UNIVERSIDADE PAULISTA - UNIP

ANALYSIS *IN VITRO* OF MACROPHAGES INFECTED BY *Encephalitozoon cuniculi* AFTER TREATMENT WITH HIGH DILUTIONS OF PHOSPHORUS

Dissertação apresentada ao Programa de Pós-Graduação em Patologia Ambiental e Experimental da Universidade Paulista – UNIP, para obtenção do título de Mestre em Patologia Ambiental e Experimental.

MIRIAN YAEKO DIAS DE OLIVEIRA NAGAI

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BANCA EXAMINADORA:

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SUMÁRIO

Artigo científico a ser submetido à revista *Cytokine*

ANALYSIS *IN VITRO* OF MACROPHAGES INFECTED BY *Encephalitozoon cuniculi* AFTER TREATMENT WITH HIGH DILUTIONS OF PHOSPHORUS

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Abstract

Encephalitozoon cuniculi (*E.cuniculi*) is a fungus that acts as an intracellular pathogen and infects different cell types. In the rabbit and immunosuppressed animals from other species, including humans, it parasites neural tissues and cause a very characteristic neurological syndrome, being this infection a zoonotic concern. Herein, the aim was to evaluate the activity of macrophages infected with *E. cuniculi* after treatment with high dilutions of phosphorus *in vitro*. For this, co-cultures of macrophages RAW 264.7 and *E. cuniculi* exposed to different high dilutions of Phosphorus were analyzed according the following parameters: phagocytosis, lysosome activity, cytokines/chemokines production and ultrastructure analysis. The control solution was the vehicle used to produce the matrix of the tested medicines (30% alcohol diluted 1:100 in water, being 0.06% the final alcohol concentration). Evaluations were performed at 01 and 24 hours post-infection. Phagocytosis and the lysosome activity were evaluated using, respectively, calcofluor and acridine orange staining methods, followed by automatic image analysis (Ofline Metamorph®). The cytokine production was evaluated using the MAGPIX-LUMINEX system. The experiments were done in triplicate. The vehicle itself could increase IL6, TNF alpha and MCP1 production ($p<0.04$), reducing the number of phagocyted parasites ($p<0.001$). A transitory increase of RANTES was seen after 1 hour of treatment with Phosphorus 200cH, followed by increase of the lysosome activity in the first and 24 hours of treatment ($p<0.002$). The ultra-structural features of internalized parasites after Phosphorus 200cH treatment show evidences of more intense parasite digestion activity in relation to the cells treated with the vehicle only. The results show that the treatment of infected macrophages with Phosphorus 200cH causes significant changes in their activity, suggesting some mechanisms to understand the clinical improvement of sick animals, seen after the treatment with this medicine.

Key words: *Encephalitozoon cuniculi*, macrophages, Phosphorus, *in vitro* models, homeopathy.

1. Introduction

The microsporidian *Encephalitozoon cuniculi* is an extremely important pathogen for your zoonotic and opportunist power. It affects both domestic rabbits as laboratory, causing a severe and chronic granulomatous interstitial nephritis with fibrosis and granulomatous encephalitis [1]. Since, in Europe and in Asian countries there is a tradition of consuming the meat of domestic rabbit (*Oryctolagus cuniculus*), for reasons of public health, is very important to monitor possible infections of the meat or the transmission from animals to man [2].

The encephalitozoonosis in humans cause local infections able to spread to systemic infections. The competence of the host immune response and the species involved is what directs the pathogenic aspects of the disease. [3,4]. According to Wasson, rabbits infected with *E. cuniculi* via ingestion or inhalation of spores, as well as via transplacental. During the acute phase of the disease, which lasts an average of 30 days, the microsporidian replicates in the lungs, liver and kidneys [5]. This phase is usually asymptomatic, and lesions are limited [6]. We can classify the clinical signs in 3 groups: neuronal signals with vestibular syndrome (including head tilt), kidney failure and eye injuries. Among the neuronal signals, we can highlight: vestibular syndrome, weakness, paresis of the hind limbs and ataxia. It is not easy to diagnose encefalitozoonosis *in vivo*, but is crucial to determine the possible zoonotic risks [7-8].

According to Abu-Akkada and Oda, the oral administration of Fenbendazole was only effective in a prophylactic way, in a context of an experimental infection model, but without significant effects when it was used therapeutically [9]. In fact, there is no treatment for encefalitozoonosis. Considering the difficulty to establish an effective therapeutic protocol, the search for alternatives is crucial.

Face to this scenario, we hypothesize that homeopathy could be a viable therapeutic possibility. Several studies have been carried out showing the effects of homeopathic medicines in animals of different species [10, 11], under different nosologically conditions, however, the understanding of the mechanisms involved, is still very poor [12]. Since *in vitro* studies have more chances to observe intracellular mechanisms involved, especially when the macrophage interaction and the pathogen is essential in pathogenesis of the disease, we have

chosen this cell type as a model. Indeed, our team have previously demonstrated the involvement of macrophages and their modulation in the activity of different homeopathic treatments, after experimental infections. These experimental studies have shown that high diluted medicines do not exterminate the microorganisms in their hosts, but facilitates the organization of the immune response to a more effective pattern, improving the process of adaptation of the organism faster than it could be done naturally [13-23].

The homeopathic medicine chosen to be tested was elected by the traditional concept of "genius epidemicus", in which, a population is affected by an infectious disease and is treated with a single homeopathic medicine, considering the main symptoms expressed by most of its members [24,25]. The previous clinical experience observed by one of the co-authors of this paper endorsed our choice. A property of 40 rabbits presented 03 animals affected by encefalitozoonosis. All of 40 rabbits were treated with *Phosphorus* (*Phos*) 200cH, weekly, and the symptoms of the 03 sick animals disappeared in few weeks. *Phos* was the chosen due to its similarity of symptoms described in the homeopathic *materia medica* and those described in the rabbits bearing encephalitozoonosis. The three most important symptoms are: cataract, weakness of limbs and torticollis [7, 8]. This criterion of choosing the medicine is also a way to challenge the "similia principle" in an experimental condition. Thus, since there are no effective therapeutic protocols to this disease, this finding was very suggestive and inspired the organization of this *in vitro* systematic study. An important remark is that Homeopathy is an official veterinarian specialization in Brazil, since 1997.

The aim of this study was evaluating the mechanisms of interaction between macrophages and *E. cuniculi in vitro* after the treatment with different homeopathic dilutions of *Phosphorus*.

2. Materials and methods

2.1. Preparation of homeopathic medicine, quality control and blinding

Phos itself is an insoluble substance, so it must be tritured in lactose under heat, in a serial sequence, before to be diluted in 70% hydro-alcoholic solution. This drug was prepared in accordance with the guidelines described in Brazilian Homeopathic Pharmacopoeia – 3rd Edition, 2011 [26]. After sequential 1:100 dilutions, followed by 100 succussions (vertical agitations), the matrix dilutions were prepared using 30% ethanol solution (*Phos* 5 cH, 29 cH and 199 cH). This step of the preparation was made in a commercial pharmacy accredited by the National Health Surveillance Agency – Brazil (ANVISA). The final dilutions (*Phos* 6 cH, 30 cH and 200 cH) were made in our own laboratory, using pure water under sterile conditions, one day before the cell assays. Before to be used in the cell cultures, this final dilution was still filtered in a 22 micra filter (Millipore®) and the flasks received **codes** by the staff not involved in the experiment laboratory, with the aim **to warrant blinded procedures** until the statistical analyses were completed.

After coding and before using the medicines in the experiments, a fast test of quality control was made. Samples of each dilution were examined in a scanning electronic microscope to proceed the identification of suspended microparticles, followed by the analysis of their chemical nature using a EDS (Energy Dispersion Spectroscopy) system. Since contaminant micro and nanoparticles are expected in the water, this analysis was useful to eliminate any possibility of a predominant element be the responsible by the effects described. The identification of suspended contaminant microparticles contained in the tested solutions was performed by means of the micro-evaporation method, followed by scanning electron microscopy (SEM) under 10-kV working conditions and by energy dispersive X-ray spectroscopy (EDX) (JSM 6510, JEOL Ltd, Tokyo, Japan). The details of this procedure and the elements found are described in **Box 1**.

