Suppression of T and B Cell Responses by *Pterodon pubescens* Seeds Ethanolic Extract

1C.C. Cardoso, 1A.C. Pinto, 1P.R. Marques, 1C.R.M. Gayer, 2M.I.R. Afel, 1M.G.P. Coelho and 1K.C.C. Sabino

Departamento de Bioquimica,
Departamento de Histologia e Embriologia, do Instituto de Biologia Roberto Alcantara Gomes,
Universidade do Estado do Rio de Janeiro, Av. Professor Manoel de Abreu, 444, 4º andar,
CEP-20550-170, Rio de Janeiro, RJ, Brasil

Abstract: The anti-artritic property of hydro alcoholic extract of *Pterodon pubescens* seeds was previously demonstrated using the Collagen Induced Arthritis (CIA) in mice, the most similar arthritis experimental model to human rheumatoid arthritis. This disease is characterized by chronic inflamed joints resulting from exacerbated functions of macrophages and T and B lymphocytes. Anti-inflammatory and antinociceptive activities by ethanolic extract of *Pterodon pubescens* seeds (EEPp) have been also reported. This study describes the effects of EEPp on T and B lymphocytes functions from healthy mice. Delayed Type Hypersensitivity (DTH), *in vivo* antibody production, T and B lymphocyte proliferation and NO production were determined. Mice treated orally for 7 days with EEPp had inhibited 58% of B cell antibody production (10^{-2} mg kg^{-1} b.wt.) and 33% of the DTH response (10^{-3} mg kg^{-1} b.wt.), also reducing tissue leukocyte infiltration. EEPp (10^{-2} mg mL^{-1}) also inhibited *in vitro* T (89%) and B (68%) lymphocytes proliferation and NO production (53%) by macrophage cell line 1774. The immunosuppression here described for EEPp supports its potential therapeutic use to control exacerbated humoral and/or cellular immune response as in autoimmune and chronic inflammatory diseases.

Key words: *Pterodon pubescens*, delayed type hypersensitivity, T cell, B cell, suppression

INTRODUCTION

*Pterodon pubescens* Benth. (Leguminosae-Papilionoideae), popularly known as Sucupira branca, is a native tree widely distributed over the central region of Brazil, which seeds are used in folk medicine as wine infusions to treat rheumatic and inflammatory diseases. Toxicological studies of the seeds ethanolic extract (oil) did not demonstrate mutagenicity or acute toxicity (Sabino *et al.*, 1999a) in mice.

Rheumatoid arthritis is an auto-immune disease characterized by chronic inflamed joints and exacerbated functions of macrophages and T and B lymphocytes. In spite of the efficacy of immunosuppressive drugs, it is still important to develop new anti-rheumatic strategies to avoid the side effects of the classical therapy, which often leads to treatment interruption. The experimental study with *P. pubescens* has already demonstrated scientifically effects related in folk medicine and may lead to identification and isolation of new compounds to treat rheumatoid arthritis, other auto-immune diseases and chronic inflammatory pathologies. The anti-arthritic effect of hydro-alcoholic extract of *Pterodon pubescens* seeds has been experimentally demonstrated using the collagen-induced arthritis (CIA) model (Sabino *et al.*, 1999b; Coelho *et al.*, 2004). Sub-acute toxic effects were not observed with this extract in terms of hematological, histopathological, clinical and biochemical parameters analysis in arthritis mice (Coelho *et al.*, 2004). Further analysis showed anti-inflammatory (Silva *et al.*, 2004) and antinociceptive effects to ethanolic extract of *P. pubescens* seeds (EEPp) (Coelho *et al.*, 2005). Nevertheless, the effects of this extract on immune response of healthy animals remain unknown. In this study, experiments were designed to determine whether oral treatment of healthy mice with EEPp can modulate T and B cells immune response by evaluating the delayed type hypersensitivity (DTH) response, *in vivo* antibody production, *in vitro* T and B lymphocyte proliferation and NO production.

Corresponding Author: K.C.C. Sabino, Departamento de Bioquimica, Instituto de Biologia, Centro Biomédico,
Universidade do Estado do Rio de Janeiro, Av. Professor Manoel de Abreu, 444, 4º andar,
CEP-20550-170, Rio de Janeiro, RJ, Brasil Tel: 55(021)25876143 Fax: 55(021)25876136
2308
MATERIALS AND METHODS

Chemicals: LPS, Con A, SDS, MTT and RPMI 1640, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal Calf Serum (FCS) was purchased from Cultilab, Brazil. 3H-Tdr was purchased from Amersham Biosciences, Brazil and Sheep Red Blood Cells (SRBC) from EBE-FARMA Biológica e Agropecuaria Ltda (RJ, Brazil). All other chemicals and reagents were of the highest grade available.

