruces), laurarodriganez@gmail.com (L. Rodrigáñez Riesco), jgarciav@salud.madrid.org (J.A. García-Vela), rsanzf@salud.madrid.org, ricardosanz.orl@gmail.com (R. Sanz-Fernández).

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IL-2 and TNF-α production in the central nervous system of rats [12]. It has been shown to be useful in the prevention of infectious processes, as well as reducing recurring episodes in athletes [18]. Even some authors hypothesize with the participation of anapsos in mechanisms of tissue repair after brain damage [19].

The aim of our paper was to assess the immunomodulator activity of Anapsos® by testing its modulator effect on tonsillar tissue. We evaluate the Anapsos ability to modulate the immunity by cytokines, immunoglobulins determinations and the kind of immune cells activated (Natural Killer cells or T lymphocytes).

2. Methods

2.1. Childs palatine tonsils

In total, 20 children of both sexes (4–12 years old) with a history of recurrent tonsillitis, and undergoing tonsil surgery were included. Forty-eight per cent were operated with partial tonsil resection/tonsillotomy (TT) and 52% with total tonsillectomy (TE), all in day surgery. Tonsils were obtained under general anaesthesia. Patients who did not fulfil criteria, who had surgery contraindications, whose clinical history was incomplete or those who have been diagnosed of a malignant tumour were excluded.

2.2. Isolation of peripheral blood mononuclear cells

Peripheral blood (15 ml) was collected from each of 20 donors into EDTA-containing vacutainer tubes (BD Biosciences). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation using Ficoll-Paque Plus® (GE Healthcare Bio-Science AB). Cells were cultured in RPMI 1640 (Lonza) containing 10% FBS and 1% streptomycin as above The cells were cultured at a 2.5×10^6 cell/ml density.

2.3. Preparation of the extract

Polypodium leucotomos rhizomes were harvested at a 2000 height in the Experimental and Ecological Recuperation Plantations in Guatemala, property of A.S.A.C. Pharmaceutics. After they had been examined, the rhizomes were dehydrated at 50 °C for 48 h. The extract obtained (Anapsos) was filtered freeze-died and granted by A.S.A.C. for the present research job. We used three different concentrations of Anapsos: 150, 300 and 1000 μg/ml.

2.4. Experimental palatine in vitro model design

The fresh surgical specimens of tonsil tissue were divided into blocks of uniform size (small pieces of 2 mm × 2 mm x1mm). Blocks were cultivated on purified Equispon foam (absorbable gelatin foam which gives it a uniform porosity EQUIMEDICAL®) in 6-well plates with medium–air interface following a Grivel [20] protocol slightly modified by us. Tonsils tissues were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Sigma-Aldrich). Some plates were incubated during 24 h while others were incubated for a 48 h period of time (Fig. 1).

We did a prospective study with palatine tonsils tissue cultures from 20 patients who underwent amigdalectomy. Tissue cultures were compared under three different experimental conditions: Control group (tissue with culture medium), PBMC group (tissue culture with patient's PBMC) and Anapsos group (tissue culture with patient's PBMC and Anapsos).

2.5. Cytokine quantification

Cell-free conditioned medium from each treatment group was collected and used to measure the levels of IL-1β, IL-2, IL-6, IL-10, IFN-γ and TNF-α using commercial enzyme-linked immunosorbent assay (ELISA) kits (Gen-Probe Diaclone SAS). After development of the colorimetric reaction, the absorbance at 450 nm was measured using a microplate reader (GENios Plus, TECAN).

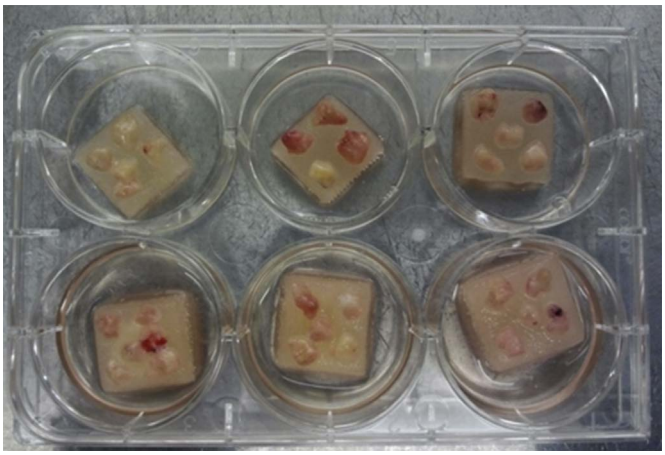

Fig. 1. Tonsils explants cultured on Equispon foam. We observed a 6-well plate with the tonsil tissue under the three experimental conditions: Control group (tissue with culture medium), PBMC group (tissue culture with patient's PBMCs) and Anapsos group (tissue culture with patient's PBMCs and Anapsos).

