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PHYTOHAEMAGGLUTININ'S EFFECT ON *NEOSPORA CANINUM* ANTIGEN PRODUCTION

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ABSTRACT: The parasite *Neospora caninum* affects mainly cattle and dogs. This study aimed to evaluate the effect of phytohaemagglutinin (PHE) in antigen production of *N. caninum* NC-1 strain in gerbils (*Meriones unguiculatus*) and in vitro. 20 gerbils were used, 10 inoculated intraperitoneally with 1 x 106 tachyzoites and 10 with 1 x 106 tachyzoites plus 300 µL/mL of PHE. 16 bottles of Vero cell culture were inoculated, 8 with 1.5 x 105 tachyzoites and 8 with 1.5 x 105 tachyzoites plus 30 µL/mL of PHE. Serology of gerbils was performed on day 0 and before euthanasia. Tachyzoites present in peritoneal fluid and cell culture bottles were quantified by Neubauer chamber and by real-time PCR (qPCR). PHE has not interfered in the production of tachyzoites of *N. caninum* in intraperitoneal inoculated gerbils and the effect of PHE in cell culture had a negative impact, considering the qPCR technique as the gold standard.
Key Words: cell culture, gerbil, *Neospora caninum*

EFEITO DA FITOHEMAGLUTININA NA PRODUÇÃO ANTIGÊNCIA DE *NEOSPORA CANINUM*

RESUMO: O parasito *Neospora caninum* acomete principalmente bovinos e cães. A realização deste estudo teve como objetivo avaliar o efeito da fitohemaglutinina (FHE) na produção de antígenos de *N. caninum* cepa NC-1 em gerbils (*Meriones unguiculatus*) e *in vitro*. 20 gerbils foram utilizados, 10 foram inoculados intraperitonealmente com 10^6 taquizoítos e 10 com 10^6 taquizoítos acrescidos de 300µL/mL de FHE. 16 garrafas de cultura de células Vero foram inoculadas, 8 com 1,5 x 10^5 taquizoítos e 8 com 1,5 x 10^5 taquizoítos acrescidos de 30 µL/mL de FHE. Sorologia dos gerbils foi realizada no dia 0 e antes da eutanásia. Taquizoítos presentes no lavado peritoneal e garrafas de cultura de células foram quantificadas em câmara de Neubauer e por PCR em tempo real (qPCR). FHE não interferiu na produção de taquizoítos de *N. caninum* em gerbils inoculados intraperitonealmente e o efeito da FHE em cultura de células teve um impacto negativo, considerando-se a técnica qPCR como o padrão-ouro.
Palavras-chave: cultura celular; gerbil; *Neospora caninum*

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INTRODUCTION

The parasite *Neospora caninum* has been discovered and described in 1984 and 1988, respectively. It mainly affects cattle and dogs, but serologic evidence are described in other species, such as buffalo, nonhuman primates, camels, wild canids and cats, and humans (Barr *et al.*, 1994; Ho *et al.*, 1997; Dubey, 2003; Dubey e Thulliez, 2005; Lobato *et al.*, 2006; McCann *et al.*, 2008; Robert-Gangneux; Klein, 2009; Bouer *et al.*, 2010). Dog, however, plays a major role by being considered as both final and intermediate host of the parasite, which can present itself in three forms: tachyzoites, tissue cysts (in intermediate hosts) and oocysts (eliminated only by the definitive host and sporulated in the environment) (McAllister *et al.*, 1998; Lindsay *et al.*, 1999; Dubey, 2003).

Diagnosis of neosporosis is currently based on serological, molecular methods or isolation of the agent. Regarding serological methods, some of the available options are the indirect immunofluorescence assay (IFA), the direct agglutination test (NAT) and enzyme-linked immunosorbent assay (ELISA) (Björkman e Uggla, 1999). Regarding isolation or bioassay, the gerbils (Meriones *unguiculatus*) are considered the best experimental models for acute neosporosis, due to its highly susceptibility to infection by the agent, not requiring the use of immunosuppressant's (Lindsay e Dubey, 1989; Gondim *et al.*, 1999; Dubey and Lindsay, 2000; Gondim *et al.*, 2001; Pipano *et al.*, 2002; Ramamoorthy *et al.*, 2005).

Direct agglutination test is traditionally made by inoculation of *N. caninum* in immunosuppressed mice added of sarcoma TG-180 cells to increase the antigen production (Romand *et al.*, 1998), though alternative methods are described, such as antigen production in cell culture (Peckham *et al.*, 1998).

It is speculated that some substances with mitogenic properties may facilitate an increase on production of tachyzoites, such as phytohaemagglutinin (PHE). PHE is a lectin purified from the bean *Phaseolus vulgaris* with mitogenic properties (Börjeson *et al.*, 1964), commonly used for stimulation of mitosis in leukocytes, lymphocytes and also some microorganisms such as amoebe and acanthamoeba (Agrell, 1966; Agrell, 1967). In this context, the aim of this study was to evaluate phytohemagglitinin's effect on *N. caninum* NC-1 strain in two models of antigen production: in gerbils and in cell culture, for direct agglutination test antigen production without the need of immunosuppressant. Gerbils were used as an alternative model animal of antigen production susceptible to inoculation of *N. caninum* without the interference of any other external substances.