Box 1. Methodological steps of microparticle profile of the tested medicines using a SEM+EDS system.

Micro-tubes containing samples of each tested solution were centrifuged at 5000 rpm for 30 minutes for the microparticles to sediment. A single drop of 10 µl was harvested from the bottom of each tube and placed on a stub. The stubs were previously cleansed with 70% acetone and dried in a closed recipient. The stubs were then kept within a closed Petri dish to ensure that only particles obtained through centrifugation of the tested solutions were analyzed. The drops were left to dry naturally on the stubs up to the time of microscopic analysis. Images were taken with a 10-µm scale. The samples were harvested only once from each tube, because in a pilot study we found that the second and third drops were almost particle-free. Thirty microparticles deposited on the stubs were randomly chosen for EDX analysis; for samples that did not reach this number, all the deposited particles were analyzed. As the stubs were made of copper, this element was not considered in analysis to avoid false positive results.

This quality control revealed that vehicle and *Phos* 200cH presented microparticles in suspension measuring between 0.5 to 1.0 µM. Traces of Cl, Pr, Ca and Na (1 to 8%), Zn and Si (7 to 14%), Au (4 to 14%) were randomly found in the composition of particles isolated from the vehicle and traces of Na (35-86%), Zn (15%), Pr (0-12%) and Pb (2-27%) were seen in the composition of particles obtained from *Phos* 200cH. This random distribution of elements in the microparticles was also seen in the other dilutions, such as *Phos* 6cH, presenting particles of 1 to 5 µM containing S (12%), Ca (4-48%), Pr (1-23%), Zn (2-6%), Au (3-14%), Pb (50-70%); and *Phos* 30cH, presenting particles of 1 to 5 µM containing Ca (7-43%), Au (14-22%), Al (0.5 – 4%). This random distribution of elements among the particles, in all tested preparations, shows that no predominant element was present, which could be related to the observed biological effects.

After all these steps, the treatment of infected cells was performed according to the following design: two experimental series were performed, being the first one after 1 hour of infection and the second one analyzed after 24 hours, to verify the internalization of the fungi by the macrophages in different steps. These times were chosen due to the fast digestion activity of RAW 264.7 cells. For each 24 wells plate, wells were distributed in parts: 2 wells containing only macrophages and other 2 wells containing macrophages + *E. cuniculi*, both without

treatment. These samples were used to define the baseline values for each studied parameter and were not included in the statistical analysis. The remaining wells were divided in such a way that each medicine and the vehicle (samples A, B, C, D) were tested in quadruplicate. The volume of the medicines and vehicle was equal to 20% of the total liquid volume in each well (500 µl), being 34µl of *E. cuniculi* suspension + 100 µl medicine + 366 µl RPMI with 10% BFS.

2.2. Culture of macrophages

RAW 264.7 lineage macrophages were purchased from the Cell Bank of Rio de Janeiro and kept frozen in a -80°C freezer. After thawing, they were expanded in RPMI medium (Cultilab, Campinas, Brazil) enriched with 10% BFS in cell culture bottles. For plating the cells, round coverslips were added to the 24-well sterile plates, being one coverslip for each well.

When the confluence of the cells (macrophages) reached 90% in the bottles, the cells were washed with serum-free PBS for the removal of any dead cells in the supernatant. Then, 5ml of RPMI medium were added to make the cell detachment, using a cell scraper. With a Pasteur pipette, the bottle was washed, up to the complete removal of detached cells. The cells were transferred to a Falcon tube and their viability was checked automatically, in the Countess® automatic counting (Life Tech, USA), using 0.4% Trypan Blue dye.

The cells were held on ice and centrifuged for 3 min at 1000 rpm. The supernatant was discarded, and the pellet was homogenized in RPMI. After suspension, cells were plated out (5×10^5 macrophages per coverslip) in 50 microliters of RPMI. They were dispensed in the center of the coverslip and gently moved for a homogeneous distribution of the cells. Before the incubation, 250 µl of RPMI enriched with 10% BFS were added to each well. The plate was incubated at 37°C for at least 2 hours, for complete adherence of the macrophages. Then, the inclusion of *E. cuniculi* spores was performed, in a 2:1 ratio, in relation to macrophages.

E. cuniculi spores were previously cultured in RK (rabbit kidney) cells. RK cells were kept in Eagle´s medium (Cultilab, Campinas, Brazil) supplemented with bovine fetal serum (BFS) (Cultilab, Campinas, Brazil), non-essential amino acids and 10% pyruvate, with penicillin-streptomycin (Sigma-Aldrich, St Louis, USA) and incubated with 5% CO_2 at 37^{o}C. Every

seven days, cultures supernatants were collected and centrifuged for 30 minutes at 500 g to obtain spores, that were further kept at 4°C in PBS 1x. Spores of *E. cuniculi* were counted in a Neubauer chamber [3], before to be incubated with macrophages. The cells were treated at the moment of infection, according to the protocol described above.

2.3. Analysis of parasite internalization

The analyses of the number of parasites internalized (by endocytosis and phagocytosis) was measured from digital photomicrographs made in a fluorescence microscope (OLYMPUS 60i, Japan) of the non-fixed cells adhered to the coverslip and stained by Calcofluor – White Stain (Sigma®) (10 microliter per coverslip) after 1 and 24 hours of infection and treatment. In this method, the spores become blue and fluorescent, due the capacity of the dye to interact with their chitin wall. Ten microscopic fields by each coverslip were recorded using a 40x objective, then, the captured images were analyzed by the manual counting tool in the Offline Metamorph® software (Molecular Devices, USA), to calculate the number of internalized spores per macrophage.

2.4. Determination of cytokines and chemokines

Culture supernatants were harvested after different times, for subsequent centrifuging and freezing at -80°C. The dosage was made by the MAGPIX method-Luminex (MILLIPORE®, USA; Kit e-Bioscience, USA), following the manufacturer's instructions. The Kit includes the detection of the following cytokines: IL-1 alpha, IL-1 beta, IFN-gamma, IL-10, IL-6, VEGF-, TNF-alpha, RANTES/CCL5, GM-CSF, IL12-p40, IL12-p70, MIP -1 beta/CCL4, MCP-1/CCL2. Those peptides that exhibit values bellow the detection limits of the method (defined in the manufacturer manual) were excluded from the statistical analysis.

2.5. Lysosome activity by fluorescence microscopy

After 1 and 24 hours, coverslips containing adherent macrophages infected with *E. cuniculi* were incubated with acridine orange dye, for 20 minutes at 37°C, to check the lysosome activity in real time. After incubation, coverslips were washed in PBS and mounted on slides, using a drop of PBS as adherent. The quantitative analysis was automatically made by Offline Metamorph® software (Molecular Devices, USA), in which the brightness intensity of the

orange color exhibited by the vacuoles was measured. To warrant trustful results, the color filter of the software was standardized and adjusted to: measurement of light area; color model: HIS; Hue: 19-20; Intensity: 89-90. The principle of this method is based on the capacity of the dye to increase its orange intensity in acid mediums.

2.6. TEM

A replication of the experiment was made after the opening of the codes and two treatments were chosen to be submitted to the transmission electronic microscopy (TEM), that are: vehicle and *Phos* 200cH. At this time, the treatment was made in the culture bottle instead of plates, in such a way that, after 24 hours of treatment, the cells were detached from the bottom of the bottle with a cell scraper and suspended in cacodylate buffer (0.2 M, pH = 7.2) for 10 h at 4^{o}C and fixed in 2% glutaraldehyde. After centrifugation (1500 RPM for 5 minutes), the pellet obtained in the bottom of each microtube was post fixed in buffered 1% OsO_4 for 2 hours, treated in crescent concentrations of propylene oxide and embedded in Epon resin. The material was previously checked in an optical microscope after the preparation of semi-slim cuts, stained with toluidine blue, aqueous uranyl citrate and lead citrate. The ultra-structural images were captured in an electronic microscope Tecnai FEI G20, with tomographic capacity, equipped with a high-resolution camera (Eagle 4kx4k).