2.6. Flow cytometry analysis

PBMCs (1×10^5 cells/mL) were evaluated for using flow cytometry with the following panel of fluorochrome-conjugated antibodies: CD3-FITC, CD8-PE, CD45-PerCP and CD4-APC (BD Multitest™, BD Biosciences). Appropriate isotype antibodies were used to control for nonspecific staining. Immunostained cells were analyzed on a FACSCalibur flow cytometer using CellQUEST software (Becton Dickinson).

2.7. Indirect immunofluorescence for IgM, IgD, IgG4

Paraformaldehyde (4%) fixed paraffin-embedded tonsils sections (5 μm thick) were mounted on poly-L-lysine-coated glass slides. After dewaxing, sections were blocked for 1 h at 37 °C and incubated overnight at 4 °C with antibody anti-IgD (Rabbit polyclonal), anti-IgM (Rabbit monoclonal) and anti-Ig4 (Rabbit monoclonal) dilution: 1:1000 (Abcam). Following washes, the sections were incubated with secondary Alexa Fluor 546-conjugated goat anti-rabbit antibody (dilution 1:250; Molecular Probes) for 45 min at 37 °C. After, cells were stained for 5 min with 300 nM of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich. Cells were scored for fluorescence using an Olympus BX51 fluorescence microscope (Olympus, Japan) and the specificity was evaluated by omission of the primary antibody.

2.8. Statistics

All statistical calculations were performed using the SPSS Windows Release 16.0 software package. Data are presented as arithmetic mean values ± SEM. Parametric test (Student's *t*-test) were used to compare the three different groups. Statistical significance was defined as a P-value < 0.05.

2.9. Ethical considerations/human subject protection

This study was approved by the Clinical Research and Ethics Committee of University Hospital of Getafe and all participants gave their written consent.

