

## EFFECT OF ANAPSOS® IN A MURINE MODEL OF EXPERIMENTAL TRICHOMONIASIS

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### Summary:

Immunomodulator effect of Anapsos® (*Polypodium leucotomos* extract) in NMRI (US Naval Medical Research Institute) outbred mice infected by the intraperitoneal route with  $10^7$  *Trichomonas vaginalis* has been tested. Gross histopathologic changes in abdominal organs and mortality rate, as a consequence of the pathogenicity of the protozoa and the immune response of the host, were evaluated. Among the different treatment regimes assayed, Anapsos® at doses of 20 mg/Kg/day administered for 10 days before infection decreases the parasite pathogenicity index (PI) in the treated animals when compared to those of the untreated control group. The immunosuppressor treatments with azathioprine (100 mg/Kg/day  $\times$  1), cyclophosphamide (100 mg/Kg/day  $\times$  1), and FK-506 (10 mg/Kg/day  $\times$  10) significantly decreased the PI, while an immunostimulant treatment with glycoposphopeptical (13 mg/Kg/day  $\times$  10) increased it. These assays have shown the usefulness of the murine model of experimental trichomoniasis for the study of immunomodulator activity of natural or synthetic drugs.

**KEY WORDS:** immunomodulation, *Polypodium leucotomos*, *Trichomonas vaginalis*, FK-506, cyclophosphamide, glycoposphopeptical.

### Résumé : EFFET PHARMACOLOGIQUE DE L'ANAPSOS® DANS UN MODÈLE DE TRICHOMONIASIS EXPÉRIMENTAL

La modulation de la réponse immunitaire dans un modèle de pathogénie expérimentale de *Trichomonas vaginalis* ( $10^7$  trichomonades inoculés dans le péritoine) chez la souris NMRI a été examinée par l'étude des changements histopathologiques sur les organes abdominaux et la mortalité après le traitement avec un extrait de *Polypodium leucotomos* (Anapsos®). Parmi les différents types de traitement analysés, l'Anapsos® (20 mg/Kg/jour administrés pendant 10 jours avant l'infestation) induit une réduction quantitative des lésions abdominales évaluées chez les animaux traités par comparaison aux non traités. D'autre part, les traitements immunosuppresseurs avec l'azathioprine (100 mg/Kg/jour  $\times$  1), le cyclophosphamide (100 mg/Kg/jour  $\times$  1), et le FK-506 (10 mg/Kg/jour  $\times$  10) diminuent de manière significative la pathogénicité de *T. vaginalis* (PI), tandis que le traitement avec glycoposphopeptical (13 mg/Kg/jour  $\times$  10), un agent immunostimulant, l'augmente. Les essais effectués ont montré l'utilité d'un modèle de trichomoniasis expérimental pour l'évaluation de l'immunomodulation des drogues naturelles ou synthétiques.

**MOTS CLÉS :** immunomodulation, *Trichomonas vaginalis*, *Polypodium leucotomos*, Anapsos®, FK-506, cyclophosphamide, glycoposphopeptical.

## INTRODUCTION

*Trichomonas vaginalis* is a parasitic protozoan that is the cause of trichomoniasis, the most common sexually transmitted diseases in the world (Thomason & Gelbart, 1989). In women, trichomonal vaginitis is characterized by inflammation of vaginal epithelium, foul-smelling, discharge, and tissue cytopathology (Alderete, 1983). Most infected men are asymptomatic, although disease manifestations such as urethritis, prostatitis, balanoposthitis have been well documented. A major problem with diagnosis and control of this disease is the dramatic variation in host symptomatology. Because the possible mechanisms of disease pathogenesis are not well known, studies aimed at understanding the biology of *T. vaginalis* must be performed.

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The most commonly used method to evaluate parasite infection and the virulence of strains consists of infecting laboratory animals (Honigberg, 1978; Kulda, 1990). Differences in pathogenicity levels among isolates can be demonstrated by intraperitoneal inoculation (Bogovsky & Teras, 1958; Teras & Roigas, 1966; Cavier *et al.*, 1972), or subcutaneous inoculation (Honigberg, 1961) in mice. In this laboratory, we have standardized and validated an intraperitoneal model of murine trichomoniasis for studies of pathogenicity and chemotherapy (Nogal-Ruiz *et al.*, 1997). Briefly, intraperitoneal injection of  $10^7$  trichomonads in mice is required to cause mortality, production of ascitic fluid, fibrinopurulent peritonitis with abscesses, and visceral (especially pancreatic and hepatic) necrosis. The extent of which is proportional to the level of virulence of inoculated strain. This model has allowed us to investigate the foundations of induced immune response in host-parasite interaction (Nogal-Ruiz *et al.*, 2003), and the immunomodulator effect of drugs, such as Anapsos®.