2.7. Statistical analysis

The statistical analysis was performed using IBM SPSS programs, version 21.0 and GraphPad Prism, version 6.0, for Windows™. Normality was assessed using Shapiro-Wilk test and evaluated by inspection of quartile-quartile plots (Q-Q plots). The homogeneity of the variances was evaluated by Levene test and Welch correction was applied to ANOVA in cases of non-homogeneity. Outliers were evaluated by Q-Q plot inspection and removed if necessary.

The results were evaluated through one-way ANOVA and post-test of Dunnett or Games-Howell, according to the case. The partial eta-square (η^2), a measure of effect size, has also been reported. This parameter is particularly useful for variables presenting large deviation values and p-value analyzes that could lead to type I errors (rejection of a true null hypothesis) or type II errors (retention of false null hypotheses). Results were presented as

mean ± standard error and values of $p<0.05$ were considered significant. Figures were made using Prism 5.0 software.

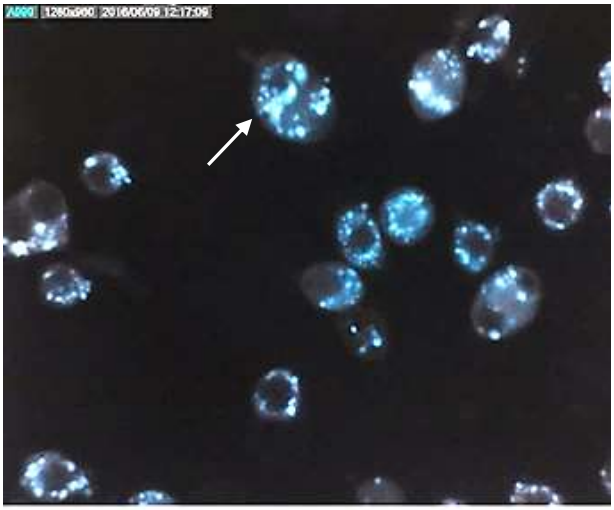
3. Results

The internalized micro-organisms presented different patterns in function of the treatment. As seen in **Figure 1**, the number and size of internalized fungi are very reduced in all treatments after 24 hours, including vehicle, being more evident in *Phos* 200 cH treated cells.

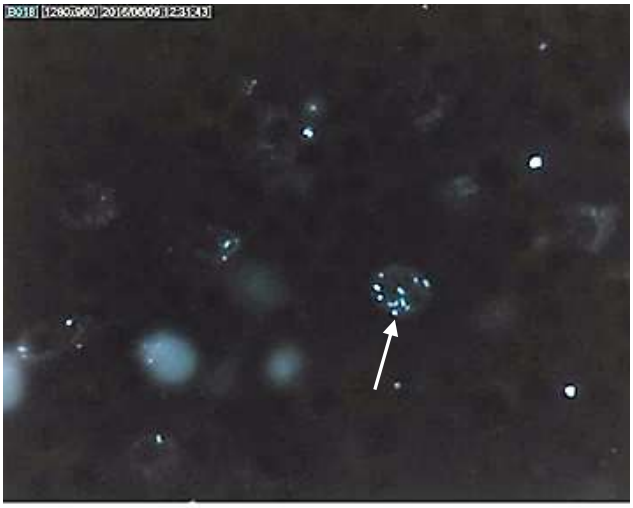
The quantification of this parameter in the image analysis system confirmed this observation (**Figure 2**). The images obtained from TEM and semi-slim cuts also corroborate these findings (**Figures 3 and 4**). Thus, the ultra-structure seen in *Phos* 200cH treated cells shows no undigested parasite. Instead, thinly and abundant parasite debris were seen in the cytoplasm of these cells, some of them presenting degeneration morphology. On the other hand, in vehicle treated cells many preserved units of fungi could be seen attached to the external surface of the cells (**Figure 4**).

The acridine orange assay put in evidence the increase of brightness intensity in fluorescent cytoplasmic vacuoles of cells treated with *Phos* 200 cH, in relation to the other treatments and to the untreated control. This pattern was seen in 1 and 24 hours of observation (**Figures 5 and 6**).

The increase in IL-6, MIP 1β, TNFα and MCP-1 in the supernatant was seen after 1 hour of observation (and 24 hours for MCP-1) in vehicle-treated cells, being considered as unspecific vehicle-dependent effect. The transitory peak of RANTES, instead, can be seen as specific effect, since only *Phos* 200 cH could exhibit this pattern (**Tables 1 and 2; Figure 7**).

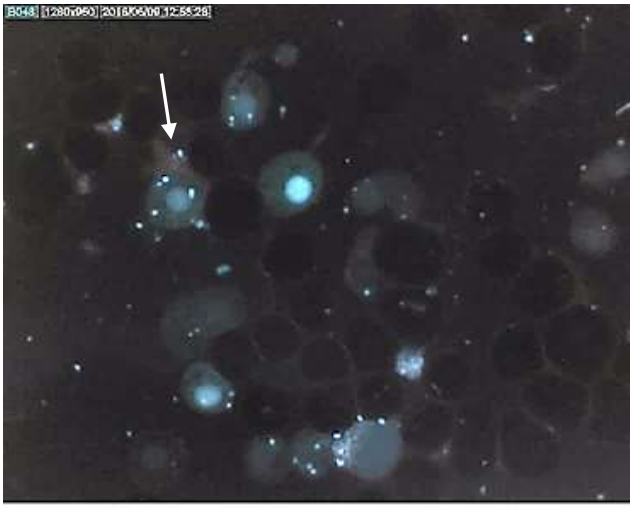


Untreated control

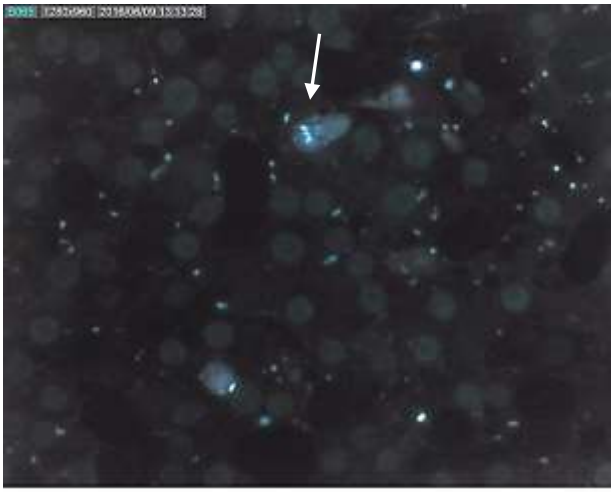


Vehicle

***Phos* 6cH**



***Phos* 30cH**



***Phos* 200cH**

Figure 1. Photomicrograph of macrophages adhered on coverslips and infected with *E. cuniculi* after 24 hours - untreated control or treated with vehicle or treated with *Phos* in different dilutions. Arrows: spores marked by the blue fluorescent dye Calcofluor-White, due to their chitin wall. Objective 200x.