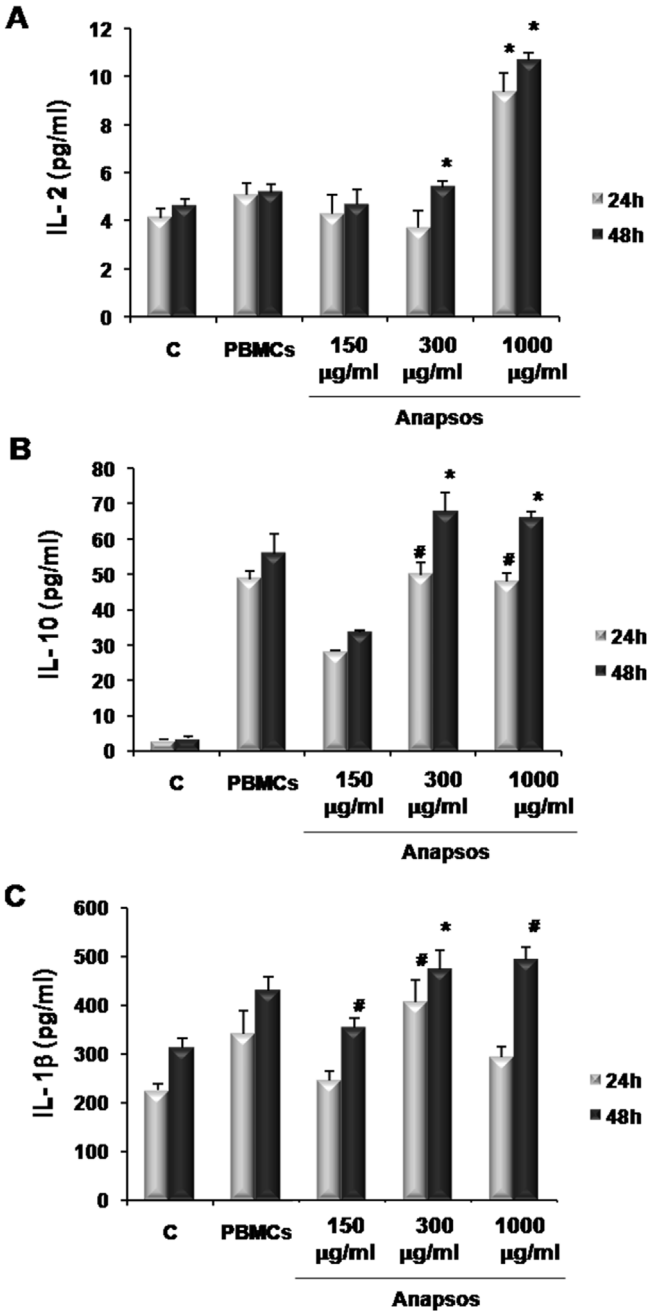

Fig. 2. Anapsos treatment showed an increase of cytokine levels in culture tonsils explants supernatants. Tonsil tissue co-cultured with PBMCs (1×10^6 cells/ml) and PBMCs activated with Anapsos (150, 300 and 1000 µg/ml) for 24 and 48 h. The supernatants were collected and the following cytokines measured by ELISA: IL-2 (A), IL-10 (B) y IL-1β (C). Results are expressed in pg/ml; $^{*}P \leq 0.05$ vs. Control (C) and PBMCs; $^{\#}P \leq 0.05$ vs. Control (C).