Animals: Female SW mice, 3-6 months old, 30-40 g body weight (b.wt.), were used to evaluate in vivo immune response. They were kept with free access to water and food, housed under standard laboratory conditions, with 12 h light/dark cycle. All experiments were performed under the consent and surveillance of the Biomedical Center Ethical Committee of UERJ, RJ, Brazil, for the use of animals in research.

Plant material and extract preparation: Pterodon pubescens Benth. seeds were collected by Luciana Pontes Coelho at the State of Goiás, Brazil. The taxonomic identity was confirmed by Haroldo Cavalcante de Lima, from the Departamento de Botânica Sistemática do Jardim Botânico do Estado do Rio de Janeiro, Brazil, where a voucher specimen collected has been deposited (RB 350279). The seeds were pulverized in liquid nitrogen and submitted to 100% absolute ethanol extraction (15 g 100 mL⁻¹) at room temperature for 15 days, in a dark condition. After complete ethanol evaporation, viscous, brown and fragrant oil was obtained, with 50% efficiency in weight (w/w).

In vivo assays: Mice were divided in seven groups and treated daily for 7 days by gavage (50 μL) with crescent doses of EEP (10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ and 1 mg kg⁻¹ b.wt.). DTH control group was treated with vehicle (0.5% Tween 20 in 15% ethanol/water solution) and a treatment group received dexamethasone 1 mg kg⁻¹ b.wt. DTH response was induced by priming mice with a subcutaneous injection of 1×10⁷ SRBC in the neck on day 2. On day 7, mice were challenged with 1×10⁸ SRBC injected subcutaneously on the Left Hind Footpad (LHF). The increase in the footpad volume was determined 24 h after challenge by plethysmometry using the Right Hind Footpad (RHF) as control. The Edema Index (EI) was calculated as [(LHF-RHF)/RHF] > 100. After anesthesia, animals were euthanized and the inflamed paws were excised and fixed in 10% buffered formalin solution for routine histological processing. Popliteal lymph-nodes of the inflamed paws were also collected for relative weight determination (RW = lymph node weight (mg)/body weight (g)). For humoral immune response evaluation, all groups received 2.5×10⁸ SRBC intraperitoneally (i.p.) on day 1. The blood was collected on day 8 and the haemagglutination titer was determined as described by Millan et al. (1996).

In vitro assays: After animal anesthesia and euthanizing, the spleen of healthy mice was aseptically removed and the splenocytes isolated. After haemolysis with hypotonic ammonium chloride buffer (0.16 M NH₄Cl, 0.1 mM EDTA and 10 mM KHCO₃, pH 7.4), cells were washed and resuspended in complete RPMI medium (5% FCS, L-glutamine 2 mM, 2-mercaptoethanol 0.05 mM, penicillin 100 UI mL⁻¹, streptomycin 0.1 mg mL⁻¹). The cellular viability was determined by Trypan Blue dye (0.2%) exclusion. Cells (2×10⁶ mL⁻¹) were incubated with Con A or LPS (5×10⁻³ mg mL⁻¹) in 96-well flat-bottom plates (200 μL) in the absence or presence of EEPp at 10⁻³ and 10⁻² mg mL⁻¹ for 72 h, at 37°C in 5% CO₂ and humidified atmosphere. To assess DNA synthesis, 25 μL of [³H]-thymidine 10 μCi mL⁻¹ were added for the last 24 h of culture and the [³H]-Tdr radioactivity was determined by liquid scintillation. Cultures were set up in triplicates.