MATERIAL AND METHODS

Parasites, study design and fluid samples collection

A non-random experimental study was design. The present study was approved in the Ethics Committee of Animal Use of São Paulo State University. The number of animals and culture bottles needed was calculated using the software G*Power (Universität Kiel, Germany).

Tachyzoites of *N. caninum* NC-1 were grown in 25cm^3 plastic bottles containing Vero cell line in RPMI 1640 media supplemented with 5% antibiotic and antimycotic solution (Invitrogen, Cat. n. 15240-096). Twenty gerbils (*Meriones unguiculatus*) were inoculated, 12 females and 8 males, aged 60 to 90 days. For intraperitoneal inoculation, the tachyzoites were harvested from culture

bottles, centrifuged at 1,600 G for 10 minutes and resuspended in sterile phosphate buffered solution pH 7.2.

Concentration of inoculums was 1 x 10^6 tachyzoites per gerbil, in agreement with previous studies (Gondim *et al.*, 1999; Pipano *et al.*, 2002). PHE (Cultilab) was added to the inoculums of the PHE group animals at the volume of 300 µL/mL suspension of tachyzoites (150µL per gerbil). All animals were examined daily to detect possible signs of disease. Euthanasia was performed in chamber saturated with isofluorane when animals were suffering from acute neosporosis. All animals had their blood taken via retro-orbital sinus through heparinized capillary of 0.9mm under general anesthesia with isofluorane at the first day of the experiment and prior to euthanasia to confirm infection via indirect fluorescent antibody test. Serum was obtained by blood centrifugation at 1,600 G for 10 minutes. Peritoneal fluid was obtained immediately after euthanasia of the gerbils by inoculation of 5mL of 10% saline supplemented with antibiotics (penicillin and streptomycin) in the abdominal cavity and recovery using disposable syringe. Peritoneal fluid was immediately counted in Neubauer chamber and centrifuged at 1,600 G for 10 minutes, resuspended in the same initial volume with ultrapure water and stored in microtubes free of DNase and RNase at -20°C for analysis by real time PCR (qPCR).

For inoculation in cell cultures, the parasites obtained from previous cell culture were harvested and directly inoculated in bottles containing confluent monolayer of Vero cell line in a volume of 0.3 mL of inoculums containing 468,000 tachyzoites per mL (1,4 x 10^5 tachyzoites per bottle). PHE was added in the PHE group bottles at the volume of 30 µL/mL of medium in cell culture (180µL per bottle).

Indirect Fluorescent Antibody Test - IFAT

For detection of antibodies anti-*N. caninum* it was used IFAT (Dubey *et al.*, 1988) at the dilutions 1:25, 1:50, 1:100, 1:200 and 1:400 with anti-mouse conjugated at 1:100 dilution (Mouse immunoglobulins, DAKO A/S, Denmark, Code No. F0232).

Tachyzoites quantification

Quantification of tachyzoites was performed by counting in Neubauer chamber and by qPCR (Leon *et al.*, 2012). Genomic DNA was extracted from peritoneal suspensions and cell culture using the DNA extraction kit "Illustra tissue & cells genomicPrep Mini Spin®" (GE Healthcare, USA) and measured by spectrophotometer (GE Healthcare, USA). All samples were extracted without duplicates, but qPCR of each sample was performed in triplicate. For each reaction it was used a standard curve determined by counting the number of previously cell culture cultivated tachyzoites in Neubauer chamber, in serial dilution, starting from concentration 10^6, 10^5, 10^4, 10^3, 10^2,10^1 and 10^0.

DNA of *N. caninum* was amplified using primers Np6 (5'-CAGTCAACCTACGTCTTCT-3') and Np21 (5'-GTGCGTCCAATCCTGTAAC-3') that amplifies a region of 328 base pairs (bp) (Yamage *et al.*, 1996). Each reaction was performed in a final volume of 25µL containing 12.5µL of Power SYBR® Green PCR master Mix (Applied Biosystems, USA), 5 µM of each oligonucleotide, 8.5µL of ultrapure water and 2µL of sample. The reactions were performed in StepOnePlus Real-Time PCR System thermocycler (Applied Biosystems, USA), according to the supplier's protocol, using initial denaturation at 95°C for 10 minutes followed by 40 cycles of 95ºC for 15 seconds and 60°C for 1 minute, and a final cycle of 95°C for 15 seconds.

Results were viewed and analyzed with the program StepOne™ Software v2.1.

Neubauer chamber counting was performed using the following formula: number of cells/mL = (total number of cells/number of quadrants counted) x dilution factor x 10.000.