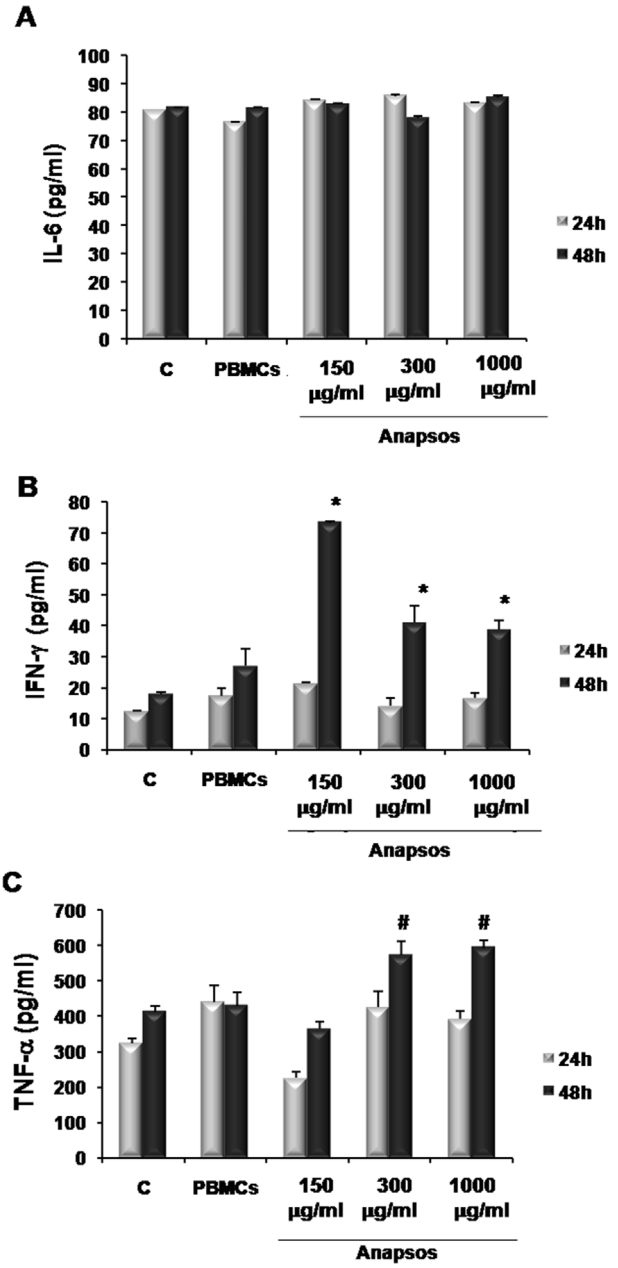

Fig. 3. Anapsos treatment showed an increase of other cytokine levels in culture tonsils explants supernatants. Tonsil tissue co-cultured with PBMCs (1×10^6 cells/ml) and PBMCs activated with Anapsos (150, 300 and 1000 µg/ml) for 24 and 48 h. The supernatants were collected and the following cytokines measured by ELISA: IL-6 (A), INF-γ (B) y TNF-α (C). Results are expressed in pg/ml; $^{*}P \leq 0.05$ vs. Control (C) and PBMCs; $^{\#}P \leq 0.05$ vs. Control (C).

3. Results

3.1. Anapsos treatment showed an increase of cytokine levels in culture tonsils explants supernatant

Anapsos increased IL-2 levels significantly ($P \leq 0.05$) in the treated group in comparison with unstimulated groups (PBMC and control groups) after 24 h at a 1000 µg/ml Anapsos concentration, and after 48 h at a 300 µg/ml solution. With the 150 µg/ml solution we did not find any statistical difference neither after 24 or 48 h culture (Fig. 2A).

In what IL-10 refers, its levels increased significantly ($P \leq 0.05$) in Anapsos group at a 300 µg/ml concentration after 24 and 48 h culture in relation to untreated cells (PBMC and control groups) (Fig. 2B). We do find similar results with de 1000 µg/ml concentration, after 48 h culture. In contrast, at a 1000 µg/ml concentration after 24 h culture, we found significant differences with the control group but not with PBMC group (Fig. 2B). The 150 µg/ml concentration of Anapsos increased the levels of IL-10 in comparison to the control group at both 24 and at 48 h of incubation (Fig. 2B).

The levels of IL-1β increase significantly in Anapsos group compared to control group at the three concentrations used and the two incubation times, 24 and 48 h. Just the 1000 µg/ml concentration increased IL-1β significantly with Anapsos treatment in comparison with PBMC group (Fig. 2C).

IL-6 levels did not have significant variations in between the three

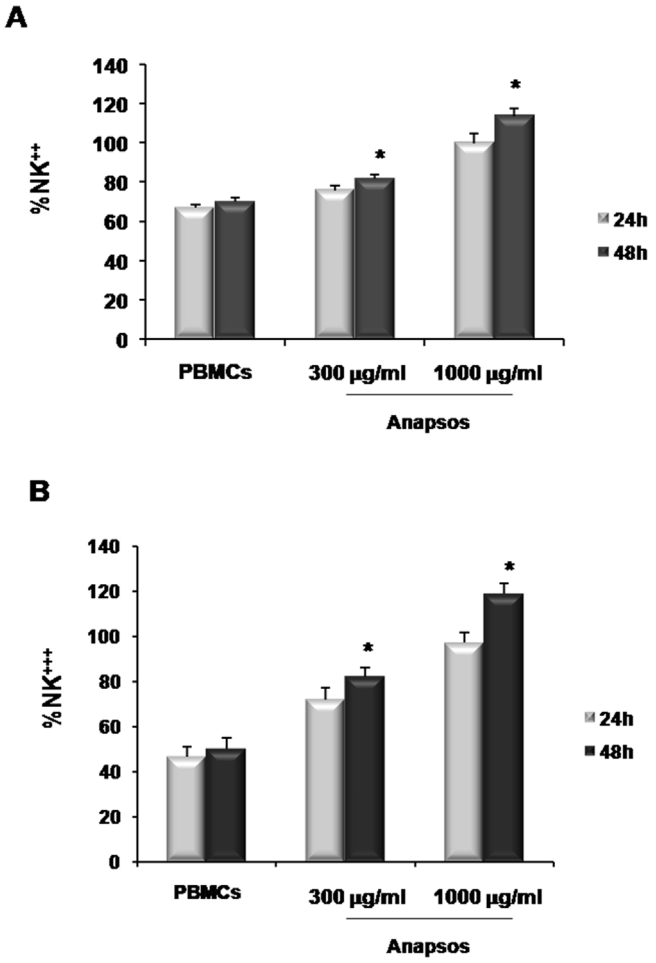

Fig. 4. Anapsos activate Natural Killers Cells. (A) Subpopulation NK^{++} and (B) Subpopulation NK^{+++}. Tonsil tissue co-cultured with PBMCs (1×10^6 cells/ml) and PBMCs activated with Anapsos (300 and 1000 µg/ml) for 24 and 48 h. The lymphocyte population was determined by flow cytometry. The data represent the mean ± SEM of at least twenty independent experiments. Results are expressed in %; *$P \leq 0.05$ vs. PBMCs.

groups: Control group, PBMC group and Anapsos group (Fig. 3A).