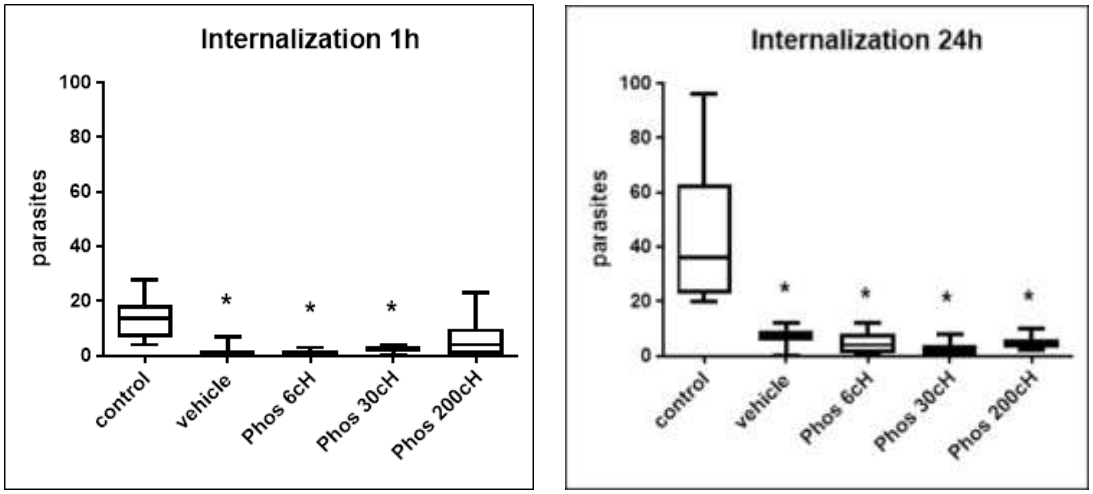


Figure 2. Number of spores inside macrophages marked with Calcofluor-White staining method and observed at a fluorescence microscope. The counting was made by the Offline Metamorph® software, using a manual counting system. After one hour: *ANOVA, Games-Howell, F (4, 25.422) = 9.663, p<0.001, η^2 = 0.486 in relation to control. After 24 hours: * ANOVA, Games-Howell, F (4, 21.232) = 9.387, p<0.001, η^2 = 0.744. Values are represented by mean and standard error. Control = untreated infected macrophages.

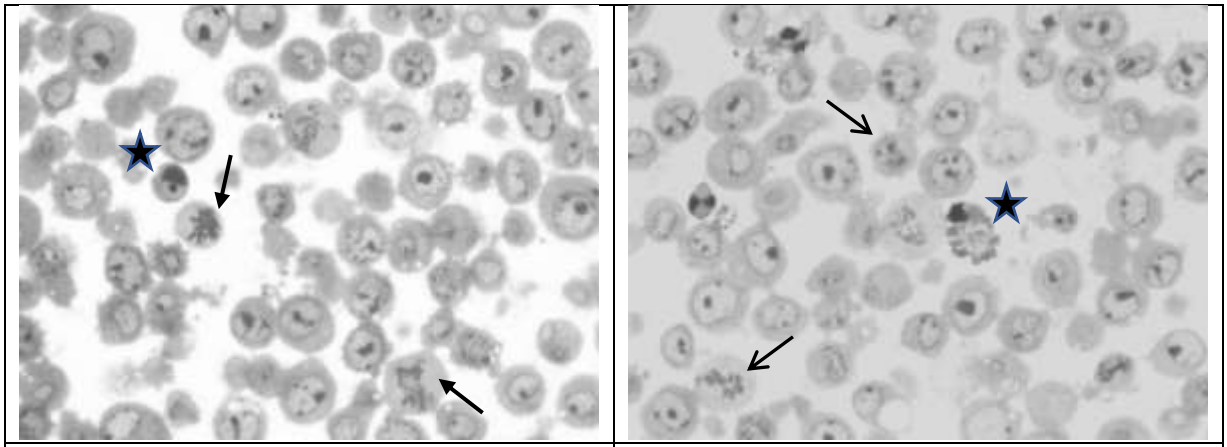


Figure 3. Semi-slim cuts of the macrophage pellet obtained from a fixed cell pellet embedded in Epon resin. The cuts were stained by toluidine blue, aqueous uranyl citrate and lead citrate. A) Untreated control; B) Phos 200 cH treated cells. Objective: 100x. Arrows: infected cells. Stars: degenerated, probably apoptotic cells.

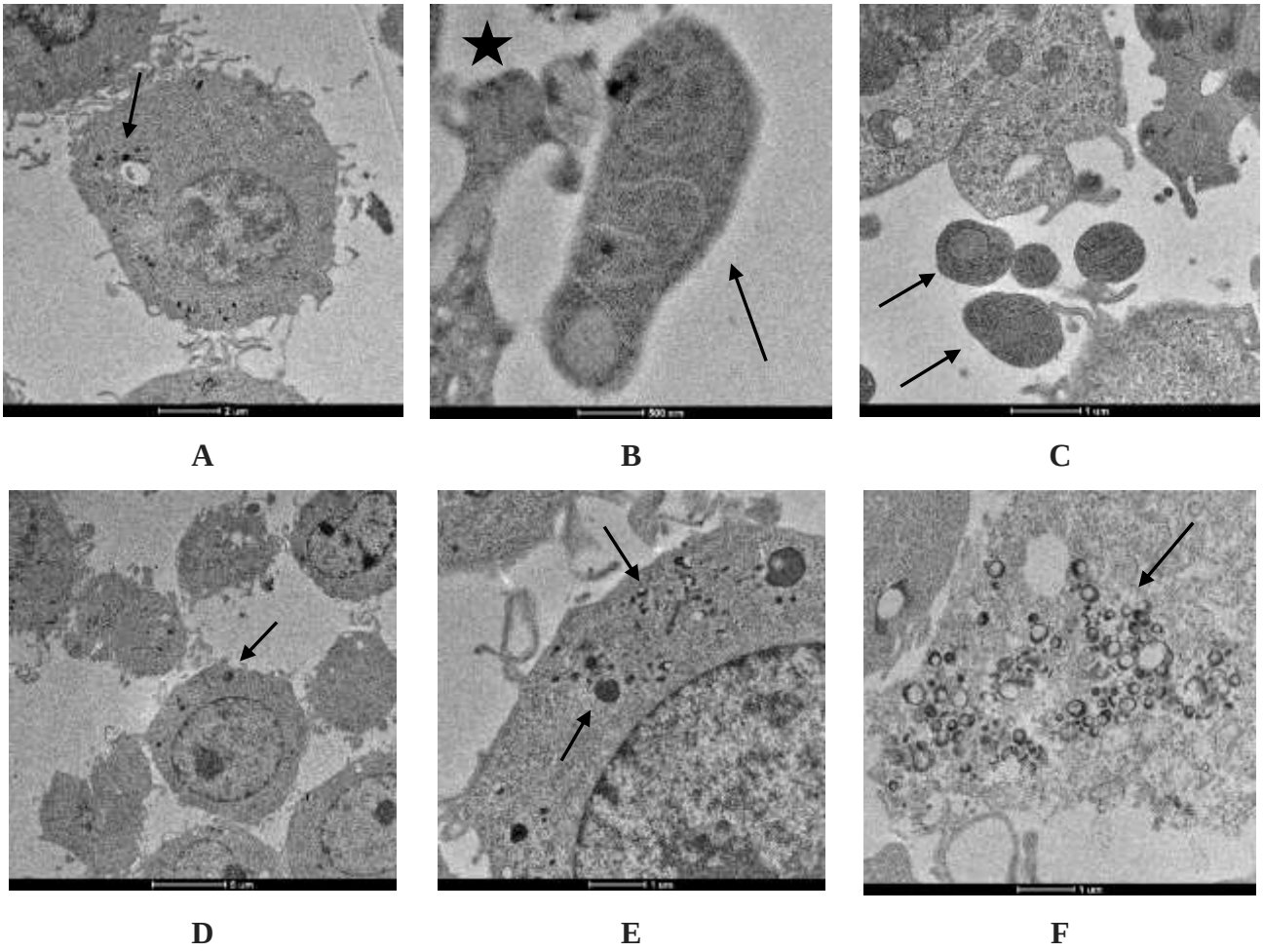
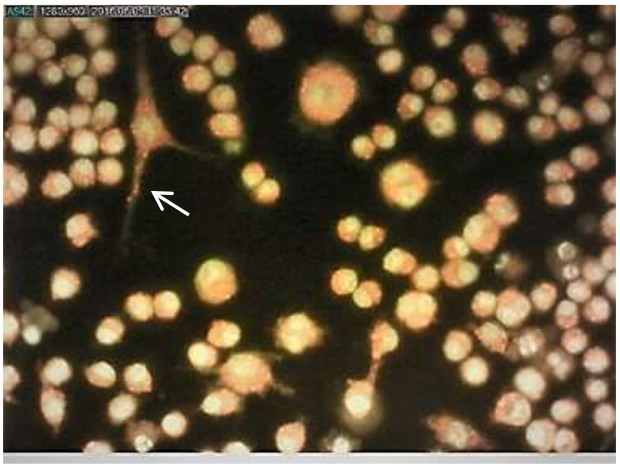