Anapsos® is an aqueous extract of the rhizome of the fern *Polypodium leucotomos*. Clinical effects have been

described in neoplastic (Horvath *et al.*, 1967), or autoimmune diseases such as atopic dermatitis (Jiménez *et al.*, 1986, 1987), psoriasis (Hernán *et al.*, 1974; Jiménez *et al.*, 1987; Thomas-Barry, 1999) and vitiligo (Mohammad, 1989). Further approaches to immunomodulator effects of Anapsos<sup>®</sup> have been attempted in experimental animals infected by parasites (Cuéllar del Hoyo *et al.*, 1997; Dea Ayuela *et al.*, 1999).

The aim of our paper was to assess the immunomodulator activity of Anapsos<sup>®</sup> by testing its modulator effect on the experimental pathogenicity of *T. vaginalis* in a murine model, such activity was measured by the capacity of this drug to diminish or clear abdominal lesions (or mortality) in animals, by comparison with untreated control group. Moreover, the effect of immunosuppressor (azathioprine, cyclophosphamide, and FK-506) and immunostimulant (thymostimulin and glycoprophosphopeptical) drugs in this experimental model has been tested.

## MATERIALS AND METHODS

### ORGANISM AND CULTURE

*Trichomonas vaginalis* strain C1-NIH isolated from human adult female with acute vaginitis was obtained from the American Type Culture Collection (reference n° 30001). Parasites are maintained in our laboratory by cryopreservation in liquid nitrogen. Protozoa were cultivated at 37°C with 5% CO<sub>2</sub> in Diamond medium (Trypticase-Yeast extract-Maltose) pH 6.2, supplemented with 10% heat inactivated horse serum, penicillin G (100 IU/ml) and streptomycin (100 mg/ml) (Diamond, 1957).

### MICE

NMRI (US Naval Medical Research Institute) mice from IFFA Credo (Criffa SA, Spain) under standard conditions in our laboratory were used for experimental infections. All animals were maintained in a temperature and humidity controlled environment with a 12 h light/dark cycle and given water and food *ad libitum*. NMRI mice weighed 20-25 g at the beginning of the experiments.

### EXPERIMENTAL DESIGN

The first series of experiences was performed in order to determine the optimum dose of Anapsos<sup>®</sup> and the most appropriate inoculation pattern (preinfection or postinfection treatment) in our experimental trichomoniasis model. Three experimental groups were treated intraperitoneally with daily Anapsos<sup>®</sup> doses of 4, 20, and 40 mg/Kg/day, respectively, for 10 days

before infection. Another experimental group was treated on days 3-7 after infection, and a second group was treated on days 10-14 after infection, both groups received five doses of 20 mg Anapsos<sup>®</sup>/Kg/day. In all cases, mice were infected intraperitoneally with a single dose of 10<sup>7</sup> trichomonads from axenic cultures in fresh Diamond medium. Moreover, one group of animals for each experiment was infected, but not drug treated, and used as infection control group. Both experimental and control groups consisted of ten mice. Another series of experiments was performed with reference drugs. The following drugs were administered *per os*, before infection at the single or multiple daily doses indicated: azathioprine (Imurel<sup>®</sup>, Gayoso Wellcome SA, Madrid, Spain) and cyclophosphamide (Genoxal<sup>®</sup>, Asta Médica SA, Madrid, Spain) at 100 mg/Kg/day × 1, and FK-506 (Prograf<sup>®</sup>, Fujisawa Ireland Ltd, Killorglin, Ireland) at 10 mg/Kg/day × 10, were used as immunosuppressor agents. Thymostimulin (TP-1<sup>®</sup>, Serono SA, Madrid, Spain) at 24 mg/Kg/day × 5, and glycoprophosphopeptical (Immunoferon<sup>®</sup>, Laboratorios Cantabria SA, Santander, Spain) at 13 mg/Kg/day × 10 were used as immunostimulant agents.