IFN-γ levels also raised in a statistically significant way ($P \leq 0.05$) in relation to control group and PBMC group with Anapsos extract after 48 h, at 150 µg/ml, 300 µg/ml and 1000 µg/ml concentrations (Fig. 3B).

Anapsos increased significantly ($P \leq 0.05$) TNF-α in comparison with control group, but this raise was not significant compared to PBMC group, after 48 h incubation at 300 and 1000µg/ml concentrations. Nevertheless, the levels of these cytokine rise in every group (Fig. 3C).

3.2. Anapsos activate Natural Killers Cells

In order to determine the immunomodulatory capacity of Anapsos, we measured different cell populations by flow cytometry in the supernatants of the tonsillar tissue cultures. As we can see in the image (Fig. 4), Anapsos treatment at the two commercialized concentrations (300 and 1000 µg/ml), activates significantly de Natural Killer cells (NK); NK^{++}: $CD7^{++}/CD3^{-}/CD56^{+++}/CD38^{+++}$ as well as the most active NK^{+++}: $CD7^{+++}/CD3^{-}/CD56^{+++}/CD38^{+++}/CD69$. The 150 µg/ml did not activate the NK (Data not shown). Once the lymphocytes contact with Anapsos, we observed the lymphocyte activation in two facts: not only they released cytokines to the culture medium but they also increased the cell population. However, no other significant differences were found between treated or not groups with Anapsos in populations of B and T lymphocytes.

3.3. Anapsos activate different immunoglobulins from tonsillar tissue

The treatment with Anapsos at a 300 and at 1000 µg/ml concentrations increases significantly IgM, IgD and IgG-4 levels, in comparison to control and PBMC groups in tonsillar tissue, both at 24 and 48 h. Thus, increased fluorescence was observed in the tonsillar tissue of Anapsos group, but not in the tonsillar tissue of control and PBMC groups (Fig. 5). We did not find any significant difference with the 150 µg/ml solution in between the three groups (Data not shown).

4. Discussion

The development of new treatments, which help to promote the immunological system, seems to be important to help children to overcome recurrent tonsillitis episodes and therefore decrease the number of surgical procedures. In our search for a pharmacological intervention in the recurrent tonsillitis problem, we proposed using *Polypodium leucotomos* (Anapsos) based on their immunomodulator properties [6–16].

In vitro and in vivo animal and human studies have demonstrated the beneficial effects of *P. leucotomos*. Together, these data indicate that extracts of this unique plant utilize multiple mechanisms of therapeutic activity that include of various skin disorders and conditions including atopic dermatitis [21], vitiligo [22], sun protection from ultraviolet radiation [23], antitumor effects [6] and immunoregulatory effects [6–16]. The in vivo studies show that the effects of *P. leucotomos* extract are not just theoretical; indeed, benefit has been demonstrated in animals and in humans. This agent which can be taken orally and has not been noted to have serious adverse reactions [24]. Winkelmann et al., 2015 included in their review 19 studies in human sciences and 6 in basic sciences that covered more than 40 years of research. Oral *P. leucotomos* was administered at daily doses ranging from 120 mg to 1080 mg. No adverse effects were reported in laboratory studies. In humans, side effects (gastrointestinal complaints and pruritus) were mild to moderate and found only in very small numbers of patients overall (16/1016 [2%]). This review concludes *P. leucotomos* is well tolerated at all doses administered and associated with a negligible risk of side effects [25]. In children and adolescents, Ramírez-Bosca et al., 2012 performed a phase IV randomized, double-blind, placebo controlled, multicenter trial involving 105 patients aged between 2 and 17 years who were receiving topical corticosteroids to treat moderate atopic dermatitis. The patients were randomized to receive, in addition to their standard treatment, *P. leucotomos* extract or placebo (both in capsule form) for 6 months. Long-term treatment with *P. leucotomos* extract has benefits for children and adolescents with atopic dermatitis who require pharmacologic treatment to reduce inflammation and relieve itching [26].