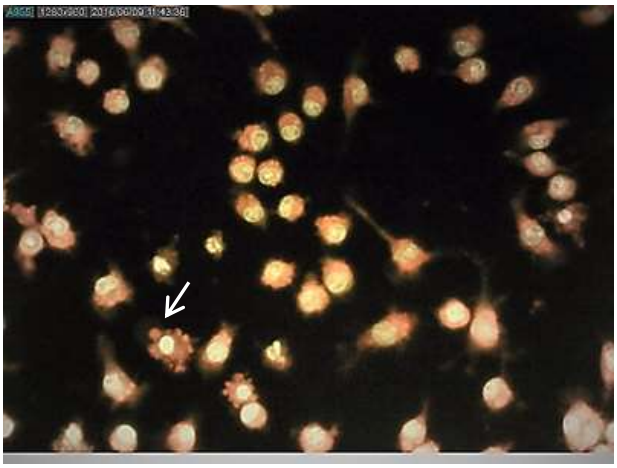
Figure 4. Ultramicrography of macrophages infected with *E. cuniculi* and treated with vehicle and *Phos* 200cH. A, B and C are images captured from cells treated with vehicle; D, E, F are images captured from cells treated with *Phos* 200cH. (A) Macrophage treated with vehicle containing multiple vacuoles of digestion with debris of the parasites inside. Magnificence: 3500x; (B) *E. cuniculi* attached to a macrophage membrane at the time of internalization. Magnificence: 14500x; (C) *E. cuniculi* attached to a macrophage membrane at the time of internalization. Magnificence: 7800x; (D) Macrophages treated with *Phos* 200cH containing multiple vacuoles of digestion with debris of the parasites inside. Magnificence: 1700x; (E) Macrophage treated with *Phos* 200cH containing multiple vacuoles of digestion with thinly debris of the parasites inside. Magnificence: 6500x; (F) Detail of multiple and thinly debris of parasites inside a degenerated macrophage treated with *Phos* 200cH. Magnificence: 6500x. Arrows: parasites (units and debris). Star: macrophage pseudopods.

Untreated control

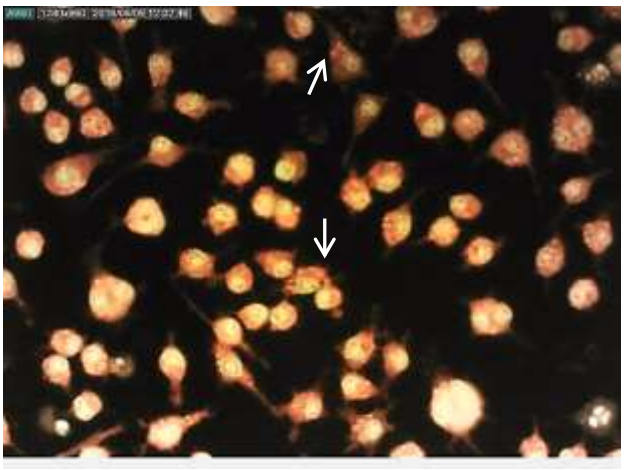


Vehicle

***Phos* 6cH**



***Phos* 30cH**



***Phos* 200cH**

Figure 5. Photomicrograph of macrophages adhered on coverslips and infected with *E. cuniculi* after 24 hours - untreated control or treated with vehicle or treated with *Phos* in different dilutions. The cells cytoplasm exhibits different degrees of brightness due to the orange fluorescent stained lysosomes (arrows), marked by the Acridine orange dye, after 20 minutes of incubation at 37°C. Objective 200x.

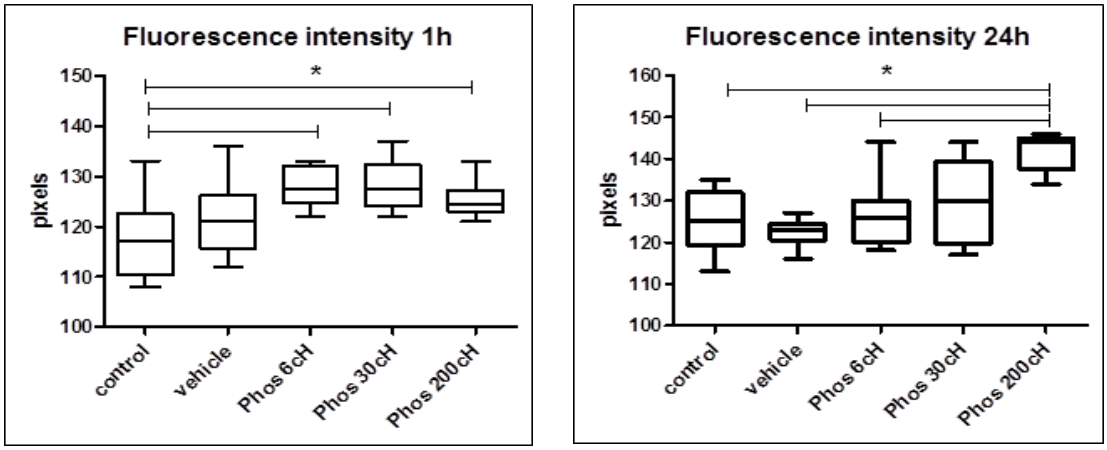
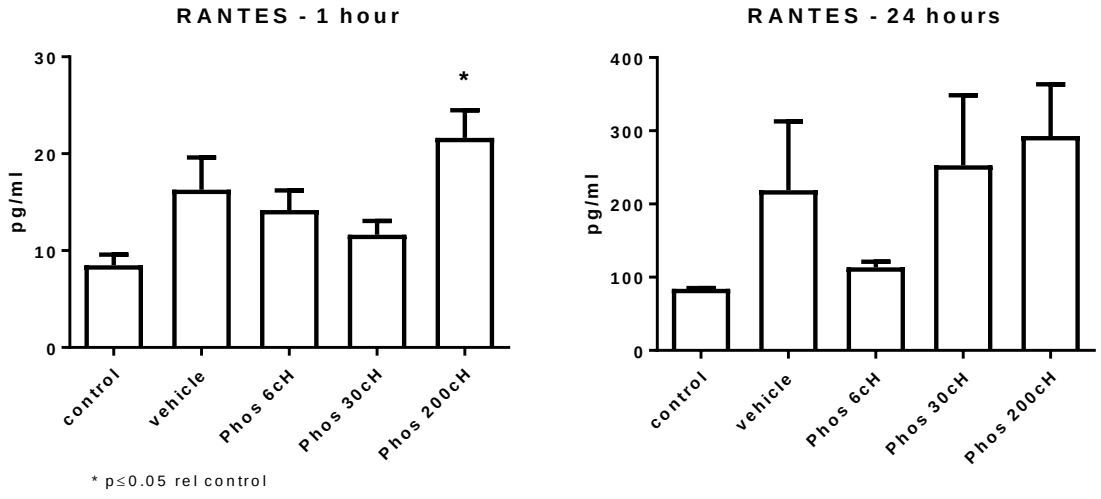


Figure 6. Average of fluorescence intensity (pixels) of macrophages stained with acridine orange and observed at a fluorescence microscope. The measures were made by the Metamorph® off-line software, using automatic counting system. After one hour: *ANOVA, Games-Howell, F (4, 23.653) = 6.060, p=0.002, η2 = 0.390 in relation to control. After 24 hours: * ANOVA, Games-Howell, F (4, 21.150) = 53.624, p<0.001, η2 = 0.522. Values are represented by mean and standard error.



A **B**

Figure 7. Concentration of relevant peptides in the supernatant of macrophage infected with *E. cuniculi* after 1 hour (A) and 24 hours (B) of treatment with different homeopathic dilutions of Phosphorus. (A) *ANOVA, Tukey, F (4, 11) = 3.372, p=0.05, η2 = 0.574 in relation to control. (B) ANOVA, Games-Howell, F (4, 4.027) = 4.585, p=0.084, η2 = 0.378. Values are represented by mean and standard error.