### PATHOGENICITY DETERMINATION

A pathogenicity index (PI) was determined following the criteria recommended by Toyos (1974) and revised by Nogal Ruiz *et al.* (1997). Briefly, mortality, ascites and the gross damage produced to the peritoneum, spleen, pancreas and stomach, and to the visceral (distal) and diaphragmatic (proximal) liver faces were rated according to Table I.

Both the mice that succumbed to the infection and those sacrificed under ether anesthesia at day 15 post-infection were examined at necropsy for the presence of trichomonads and for gross-pathologic changes in the abdominal cavity. The PI was then calculated as the arithmetic mean of the values for each parameter, 100 being the maximum value.

### STATISTICAL ANALYSIS

Statistical analysis was performed using the non-parametric Mann-Whitney U test. Analysis assumes that two-independent samples come from populations having the same distribution. A probability  $p < 0.05$  was considered indicative of statistical significance.

### PREPARATION OF *POLYPODIUM LEUCOTOMOS* EXTRACT (ANAPSOS<sup>®</sup>)

*Polypodium leucotomos* Poir, 1804 (Polypodiaceae) rhizomes were harvested in the Experimental and Ecological Recuperation Plantations (property of ASAC Pharmaceutical International, AIE) in Guatemala, located at an altitude of 2,000 m above sea level.

Pathological manifestations	Values assigned
<b>Mortality</b> (days p.i.)	
3	50
4	44
5	38
Every delay day	Discount 6 points
> 10	6
<b>Ascites</b>	
< 1 mL	2
1-2 mL	4
> 1 mL	6
<b>Peritoneum</b>	
1-3 SN	2
Multiple SN and/or N1	4
Multiple N1 and/or N2	6
MN affecting pelvis	8
MN affecting intestine and kidneys	10
<b>Spleen/pancreas/stomach</b>	
1-3 SN and/or splenomegaly	2
Multiple SN and/or N1	4
N2	6
N3 affecting more than 1 organ	8
N4	10
N5 affecting liver and intestine	12
<b>Visceral liver side</b>	
1-3 SN	2
N1	4
N2	6
N3	8
N4	10
<b>Diaphragmatic liver side</b>	
1-3 SN and/or colour changes	2
N1	4
N2	6
N3	8
N4	10
N5	12

SN: single necrosis; N1: necrosis of 3-5 mm diameter; N2: necrosis of 5-8 mm; MN: multiple necrosis; N3: necrosis of 8-10 mm; N4: necrosis of 10-15 mm; N5: necrosis of more than 15 mm of diameter affecting the most organ surface.

Table I. – Rating of pathological manifestations in mice infected by the intraperitoneal route with *Trichomonas vaginalis*.

Once their identity was verified by botanists from the University of San Carlos in Guatemala, the rhizomes were dehydrated at 50° C for 48 h. After grinding, the rhizomes were extracted by maceration and percolation with a mixture water-alcohol at 60° C for 48 h. After the extraction, the product was purified by adsorption chromatography and ion-exchange chromatography A column Supelcosil LC-18 (25 cm × 4.6 mm × 5 µm), and solvent system (0.3 % v/v phosphoric acid in water/water/acetonitrile 25:72:3) with a flow rate 0.85 ml/min was used. The detection was carried out by the determination of absorbance samples at 210 nm. The injection volume was 2 µl at concentration of 25 mg dried extract/ml. The extract obtained (Anapsos<sup>®</sup>) was filtered, freeze-dried in

50 mg vials and supplied by ASAC for the present research work.

## RESULTS

Figure 1a shows the PIs determined in mice treated with the different doses of Anapsos<sup>®</sup>. Animals pre-medicated with ten doses of 20 or 40 mg/Kg/day demonstrated pathogenicity scores that were 20 % lower than that of corresponding untreated infected controls ( $p < 0.05$ ). By contrast, no significant differences between the PIs of treated (10 days × 4 mg Anapsos<sup>®</sup>/Kg/day) and control groups were shown. Figure 1b shows the PIs after treatment with 5 mg Anapsos<sup>®</sup>/Kg/day during days 3-7 (w-1) or 10-14 (w-2) after experimental infection. As compared to controls, no significant differences were found in any case ( $p > 0.05$ ).