P. leucotomos extract (Armaya fuerte, Centrum laboratories, Alicante, Spain), have been shown to influence the components of the immune system. In vitro studies carried out on humans show that *P. leucotomos extract* stimulates the proliferation of peripheral blood mononuclear cells in vitro and increases interleukin-2 (IL-2) and interferon-γ secretion. It also enhances the stimulant effect of other mitogens on cytokines such as IL-10 [4,11,15,21,26–29]. In our study we have found that Anapsos extract increased IL-2 levels significantly, we have also found that IFN-γ levels also raised in a statistically significant way with the Anapsos's extract after 48 h, at the three concentrations used. Concerning IL-10, its levels were also higher in Anapsos group compared to Control and PBMC groups. In what refers to the others interleukins studied, we found that IL-6 levels did not have significant variations in between the three groups. We did not found consistent data published before about its variations with Anapsos. Nevertheless, Anapsos increased significantly IL-1β and TNF-α in comparison with control group but not compared to PBMCs group. What we have found could mean that once the inflammatory process has begun and the PBMC are stimulated, Anapsos has little benefit on these two cytokines.

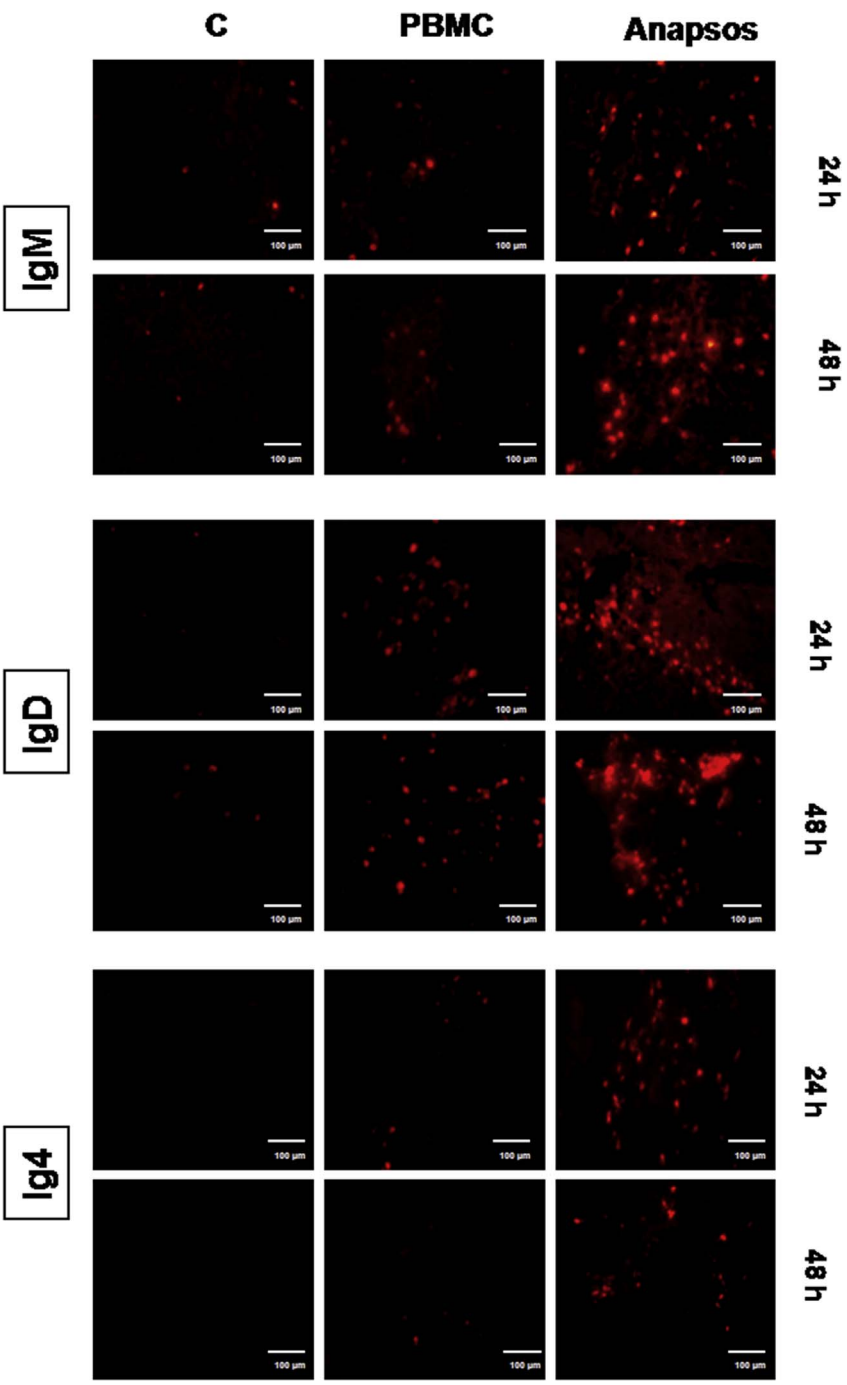

Fig. 5. Anapsos activate IgM, IgD, IgG4 from tonsillar tissue. Representative image the indirect immunofluorescence of tonsil tissue co-cultured with PBMCs (1×10^6 cells/ml) and PBMCs activated with Anapsos (300 and 1000 µg/ml) for 24 and 48 h for determinate IgM, IgD and IgG4.Sacale bar 100 µm. 40x.

These data contradicts what has been published by others [7,14] but the variations might be due to methodological differences. These results indicate a pleiotropic effect of *P. leucotomos extract* or Anapsos for different cytokines, probably due to a different way of acting on distinct areas of the immune system. The Anapsos pleiotropic ability to down-regulate the secretion of proinflammatory cytokines such as IL-1β and to up-regulate anti-inflammatory cytokines such as IL-10 [11,12].

We also found a significant increase of the Natural Killer cells activation in both kinds of NK: NK++ (CD7++/CD3-/CD56+++/CD38+++) as well as the most active NK+++ (CD7++/CD3-/CD56+++/CD38+++/CD69). Our results are consistent with what has been published by Sempere et al. [26]. In contrast, we did not find differences in the lymphocytes B and T activation by flow cytometry.