Table 1. Cytokines, chemokines and VEGF concentration in the supernatant of the macrophages infected with *E. cuniculi* after 1 hour of treatment with different homeopathic dilutions of Phosphorus. The values are represented by mean and standard deviation, level of significance and eta-squared (effect size, η2). *ANOVA, Games-Howell in relation to control.

Peptide	Mean ± Standard deviation					p value	
	UNTR CONT	VEHICLE	*Phos* 6cH	*Phos* 30cH	*Phos* 200cH	η2	
IL 1α	14,08 ±5.41	30.08 ± 7.80	2859 ± 16.63	25.95 ± 15.75	26.94 ± 17.52	**0.786**	**0.135**
IFNγ	4.94 ± 0.39	5.33 ± 0.56	4.45 ± 0.68	4.21 ± 0.76	5.50 ± 2.88	**0.464**	**0.162**
IL 6	3.52 ± 0.71	6.83 ± 1.69 *	5.79 ± 0.65	5.55 ± 1.29	5.13 ± 0.46	**0.035**	**0.582**
IL 10	21.47 ± 8.40	51.98 ± 26.02	49.57 ± 19.93	45.78 ± 24.26	50.09 ± 29.08	**0.602**	**0.191**
IL 12p40	6.23 ± 1.04	6.61 ± 2.46	5.70 ± 0.48	4.96 ± 0.55	5.95 ± 3.06	**0.502**	**0.134**
IL 12p70	8.10 ± 3.05	17.43 ± 13.12	16.69 ± 8.04	14.34 ± 8.32	5.04 ± 2.14	**0.332**	**0.319**
MCP 1	717.5 ± 21.92	1816.0 ± 128.29 *	1942.0 ± 228.93 *	1633.75 ± 131.20	1774.33 ± 721.21 *	**p<0.001**	**0.629**
MIP 1β	6036 ± 1193.59	10959.0 ± 816.67 *	10087.75 ± 732.64	8896.75 ± 633.61	7624.0 ± 5719.37	**0.062**	**0.358**
RANTES	8.49 ± 1.55	14.17 ± 4.09	14.17 ± 4.09	11.64 ± 2.85	**21.65 ± 4.01 ***	**0.054**	**0.574**
VEGF	2.65 ± 0	3.06 ± 0.41	3.13 ± 0.13	2.93 ± 0.33	3.01 ± 0.83	**0.769**	**0.131**
TNFα	1887 ± 57.98	2320.5 ± 177.48 *	2374.75 ± 331.05	2208.75 ± 99.66	1865.0 ± 1071.80	**0.046**	**0.359**

Table 2. Cytokines, chemokines and VEGF concentration in the supernatant of the macrophages infected with *E. cuniculi* after 24 hours of treatment with different homeopathic dilutions of Phosphorus. The values are represented by mean and standard deviation, level of significance and eta-squared (effect size, η2). **ANOVA, Games-Howell in relation to the other groups.

Peptide	Mean ± Standard deviation					p value	
	UNTR CONT	VEHICLE	*Phos* 6cH	*Phos* 30cH	*Phos* 200cH	η2	
IL 1α	78.19 ± 4.78	56.09 ± 26.46	72.69 ± 1.83	84.50 ± 11.31	90.01 ± 11.46	**0.332**	**0.562**
IFNγ	5.97 ± 0.22	4.02 ± 1.86	5.58 ± 1.33	6.59 ± 2.54	7.30 ± 2.56	**0.406**	**0.332**
IL 6	5.40 ± 0.27	20.36 ± 5.76	10.64 ± 6.30	23.04 ± 17.55	26.67 ± 9.28	**0.191**	**0.461**
IL 10	48.01 ± 2.20	46.59 ± 25.81	49.64 ± 16.18	54.69 ± 30.09	51.73 ± 26.98	**0.994**	**0.022**
IL 12p40	5.56 ± 0.29	5.02 ± 1.42	5.46 ± 1.40	5.92 ± 2.08	8.55 ± 2.58	**0.222**	**0.438**
IL 12p70	5.37 ± 0.80	3.88 ± 0.88	6.82 ± 3.50	7.86 ± 3.63	6.01 ± 1.25	**0.428**	**0.321**
MCP1	4981.0 ± 1544.32 **	9025.66 ± 411.07	9406.66 ± 1287.16	9736.0 ± 1681.37	9752.66 ± 1096.79	**0.014**	**0.717**
MIP 1β	16289.5 ± 331.63	14912.0 ± 2834.04	15956.66 ± 1472.71	16640.66 ± 1302.29	16252.67 ± 395.93	**0.752**	**0.175**
RANTES	84.28 ± 1.28	218.66 ± 163.22	113.38 ± 13.50	252.66 ± 165.71	292.66 ± 122.83	**0.084**	**0.378**
VEGF	2.65 ± 0.26	2.86 ± 0.32	3.00 ± 0.48	2.91 ± 0.47	3.03 ± 0.42	**0.864**	**0.121**
TNFα	3154.0 ± 216.37	2207.0 ± 936.05	7030.0 ± 5963.97	2619.0 ± 895.76	2735.66 ± 367.45	**0.450**	**0.384**

4. Discussion

A general view of the results shows that the effects of the treatments can be classified in two types: specific and unspecific, according to the effects observed for each treatment in relation to vehicle (specific vehicle independent effects) and those observed in the vehicle treated cells, in relation to untreated control (unspecific vehicle dependent effects). Thus, the unspecific vehicle-dependent effects could be listed as: reduction of the number of internalized parasites and increase of IL-6, MIP 1β, TNFα and MCP-1. The specific vehicle-independent effects would be: increase in lysosome activity after 01 and 24 hours - being the effects of *Phos* 200cH more evident after 24 hours - and the transitory peak of RANTES seen after one hour of infection in *Phos* 200cH treated cells. This peak decreased after 24 hours.

In both cases, there was no correlation between these findings and the patterns of size and chemical composition of the contaminant microparticles suspended in all homeopathic preparations. Instead, a complete random distribution of elements was seen in all medicines and vehicle, as described in methods. Although these particles are always present, as previously described by other authors [27-30], there is no evidence that they could participate of the biological effects in this case. In fact, previous results described by our group lead us to the same conclusion [31]. Anyway, the physico-chemical control of the medicines done before the execution of the experiments was useful to exclude any possibility of unspecific effects due to the predominance of contaminant elements in sufficient quantity to produce biological changes.

On the other hand, the cell mechanisms to homeopathic treatments have been very well described in the last years and are related to the epigenetic control of specific genes, in function of the nature of the medicines and dilutions [16, 17, 32-36]. In our previous experience, macrophages can respond to specific homeopathic stimuli by the expression of cytokines and chemokines involved in the experimental context, i.e., the blockage of MCP- 1 production induced by *Antimonium crudum* 30cH on macrophages infected with *Leishmania amazonensis* was in accordance to the anti-inflammatory action of this medicine *in vivo* [13, 25]. In the present study, the specific (vehicle independent) effects were seen after *Phos* 200cH treatment and are represented by changes in lysosome activity after 1 and 24 hours from treatment and a peak of RANTES (CCL5) after 1 hour of treatment that persisted after 24 hours, but without statistical significance, indicating to be a transitory effect.

This peak of RANTES has a significant biological meaning. During acute infections, the chemokin RANTES (CCL5) is expressed in different hematopoietics and non-hematopoietics cell types, which presents a very important function in the migration of memory T cells and effector T cells. Moreover, the biggest goal of HIV drugs is the RANTES receptor, CCR5, blocking the viral entry. However, problems in RANTES or their receptors (CCR5) may impair immunity in acute infections in animal models and aggravate disease in humans infected with West Nile virus (WNV) [37]. According to Kawai and colleagues, it is probable that RANTES – CCR5 interaction promotes diapedesis of Th1 adhesion cells so, the improvement of selective migration of Th1 could be explained in this case [38].