Modulation of pathogenicity scores by premedication with the immunostimulants glycofosphopeptid and

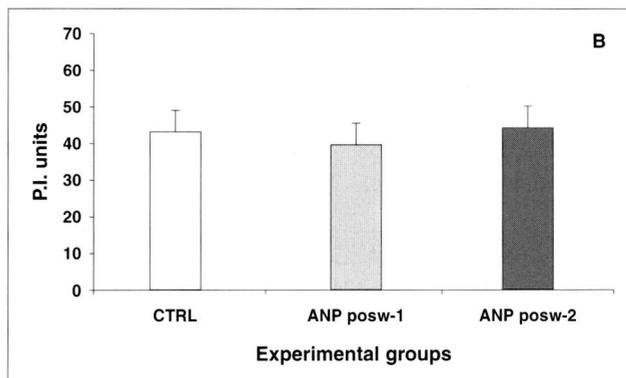
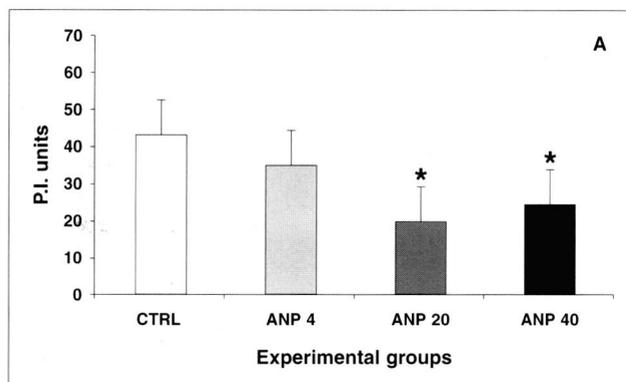


Fig. 1. – Pathogenicity indices (PIs) in mice infected with  $10^7$  *Trichomonas vaginalis* and treated with Anapsos<sup>®</sup> (ANP).

(A) Preinfection treatment for 10 days at 4, 20, and 40 mg ANP/Kg/day; CTRL: control group.

(B) Postinfection treatment with 20 mg ANP/Kg/day, on days 3-7 (ANP pos w1), and, alternatively, on days 10-14 (ANP pos w2).

The asterisk (\*) shows significant differences when compared to the untreated control group by Mann-Whitney U test ( $p < 0.05$ ).

Treatments	Pathological manifestations					
	M	A	P	S/P/S	L (V)	L (D)
ANP4	3.2 ± 4.7	1.2 ± 1.4	6.0 ± 1.6	10.4 ± 2.5	7.2 ± 1.7	7.2 ± 3.5
ANP20	0.0 ± 0.0	0.2 ± 0.6	5.6 ± 1.8	4.8 ± 1.4	3.8 ± 1.9	5.0 ± 3.5
ANP40	0.0 ± 0.0	0.8 ± 1.4	6.4 ± 2.0	6.4 ± 2.9	4.8 ± 2.1	6.2 ± 2.7
ANP20w1	4.7 ± 7.0	0.8 ± 1.4	8.0 ± 1.4	11.3 ± 1.4	7.6 ± 2.4	6.9 ± 3.7
ANP20w2	6.0 ± 6.1	2.0 ± 2.1	8.6 ± 1.6	10.2 ± 2.2	8.2 ± 1.7	8.4 ± 2.2
CTRL	3.6 ± 3.1	2.0 ± 1.6	8.6 ± 1.9	10.2 ± 2.7	9.4 ± 1.3	9.4 ± 2.8
CPA	0.0 ± 0.0	0.4 ± 1.3	5.8 ± 2.7	8.9 ± 1.7	7.6 ± 1.3	6.2 ± 2.3
AZA	7.5 ± 10.9	2.7 ± 1.7	7.3 ± 2.8	8.2 ± 3.8	5.6 ± 2.1	7.2 ± 3.9
FK-506	5.1 ± 10.2	0.4 ± 1.3	6.4 ± 1.3	9.6 ± 1.9	7.1 ± 1.8	9.3 ± 2.0
INM	22.4 ± 6.0	1.0 ± 1.4	8.0 ± 1.9	9.6 ± 2.1	7.0 ± 1.0	5.8 ± 1.7
TP-1	5.6 ± 4.1	0.9 ± 1.4	8.9 ± 1.4	11.3 ± 1.4	9.3 ± 1.0	10.4 ± 1.7
CTRL	8.7 ± 10.3	3.1 ± 2.0	8.4 ± 1.7	9.8 ± 3.0	8.0 ± 2.8	9.1 ± 3.2

M: mortality; A: ascites; P: peritoneum; S/P/S: spleen/pancreas/stomach; L(V): visceral liver side; L(D): diaphragmatic liver side; ANP: Anapsos® groups (4, 20, and 40 mg/Kg/day); ANP20w1: postinfection treatment with Anapsos® (days 3-7); ANP20w2: postinfection treatment with Anapsos® (days 10-14); CTRL: control untreated group; CPA: cyclophosphamide group; AZA: azathioprine group; FK-506: FK-506 group; INM: glycoposphopeptical group; TP-1: thymostimulin group.