We also found a significant increase in IgM, IgD and IgG4 levels in the group treated with Anapsos fraction in comparison with other two groups after 24 and 48 h cultures. Cardelús et al. [30] also found an increase in IgM and IgG but their study was completely different to ours as they did a prospective in vivo study with patients that consulted because of pharyngo-tonsillitis. However, our results support the two concentrations commercialized [21] while others have found benefits just with one of them [16]. As described by Van den Eertwegh et al. [31], antigens will be taken up and processed by macrophages or IDC in the extrafollicular area of the palatine tonsils. Upon contact, antigen-specific T cells are activated and proliferate. Subsequently, B cells encounter the specific T cells in the extrafollicular area and T-B cell interactions with cytokine and antibody production occur [12]. In this order we could understand that, despite the fact that our study was not be able to find any differences in the number of lymphocytes, they should have proliferated and been activated as the specific

immunoglobulins have significantly increased their levels.

5. Conclusions

Our results provide for the first time convincing evidence that Anapsos can modulate remarkably cellular and humoral immune reactions in a model of tonsil explants in vitro. The stimulant effect caused by in vitro Anapsos extract on immune system cells (natural killer cells) and its effect on diverse cytokines gives it a significant immunomodulatory capacity. Polypodium leucotomos Extract or Anpasos offers an optimum security profile alone or when used with other medications, thus offering a suitable alternative for the prevention of the recurrent episodes of tonsillitis in childs who might be vulnerable to immunosuppression. Therefore, it seems appropriate to confirm these pharmacological effects of Anapsos in other in vitro studies, in addition to wide-ranging clinical trials to confirm this hypothesis.

Funding and conflict of interest disclosures

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Conflict of interests: Ph. Carolina Sánchez-Rodríguez and M.Ph. Ricardo Sanz-Fernández have worked as clinical researchers for ASAC Pharma. Dr. Laura Rodrigáñez, Dr. Keyliz Peraza and Ph. Eduardo Martín report that they have no conflict of interests.

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