The murine macrophage cell line J774 was maintained (25 cm² Corning culture flasks) in RPMI-1640 medium supplemented with streptomycin (0.1 mg mL⁻¹) and 10% FCS. Cells were plated in 96-well plastic culture plate (200 μL final volume) at 5×10⁵ cells mL⁻¹. NO production was induced with LPS (5×10⁻³ mg mL⁻¹), in the absence or presence of EEPp at 10⁻³, 10⁻² and 10⁻¹ mg mL⁻¹ for 24 h, at 37°C in 5% CO₂ and humidified atmosphere (cultures were set up in duplicates). NO production was evaluated by determining nitrite concentration, incubating equal volumes of Griess reagent (N-(1-naphthyl)-ethylenediamine dihydrochloride 0.1%, sulfanilic acid 1% in H₂PO₄ 5%) and sample (culture supernatant) or nitrite standard solution, at room temperature for 10 min. The sample nitrite concentration was determined by comparing the sample absorbance at 550 nm with the NaNO₂ standard curve. EEPp cytotoxicity to unstimulated J774 cells was also determined after culture for 24 h by MTT assay (Mosman, 1983). Briefly, after removing culture supernatant (100 μL) 10 μL of MTT solution (5 mg mL⁻¹ in phosphate buffered saline) were added to cells and then incubated for the last 2 h of culture at 37°C and in 5% CO₂. Afterwards, 100 μL of SDS solution (SDS 10%, HCl 0.01N) were added to solve the reduced formed product and the absorbance at 570 nm was determined. The absorbance obtained by incubation of cells without extract was considered as 100% of viability.
Statistical analysis: Variance analysis between groups was performed by One-Way ANOVA. Significant differences between pairs of groups were accessed by Tukey’s multiple comparison test or Student’s t test, with significance level set at p<0.05.

RESULTS AND DISCUSSION

In vivo evaluations: Groups treated with EEPp at 10^{-4} and 10^{-3} mg kg^{-1} doses showed, respectively, reductions of 33 and 34% in paw edema (Fig. 1A). As expected, the group treated with dexamethasone (1 mg kg^{-1}) showed severe DTH inhibition (83%). These EEPp doses also decreased the popliteal lymph node relative weight, showing reductions of 68% (p<0.001) and 37% (p<0.05), respectively, in relation to vehicle group (Fig. 1B), while dexamethasone induced reduction of 56%. The DTH response of animals treated with vehicle (control) showed dermis hyper trophy and severe leukocyte infiltration (Fig. 2A). This inflammatory infiltrate was clearly reduced in groups treated with EEPp, with prominent reduction in the group treated with 10^{-3} mg kg^{-1} (Fig. 2B). As expected, animals treated with dexamethasone did not show inflammatory infiltrate or tissue hypertrophy (Fig. 2D).

The antibody production against SRBC was significantly inhibited (p<0.05) in the groups treated with EEPp doses of 10^{-7}, 10^{-3}, and 10^{-7} mg kg^{-1}, showing reductions of 58, 40 and 48%, respectively. Dexamethasone inhibited 73% of this response (Fig. 3).

In vitro assays: T and B lymphocytes in vitro proliferation (Fig. 4) were stimulated with Con A and LPS, reaching 11625±2240 and 6931±354 cpm, respectively. EEPp (10^{-4} mg mL^{-1}) induced 89 and 75% inhibition of T and B lymphocyte proliferation, respectively.

After stimulation of J774 cells with LPS for 24 h, the nitrite (NO_{2}^{-}) concentration in the supernatant was 2.8±0.8 μM (Fig. 5A). Simultaneous treatment of J774 cells with LPS and EEPp did not stimulate in vitro nitrite production but it inhibited 53% (p<0.01) of its production when treated with 10^{-3} mg mL^{-1}, without significant cytotoxic effects (Fig. 5B).

The oral and daily treatment of animals with EEPp for seven days has significantly reduced both cellular and humoral immune response to the T cell-dependent antigen SRBC. The effects of EEPp on cellular immune response modulation was demonstrated using the delayed type hypersensitivity model of immune response which is characterized by an immune-inflammatory reaction developed in two phases, where macrophages and Th lymphocytes are the major participants (Kobayashi et al., 2001). EEPp reduced significantly the paw edema at lower doses. EEPp can be inhibiting the early phase of DTH (sensitization), impairing the first antigen presentation and/or inhibiting the oligoclonal expansion of lymphocytes, or the second phase (effector one), when the immune system has to develop a rapid and intense immune-inflammatory reaction through the secretion of several pro-inflammatory cytokines. Alterations in cytokine patterns after treatment of immunized mice with crude extracts of another plant have been found by
Fig. 2: Effects of *P. pubescens* ethanolic extract (EEPp) on paw histopathology of mice submitted to delayed type hypersensitivity (DTH) response to sheep red blood cell (SRBC). The animals were treated orally and daily for seven days with: (A) vehicle (control), (B) EEPp $10^{-4}$ mg kg$^{-1}$ b.w.t., (C) EEPp $10^{-3}$ mg kg$^{-1}$ b.w.t. and (D) dexamethasone 1 mg kg$^{-1}$ b.w.t. DTH was induced with $1 \times 10^8$ cells, s.c. in the neck at day 2 and challenged with $1 \times 10^7$ cells, s.c. in the left hind paw at day 7. The left hind paw was excised on 8th day and processed for routine histology. Photomicrography shows paw longitudinal sections processed and stained with eosin and hematoxylin (200 X). (ep) epidermis, (d) dermis, (li) leukocyte infiltrate, (m) muscle