Table II. – Values assigned to the pathologic manifestations in mice treated with different immunomodulator agents and infected with  $10^7$  *Trichomonas vaginalis*, as expressed by the arithmetic means and the standard error.

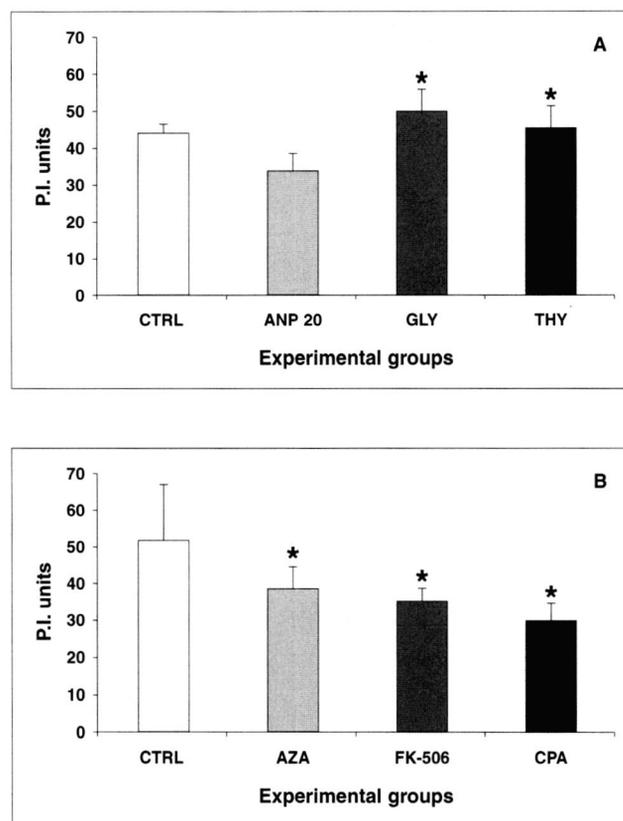


Fig. 2. – Pathogenicity indices (PIs) in mice infected with  $10^7$  *Trichomonas vaginalis* and treated with immunomodulating drugs before the infection.

(A) ANP20: Anapsos® group; GLY: glycoposphopeptical group; THY: thymostimulin group; CTRL: control group.

(B) AZA: azathioprine group; FK-506: FK-506 group; CPA: cyclophosphamide group.

The asterisk (\*) shows significant differences when compared to the untreated control group by Mann-Whitney U test ( $p < 0.05$ ).

thymostimulin (A) and the immunosuppressors azathioprine, cyclophosphamide, and FK-506 (B) yielded the results indicated in Figure 2. Significant differences ( $p < 0.05$ ) among experimental and control groups were found with the only exception of thymostimulin. Finally, Table II shows the values assigned to the pathological manifestations of the experimental and control groups treated with Anapsos® and the reference immunomodulating agents. The lesions with the highest score are usually those found in spleen/pancreas/stomach, while ascites renders the lowest values. Except for glycoposphopeptical group, the parameter “mortality” contributes to PI lesser that produce by abdominal lesions.

## DISCUSSION

The immunomodulating effect of Anapsos® has been studied in an experimental model of *T. vaginalis* by evaluation of gross histopathologic changes and mortality rate of intraperitoneally infected mice.