In the context of *E. cuniculi* infection, RANTES among other chemokines, has an important role in granulocytes and T cell migration. As reported by Fisher and colleagues, in the infection by *E. cuniculi,* the expression of countless chemokines responsible for the recruitment of granulocytes (CXCL1, CXCL2, CXCL3, CXCL5 and CXCL8) occurs, confirming the hypothesis that other cellular types are present at the infection site [39]. Patients infected with HIV1 that exhibit higher serum levels of RANTES are partially protected against the infection [40].

Herein, the transient increase of RANTES can be the result of the monoculture: as there is no NK cells or lymphocytes in co-culture with macrophages *in vitro*, no feedback for further production of RANTES was possible. Maybe *in vivo* the production of RANTES could be more durable. This is a hypothesis that must be checked in the future. Curiously, the specific increase in RANTES after the treatment with *Phos* 200cH is in accordance with the biological context involving *E. cuniculi* infection, that is, immunosuppressive conditions in which the capacity of patients to secrete RANTES is related to a better prognosis [40]. This context could be "translated" to the traditional concept of "ground" or "susceptibility", as described by Hahnemann in the beginning of the XIX century [41]. On the other hand, the fast digestion of the parasites inside macrophages could also explain the short period of RANTES identification in the supernatant.

About the increase of lysosome activity, the analysis of the infected cells using a quantitative and automatic method (Metamorph®) allowed to determine its intensity with high sensibility. This finding was corroborated by the images got by TEM, in which the parasite debris inside the cytoplasm were clearly seen. Thus, the decrease of parasite internalization induced by the vehicle itself is probably related more to the increase in parasite digestion than to the decrease in the phagocytosis or endocytosis. In this sense, intact parasites were seen only in vehicle treated cells, but in *Phos* 200cH treated cells almost all macrophages presented an impressive number of thinly fragments of parasite inside the digestion vacuoles, suggesting a more efficient capacity to do it. The reason by which the vehicle itself is able to increase the parasite digestion in relation to the untreated control is not known, but one plausible hypothesis is the generation of multiple nanobubbles inside the liquid submitted to serial succussion during the manufacture process of the remedy [42], which could stimulate the exudative activity of macrophages in the culture plate, leading to the increase of digestion

process and the production of inflammatory cytokines and chemokines, such as IL 6, MCP-1 and MIP 1beta, as seen in Tables 1 and 2.

These findings corroborate the previous clinical experience, as reported in the introduction, and give a mechanistic explanation to it. Although it is still a first systematic study about the use of *Phos* 200 cH in the treatment of encefalitozoonosis, the results open a new line of research toward further clinical controlled trials and give perspectives to an unsolved problem regarding to infections by opportunistic agents.

5. Conclusion

These *in vitro* results could explain the mechanism behind the clinical benefits of *Phos* 200 cH in cases of encefalitozoonosis due to the following findings:

a) Production of RANTES (and putative organization of Th1 response against the infected cells *in vivo*);
b) Increase of lysosome activity and increase of micro-organism digestion in the macrophages.

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7. Conflict of interest

There is no conflict of interest related to this project.

8. References

1. Rodríguez-Tovar LE, Villarreal-Marroquín A, Nevárez-Garza AM, Castillo-Velázquez U, Rodríguez-Ramírez HG, Navarro-Soto MC Zárate-Ramos JJ, Hernández-Vidal G, Trejo-Chávez A. Histochemical study of *Encephalitozoon cuniculi* spores in the kidneys of naturally infected New Zealand rabbits. **J Vet Diagn Invest**. 2017 May;29(3):269-277. doi: 10.1177/1040638716668559. Epub 2017 Feb 16.

2. Neumayerová H, Juránková J, Jeklová E, Kudláčková H, Faldyna M, Kovařčík K, Jánová E, Koudela B. Seroprevalence of *Toxoplasma gondii* and *Encephalitozoon cuniculi* in rabbits from different farming systems. **Vet Parasitol**. 2014 Aug 29;204(3-4):184-90. doi: 10.1016/j.vetpar.2014.04.020. Epub 2014 May 1.

3. Francisco Neto A, Dell'Armelina Rocha PR, Perez EC, Xavier JG, Peres GB, Spadacci-Morena DD, Alvares-Saraiva AM, Lallo MA. Diabetes mellitus increases the susceptibility to encephalitozoonosis in mice. **PLoS One.** 2017 Nov 1;12(11):e0186954. doi: 10.1371/journal.pone.0186954. eCollection 2017.

4. Nell B, Csokai J, Fuchs-Baumgartinger A, Maaß G. *Encephalitozoon cuniculi* causes focal anterior cataract and uveitis in dogs. **Tierarztl Prax Ausg K Kleintiere Heimtiere**. 2015; 43(5):337-44. doi: 10.15654/TPK-141053. Epub 2015 Sep 10.

5. Wasson K, Peper RL. Mammalian microsporidiosis. **Vet Pathol** 2000; 37:113-128.

6. Harcourt-Brown FM. Encephalitozoon cuniculi infection in rabbits. **Seminars in Avian and Exotic Pet Medicine** 2004; 13:86-93.

7. Jordan CN, Zajac AM, Lindsay DS. *Encephalitozoon cuniculi* infection in rabbits. Compendium Magazine Veterinary. Vol.28. n.2. February 2006a; 108-116.

8. Künzel F, Gruber A, Tichy A, Edelhofer R, Nell B, Hassan J, Leschnik M, Thalhammer JG, Joachim A. Clinical symptoms and diagnosis of encephalitozoonosis in pet rabbits. **Vet Parasitol** 2008; 151: 115-124.

9. Abu-Akkada SS and Oda SS. Prevention and treatment of *Encephalitozoon cuniculi* infection in immunosuppressed rabbits with fenbendazole. Iran J Vet Res. 2016 Spring; 17(2): 98–105.

10. Bonamin LV, Endler PC. Animal models for studying homeopathy and high dilutions: conceptual critical review. **Homeopathy**. 2010 Jan;99(1):37-50. doi: 10.1016/j.homp.2009.11.003. Review.

11. Bonamin LV, Cardoso TN, de Carvalho AC, Amaral JG. The use of animal models in homeopathic research-a review of 2010-2014 PubMed indexed papers. **Homeopathy**. 2015 Oct;104(4):283-91. doi: 10.1016/j.homp.2015.06.002. Review.

12. Bonamin LV. A solidez da pesquisa básica em homeopatia. **Revista de Homeopatia**. 2017. 80 (1/2): 89-97.

13. Santana FR, Dalboni LC, Nascimento KF, Konno FT, Alvares-Saraiva AM, Correia MS, Bomfim MD, Casarin RC, Perez EC, Lallo MA, Peres GB, Laurenti MD, Benites NR, Buchi DF, Bonamin LV. High dilutions of antimony modulate cytokines production and macrophage - *Leishmania (L.) amazonensis* interaction *in vitro*. **Cytokine**. 2017, 92, 33-47. doi: 10.1016/j.cyto.2017.01.004.

14. Cajueiro APB, Goma EP, Dos Santos HAM, Almeida Rodrigues I, Toma HK, Araújo SM, Bonamin LV, Gomes NBN, Castelo-Branco MTL, de Souza Dias EP, Dos Santos Pyrrho A, Holandino C. Homeopathic medicines cause Th1 predominance and induce spleen and megakaryocytes changes in BALB/c mice infected with *Leishmania infantum*. **Cytokine**. 2017 Jul;95:97-101. doi: 10.1016/j.cyto.2017.02.010.

15. Nascimento KF, de Santana FR, da Costa CRV, Kaplum V, Volpato H, Nakamura CV, Bonamin LV, de Freitas Buchi D. M1 homeopathic complex trigger effective responses against *Leishmania (L) amazonensis in vivo* and *in vitro*. **Cytokine**. 2017 Jul 21;99:80-90. doi: 10.1016/j.cyto.2017.07.001.