Fig. 3: Effects of *P. pubescens* ethanolic extract (EEPp) on mice antibody production to sheep red blood cell (SRBC). Antibody was determined by haemagglutination titer. Animals were immunized with $2.5 \times 10^6$ cells, i.p., at day 1 and sacrificed at day 8. The haemagglutination titer is considered the higher serum dilution with positive haemagglutination. Animals were treated orally and daily for seven days with vehicle (C), EEPp, or dexamethasone (DX, 1 mg kg$^{-1}$). Values represent Mean±SEM of three independent experiments (n = 6-8/group in each experiment). *p<0.05 and **p<0.01 vs. vehicle treated group (Tukey’s test)

Fig. 4: Effects of *P. pubescens* ethanolic extract (EEPp) on T and B lymphocyte proliferation. Splenocytes ($1 \times 10^6$ ml$^{-1}$) proliferation were stimulated with Concanavalin A (Con A, $10^{-3}$ mg ml$^{-1}$), a T cell mitogen and with Lipopolissacharide (LPS, $10^{-4}$ mg ml$^{-1}$), a B cell mitogen, in the absence (control) or presence of EEPp for 72 h. Proliferation was determined by $^{3}$H-thymidine incorporation as previously described. Results represent Mean±SD of 2 independent experiments with triplicates. *p<0.05 and **p<0.01, related to control by Student t-test
Fig. 5: (A) Effect of *P. pubescens* ethanolic extract (EEPp) on nitric oxide (NO) production by J774 macrophage cell line. Cells (5×10^5 mL^-1) were stimulated with lipopolysaccharide (LPS, 10^-3 mg mL^-1) in the absence (control) or presence of different EEPp concentrations for 24 h. NO was determined by nitrile determination in cell supernatant as described in methods. (B) The graphic shows the survival of non-stimulated cell treated or not with EEPp for 24 h, by MTT assay (last 2 h of culture). Results represent Mean±SD of 2 independent experiments with triplicates. *p<0.05 related to control by Student t-test.

Ohazanfari *et al.* (2002). Results of lymph nodes weight were closely related to paw edema, as significant reductions on both parameters with lower doses were observed. Reduction of the inflammatory infiltrate and of subcutaneous tissue and dermis thickness, observed in histopathological analysis of animal paw treated with EEPp at 10^-2 mg kg^-1, must be contributing to reduction of paw edema. Taken together, the observed results of lymph nodes weight and paw histopathology after EEPp treatment suggest it may be inhibiting clonal expansion of SRBC-specific lymphocytes and/or cellular migration to lymph nodes or to the site of the inflammatory response.

Modulation of B cells function by EEPp was also accessed by *in vivo* antibody production against SRBC. This *in vivo* humoral response was also inhibited by EEPp. Since SRBC is a T cell-dependent antigen, the reduced antibody production may result from impaired B and/or T cell function. The inhibition of *in vitro* proliferation of T and B cells suggests that EEPp may be impairing the function of both cells. Considering the key role of macrophages in DTH responses and that NO is an important inflammatory and immunologic mediator, we conducted experiments to verify if EEPp treatment could modulate the *in vitro* NO production by J774 macrophage cells. *In vitro* inhibition of NO production by EEPp suggests that it may be acting on the macrophage functions. Several plants with immunoregulatory effects have major effects on macrophage functions (Yamaguchi, 1992; Dhaley, 1997). Inhibition of NO synthase induction in macrophages contributes to anti-inflammatory and immunosuppressive actions of glucocorticoids (Di Rosa *et al.*, 1990). Inhibition of lymphocyte proliferation and NO production by macrophages may be contributing to EEPp immunosuppressive effects.

Here, we provided evidence that EEPp can suppress both cellular and humoral immune responses of healthy mice in lower concentrations than dexamethasone, an immunosuppressant drug. Despite the fact that the extrapolation of animal models results to a clinical situation must be treated with caution, the demonstrated EEPp immunomodulatory activity is a highly sought goal due to its potential application to treat patients with chronic immune-inflammatory or self-immune diseases.

**ACKNOWLEDGMENTS**

We thank the Laboratory LIA-BPPN personnel for technical assistance and FAPEJ, CNPq and UERJ for financial support.

**REFERENCES**