Statistical Analysis

Data were tabulated in an Excel spreadsheet and statistically analyzed in GraphPad Instat® v.3.06. Median, 25% percentile and 75% percentiles were determined and compared by nonparametric tests. Concentration of tachyzoites per mL was compared by nonparametric Wilcoxon test for antigen treated and untreated with PHE when analyzed by the same quantification method, or by nonparametric Mann-Whitney test comparing the quantification methods by treatment (PHE or control group).

Subsequently, comparisons between treated or untreated antigen according to the model of antigen production were performed by Wilcoxon test, and by Mann-Whitney test when comparing the models of antigen production to treatment (PHE or control) of the antigen. For all the analysis, it was considered a significance level (α) of 5%.

RESULTS

Clinical signs usually started from the third day post-inoculation (DPI), and because of the development of acute neosporosis by all animals. Clinical signs consisted of lethargy, poor general condition, ruffed hair, progressive weight loss and lack of eye opening at stimulus.

All gerbils used tested negative for neospora antibodies at the beginning of the experiment and only two animals seroconverted, with titres 25 and 400 (from PHE and from control group, respectively). Out of the 16 peritoneal fluids recovered, exudates volumes ranging from 1 to 3mL were present in 12 of them (6 from each group). The mean exudate volume of the animals from PHE group was 1.2mL and from control group was 1.6mL. The median, percentile and *P* values comparing the two counting techniques are shown in Table 1. When considering the quantification of tachyzoites by Neubauer chamber, statistical difference (*P*=0.0104) was found regarding treatment with PHE in gerbil inoculation, resulting in a higher concentration of tachyzoites. On the other hand, considering quantification by qPCR, this correlation could not be observed.

Table 1 - Median, 25% percentile (P25) and 75% (P75) of concentration of tachyzoites ($x10^3$) per mL by counting in Neubauer chamber and qPCR in gerbils inoculated with NC-1 strain treated or untreated with phytohaemagglutinin (PHE).

	Test used (P25:P75)			
	Neubauer ($x10^3$)	qPCR ($x10^3$)	Statistics	*P* value
Treated	173,75 (135,00:261,88)	35,53 (26,68:88,50)	W = 32,00	0,0234
Untreated	80,00 (53,13:117,50)	26,32 (17,54:40,28)	W = 36,00	0,0078
Statistics	U = 8,00	U = 20,00		
P value	0,0104	0,2345		

W = Wilcoxon non-parametric test
U = Mann-Whitney non-parametric test
P = *P* value for α = 5%

Regarding antigen production in cell cultures, the median, percentile and *P* values comparing quantification of tachyzoites by Neubauer chamber and qPCR are shown in Table 2. There was no statistical correlation between treatment with PHE and increased tachyzoites production using Neubauer chamber counting technique. When performed by qPCR, the correlation was negative, treatment with PHE decreased production of tachyzoites in cell culture.

Table 2 - Median, 25% percentile (P25) and 75% (P75) of concentration of tachyzoites ($x10^3$) per mL by counting in Neubauer chamber and qPCR in cell culture inoculated with NC-1 strain treated or untreated with phytohaemagglutinin (PHE).

	Test used (P25:P75)			
	Neubauer ($x10^3$)	qPCR ($x10^3$)	Statistics	*P* value
Treated	1035,00 (700,00:1220,50)	22,49 (98,30:72,27)	W = 36,00	0,0078
Untreated	1168,00 (1149,00:1265,50)	250,42 (106,78:472,11)	W = 36,00	0,0078
Statistics	U = 18,00	U = 6,00		
P value	0,1605	0,0047		

W = Wilcoxon non-parametric test
U = Mann-Whitney non-parametric test
P = *P* value for α = 5%

DISCUSSION

Regarding the disease in gerbils, the clinical signs presented were in agreement with previous studies (Pipano *et al.*, 2002). Infection by *N. caninum* could only be confirmed by IFAT in two animals, a predictable result since presence of antibody is not expected within 5 days of infection.

It was speculated to obtain a higher parasite load, once PHE could increase cell reproduction and, considering an intracellular pathogen, increase *N. caninum* multiplication.

Although the present study had limitations, such as non-randomized allocation of treatment with PHE, it made possible to raise hypothesis on a new possible instrument to increase antigen production of *N. caninum*. Although statistic significant effect was not observed, further studies are necessary to evaluate whether and how treatment with PHE is influenced by different concentrations. PHE might have a potential to substitute the use of immunosuppressed mice in massive antigen production of *N. caninum*.

CONCLUSION

In this study, PHE did not increase the production of *N. caninum* tachyzoites regardless of the model used (gerbils or cell culture), though showed a tendency of increasing antigen production in gerbils, which should be further studied with different PHE concentrations. In cell culture, PHE interfered negatively in the production of *N. caninum* tachyzoites.

INFORMATIVE NOTE

The present study was approved by the Ethics Committee of Animal Use of São Paulo State University under protocol number 146/2010.

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