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

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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,

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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).

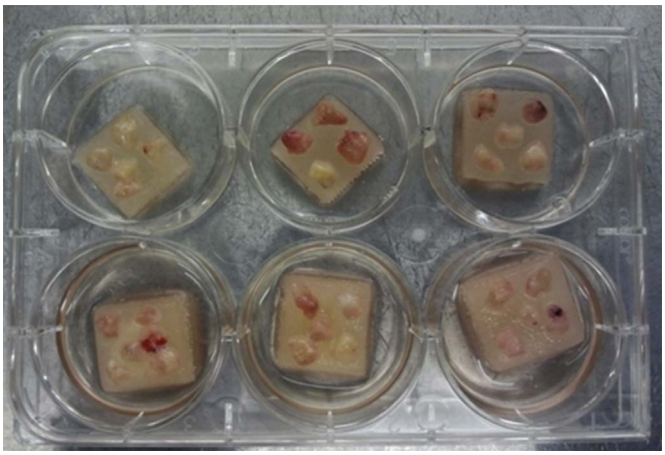

Fig. 1. Tonsils explants cultured on Equispon foam. We observed a 6-well plate with the tonsil tissue under the three experimental conditions: Control group (tissue with culture medium), PBMC group (tissue culture with patient's PBMCs) and Anapsos group (tissue culture with patient's PBMCs and Anapsos).

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.