16. Olioso D, Marzotto M, Bonafini C, Brizzi M, Bellavite P. *Arnica montana* effects on gene expression in a human macrophage cell line. Evaluation by quantitative Real-Time PCR. **Homeopathy**. 2016 May;105(2):131-47. doi: 10.1016/j.homp.2016.02.001.

17. Marzotto M, Bonafini C, Olioso D, Baruzzi A, Bettinetti L, Di Leva F, Galbiati E, Bellavite P. Arnica montana Stimulates Extracellular Matrix Gene Expression in a Macrophage Cell Line Differentiated to Wound-Healing Phenotype. **PLoS One**. 2016 Nov 10;11(11):e0166340. doi: 10.1371/journal.pone.0166340. eCollection 2016.

18. Doehring C, Sundrum A. Efficacy of homeopathy in livestock according to peer-reviewed publications from 1981 to 2014. **Vet Rec.** 2016 Dec 17;179(24):628. doi: 10.1136/vr.103779. Epub 2016 Dec 12.

19. Hahnemann, S. Organon da Arte de Curar. 3 ed. São Paulo: Bento Mure, 2007. 216 p.

20. Paula Coelho C, Motta PD, Petrillo M, de Oliveira Iovine R, Dalboni LC, Santana FR, Correia MS, Casarin RC, Carvalho VM, Bonamin LV. Homeopathic medicine Cantharis modulates uropathogenic *E. coli* (UPEC)-induced cystitis in susceptible mice. **Cytokine**. 2017 Apr; 92:103-109. doi: 10.1016/j.cyto.2017.01.014

21. Bonamin LV, Bellavite P. Immunological models in high dilution research following M Bastide. **Homeopathy**. 2015 Oct;104(4):263-8. doi: 10.1016/j.homp.2015.08.004.

22. Rodrigues de Santana F, Coelho Cde P, Cardoso TN, Laurenti MD, Perez Hurtado EC, Bonamin LV. Modulation of inflammation response to murine cutaneous Leishmaniosis by homeopathic medicines: thymulin 5cH. **Homeopathy**. 2014 Oct;103(4):275-84. doi: 10.1016/j.homp.2014.08.002.

23. Bonamin LV, Sato C, Zalla Neto R, Morante G, Cardoso TN, de Santana FR, Coelho Cde P, Osugui L, Popi AF, Hurtado EC, Mariano M. Immunomodulation of Homeopathic Thymulin 5CH in a BCG-Induced Granuloma Model. **Evid Based Complement Alternat Med.** 2013;2013:686018. doi: 10.1155/2013/686018.

24. Sato C, Listar VG, Bonamin LV. Development of broiler chickens after treatment with thymulin 5cH: a zoo technical approach. **Homeopathy**. 2012 Jan;101(1):68-73. doi: 10.1016/j.homp.2011.09.004.

25. Rodrigues de Santana F, de Paula Coelho C, Cardoso TN, Perez Hurtado EC, Roberti Benites N, Dalastra Laurenti M, Villano Bonamin L. Modulation of inflammation response to murine cutaneous Leishmaniasis by homeopathic medicines: *Antimonium crudum* 30cH. **Homeopathy**. 2014 Oct;103(4):264-74. doi: 10.1016/j.homp.2014.08.006

26. ANVISA. Farmacopeia Homeopática Brasileira 3ª edição 2011. www.anvisa.gov.br/hotsite/farmacopeiabrasileira/conteudo/3a_edicao.

27. Bell IR, Muralidharan S, Schwartz GE. Nanoparticle Characterization of Traditional Homeopathically-Manufactured Gelsemium sempervirens Medicines and Placebo Controls. **J Nanomedine Biotherapeutic. Discov** (2015) 5: 136. doi:10.4172/2155-983X.1000136

28. Chikramane PS, Suresh AK, Bellare JR, Kane SG. Extreme homeopathic dilutions retain starting materials: A nanoparticulate perspective. **Homeopathy**. 2010 Oct;99(4):231-42. doi: 10.1016/j.homp.2010.05.006.

29. Chikramane PS, Suresh AK, Kane SG, Bellare JR. Metal nanoparticle induced hormetic activation: a novel mechanism of homeopathic medicines. **Homeopathy**. 2017 Aug;106(3):135-144. doi: 10.1016/j.homp.2017.06.002. Epub 2017 Jul 21.

30. Temgire, M.K., Suresh, A.K., Kane, S.G., et al. Establishing the interfacial nano-structure and elemental composition of homeopathic medicines based on inorganic salts: a scientific approach. **Homeopathy** 2016; 105(2): 160-172.

31. Saha SK, Roy S, Khuda-Bukhsh AR. Ultra-highly diluted plant extracts of Hydrastis canadensis and Marsdenia condurango induce epigenetic modifications and alter gene expression profiles in HeLa cells in vitro. **J Integr Med**. 2015 Nov;13(6):400-11. doi: 10.1016/S2095-4964(15)60201-1.

32. Marzotto M, Olioso D, Brizzi M, Tononi P, Cristofoletti M, Bellavite P. Extreme sensitivity of gene expression in human SH-SY5Y neurocytes to ultra-low doses of Gelsemium sempervirens. **BMC Complement Altern Med**. 2014 Mar 19;14:104. doi: 10.1186/1472-6882-14-104.

33. Olioso D, Marzotto M, Moratti E, Brizzi M, Bellavite P. Effects of Gelsemium sempervirens L. on pathway-focused gene expression profiling in neuronal cells. **J Ethnopharmacol**. 2014 Apr 28;153(2):535-9. doi: 10.1016/j.jep.2014.02.048

34. Khuda-Bukhsh AR, Sikdar S. Condurango 30C Induces Epigenetic Modification of Lung Cancer-specific Tumour Suppressor Genes via Demethylation. **Forsch Komplementmed**. 2015;22(3):172-9. doi: 10.1159/000433485.

35. Saha SK, Roy S, Khuda-Bukhsh AR. Ultra-highly diluted plant extracts of *Hydrastis canadensis* and *Marsdenia condurango* induce epigenetic modifications and alter gene expression profiles in HeLa cells *in vitro*. **J Integr Med**. 2015 Nov;13(6):400-11. doi: 10.1016/S2095-4964(15)60201-1.

36. Mondal J, Das J, Shah R, Khuda-Bukhsh AR. A homeopathic nosode, Hepatitis C 30 demonstrates anticancer effect against liver cancer cells in vitro by modulating telomerase and topoisomerase II activities as also by promoting apoptosis via intrinsic mitochondrial pathway. **J Integr Med.** 2016 May;14(3):209-18. doi: 10.1016/S2095-4964(16)60251-0.

37. Crawford A, Angelosanto JM, Nadwodny KL, Blackburn SD, Wherry EJ. A role for the chemokine RANTES in regulating CD8 T cell responses during chronic viral infection. **PLoS Pathog.** 2011 Jul;7(7):e1002098. doi: 10.1371/journal.ppat.1002098.

38. Kawai T, Seki M, Hiromatsu K, Eastcott JW, Watts GF, Sugai M, Smith DJ, Porcelli SA, Taubman MA. Selective diapedesis of Th1 cells induced by endothelial cell RANTES. **J Immunol.** 1999 Sep 15;163(6):3269-78.

39. Fischer J, West J, Agochukwu N, Suire C, Hale-Donze H. Induction of host chemotactic response by *Encephalitozoon spp*. **Infect Immun**. 2007 Apr;75(4):1619-25.

40. Mutuiri SP, Kutima HL, Mwapagha LM, Munyao JK, Nyamache AK, Wanjiru I, Khamadi SA. RANTES Gene Polymorphisms Associated with HIV-1 Infections in Kenyan Population. **Dis Markers.** 2016;2016:4703854.

41. Barbosa Neto, RM. **Bases da Homeopatia**. http://www.ihjtkent.org.br/pdf/artigos/bases-da-homeopatia.pdf

42. Demangeat JL. Gas nanobubbles and aqueous nanostructures: the crucial role of dynamization. **Homeopathy**. 2015 Apr;104(2):101-15. doi: 10.1016/j.homp.2015.02.001.