In order to know the most suitable treatment regime, each of five groups of animals were treated for 10 days before or five days after the initiation of infection with different doses of Anapsos®. Administration of daily doses of 20 mg/Kg for 10 days before infection was the most active pattern in modulating the pathogenicity of *T. vaginalis*. Three independent experiments using these treatment regime resulted in a decrease of the PI around 20 %, significantly different ( $p < 0.05$ ) as compared to control. In a previous paper (Nogal Ruiz *et al.*, 2003), we have postulate that the development of abdominal lesions is compatible with an

inflammatory response regulated by Th1 cells. The mechanism involved in the decrease of lesions induced by the parasite maybe related to mediators of Th1 response. Some clinical effects of Anapsos® have been described in autoimmune diseases such as atopic dermatitis (Jiménez *et al.*, 1986, 1987), or vitiligo (Mohammad, 1989); in such patients the slight shift in CD4<sup>+</sup>/CD8<sup>+</sup> ratio observed was normalized on Anapsos® treatment due to an increase in the CD8<sup>+</sup> subpopulation. In psoriasis treatment, this drug decreases the production of inflammatory cytokines such as IL-1b (Jiménez *et al.*, 1987; Hernán *et al.*, 1974). Also, Anapsos® may have utility in therapeutic approaches to allergic disorders (Yssel *et al.*, 1994).

*In vitro* studies have shown that Anapsos® has potential neurotrophic and neuroimmunomodulating effects (Cacabelos & Takeda, 1995; Fernández-Novoa *et al.*, 1997) which are accompanied by significant modulation of the proinflammatory cytokines IL-1, IL-2, and TNF characteristic for monocytes and dendritic cells (Álvarez *et al.*, 1992). Also, human peripheral blood mononuclear cells *in vitro* co-cultured with the extract in the presence of certain mitogens have shown an increase in cell proliferation based on a T and NK cells activation induced by the Th1-like cytokines IFN-γ and IL-2 (Sempere *et al.*, 1997). The observed pleiotropic effects of Anapsos® may result from its differential effects on different sub-populations of cells participating in the CMI response.

Other approaches to assessing the immunomodulating effects of Anapsos® have been attempted in experimental animals infected by parasites (Cuéllar del Hoyo *et al.*, 1997; Dea Ayuela *et al.*, 1999). In these helminth models, this extract downregulates the Th2 responses generated against *Anisakis simplex* or *Trichinella spiralis* antigens, respectively.

Regarding to the reference drugs assayed, cyclophosphamide was found to suppress humoral (Santos & Owen, 1966) as well as cell-mediated immunity (Poulter & Turk, 1972) when given at the same time as or after the antigen. However, when given before antigenic challenge, the drug enhances cell-mediated responses (Lagrange, 1974). Azathioprine is a powerful inhibitor of primary (innate) responses including allograft rejection and graft-versus-host responses, and FK-506 exerts its immunosuppressive action by the inhibition of IL-2 (Sigal & Dumont, 1992), IL-3, IL-4, GM-CSF, TNF-α, and IFN-γ (Granelli-Piperno, 1988; Herold *et al.*, 1986; Tokuda *et al.*, 1994). FK-506 is widely used for the prophylaxis and reversal of organ allograft rejection and has recently been used as an experimental treatment for some autoimmune disorders.

In our *T. vaginalis* experimental model, azathioprine, cyclophosphamide, and FK-506 significantly decreased the PIs of experimental groups when compared with untreated control groups, independent of their immu-

nosuppression mechanisms acting at different levels. Immunosuppressor treatments had a dramatic effect on murine protective immunity to *T. vaginalis* intraperitoneal infection.

On the other hand, the immunostimulant drug glyco-phosphopeptical enhanced IL-2 synthesis and NK cell activity in the spleen, possibly can be consequence of a modification in prostaglandin synthesis (Rojo *et al.*, 1986). Likewise, thymostimulin is a polipeptidic complex whose biological activity stimulates the cellular immune response (Th1 cells). IL-2 and IFN-γ were all significantly increased in human thymostimulin treatment. Also, the administration of thymostimulin to mice resulted in considerable augmentation of NK cell activity. The activated cells responsible for the increased natural cell-mediated cytotoxicity appeared to be typical murine NK cells (Bistoni *et al.*, 1984).

Immunostimulants agents assayed in our *T. vaginalis* experimental model had unequal effects, while glyco-phosphopeptide significantly increased the PI when compared to untreated control, thymostimulin did not significantly affect the gross-pathologic changes observed in mice abdominal cavity following *T. vaginalis* infection. Both the increase of experimental pathogenicity by immunostimulants and its decrease by immunosuppressors showed the versatility of the experimental trichomoniasis model for studies of immunomodulating activity in natural and synthetic compounds.

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All animal experiments were carried out according to the European Council on applied animals experiments, published in the Guidelines 86/609 EC, and controlled in Spain by Royal Decree 223/1988 of 14<sup>th</sup> March, on the protection of animals used for research and other scientific ends.

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