TOXOPLASMA GONDII INFECTION OF CHICKEN EMBRYOS CAUSES RETINAL CHANGES AND MODULATES HSP90B1 GENE EXPRESSION: A PROMISING OCULAR TOXOPLASMOSIS MODEL

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HSP90B1 is a gene that codifies heat shock protein 108 (HSP108) that belongs to a group of proteins induced under stress situation, and it has close relation with the nervous system, especially in the retina. *Toxoplasma gondii* causes ocular toxoplasmosis that has been associated with a late manifestation of the congenital toxoplasmosis although experimental models show that morphological alterations are already present during embryological development. Here, we used 18 eyes of *Gallus domesticus* embryos in 7th and 20th embryonic days to establish a model of congenital ocular toxoplasmosis, experimentally infected in its fifth day correlating with *HSP90B1* gene expression. Embryos' eyes were histologically evaluated, and gene expression was performed by real-time polymerase chain reaction (PCR). Our data showed parasite present in the choroid, unusual migration of retinal pigment epithelium, and chorioretinal scars, and a tendency to a lower expression of the *HSP90B1* gene upon experimental infection. This is a promising model to better understand *T. gondii* etiopathogeny.

Keywords: ocular toxoplasmosis, regulation of gene expression in development, embryo development, parasitic ocular infections, retina, experimental models

Introduction

The *Toxoplasma gondii*, the etiologic agent of toxoplasmosis [1], affects one third of the world population [2–9], and it is estimated that 20% have ocular infection [1, 10]. Ocular toxoplasmosis has always been associated to congenital toxoplasmosis, where the mother contracts the parasite and transmits to the fetus; depending on the period that this infection occurs, the degree of complications varies, with more complicated cases during first months of the gestational period [2, 4–6]. Studies using animal models show that toxoplasmosis do not exhibit the same symptoms as in humans [5, 11] but understanding the infection progress might aid in the design of vaccines and improve a better prevention. On the other hand, Shin et al. [12] and Zhao et al. [13] have described a stress protein, identified as heat shock protein 108 (HSP108) coded by *HSP90B1* gene, that has close relation with the nervous system development

[14], especially in the retina, one of the principal structures affected by toxoplasmosis; HSP108 is able to be expressed on stress events as development, cell cycling, ischemia, inflammation, and infections [12, 13, 15, 16]. Hence, the aim of this study was to identify alterations in the structure and in expression of the *HSP90B1* gene of the eyes of the chicken embryos after infection with *T. gondii*.

Methods

The Research Ethics Committee (REC) of the Federal University of São Paulo (0149/12HE) approved the study.

Embryonated chicken (White Leghorn) eggs were obtained from a commercial aviaries (Porto Ferreira city, São Paulo) and divided into three groups identified as control group (A); sham group (B), that received, at the 5th embryonic day (E5), an injection of 100 µl of sterile phos-

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phate buffer solution (PBS) at the allantoic cavity [17]; and experimental group (C), which received the same as the sham group but PBS containing $1 \cdot 10^3$ tachyzoites of the ME49 strain. *T. gondii* tachyzoites were obtained from mice brain cysts provided by Instituto Oswaldo Cruz and Instituto de Medicina Tropical. Embryonated chicken eggs were incubated in an incubator, under 37 °C to 37.5 °C and estimated humidity of 40%, and in predetermined days (E7 and E20), eggs were opened and embryos were sacrificed by decapitation, and their eyes were enucleated, using left eye for histological and right eye for molecular studies.

Histological studies

After fixations, left eyes were dehydrated in a graded ethyl alcohol series and embedded for 12 h in historesin (glycol methacrylate, Technovit 7100) and ethyl alcohol (1:1); blocks were cut at a thickness of 1 µm in a semi-series (American Optical) and stained with 0.25% toluidine blue in 1% sodium borate. Slides were preserved in Erv-Mont (EasyPath®) covered by coverslips and observed in a Zeiss-Axioplan microscope [1–16, 18].

Molecular studies

Total RNA was extracted from the posterior part of the right eye by the Trizol® method [8] and was quantified

using Nanodrop (Spectrophotometer ND1000, UNISCIENCE®). All samples had their integrity tested using electrophoresis, and samples which demonstrated integrity had cDNA synthesized. cDNA, dNTP after having been treated with RQ1 RNase-Free DNase (Promega), and pDT were synthesized using Superscript® II Reverse transcriptase (Invitrogen) [13, 14].

Parasite identification

Parasite's strands were identified by electrophoresis after conventional polymerase reaction chain (PCR), using Taq polymerase, dNTP, magnesium chloride and primers SAG1 [8] (Table 1).

HSP90B1 gene

HSP90B1 gene quantification was performed by real-time PCR with SYBER® Green (Applied Biosystems) and a specific primer, denominate HSP108 designed by our group, and an endogenous primer for β-actin [19], (Table 1).

Statistical studies

Results were obtained only from descriptive statistical analysis, from parameters of mean and dispersion: simple

Table 1. Primers used for PCR

Primer	Sense	Anti-sense	Amplicon (pb)
SAG1 (de Miguel et al., 2005)	GCG CGG ATC CAT GGT CAC GGT GAC AGT A	GCG CAA GCT TTC ACG CGA CAC AAG CTG CGA	≈700
β-actin (Wen-Qiao et al., 2012)	CAG ATA CGG GTA TTG GCA TGA	AAA AGC CAA CAC CAA ACT GG	175
HSP 108	CAG ATA CGG GTA TTG GCA TGA	AAA AGC CAA CAC CAA ACT GG	156

Fig. 1. Retinoic structure of control group in E7, with RPE and NF well limited, and nuclear cells morphologically differing

Fig. 2. E20, with all layers well defined, from outer layers to internals: RPE, PH, OLM, ON, OP, IN, IP, GC, NF, and ILM

arithmetical mean and standard deviation [20], respectively, using Statistica 12 software (StataSoft®).

Fig. 3. Projection of NF and NL (arrow) with detachment of RPE

Figs 4 and 5. Parasite presence indicated by arrow boundary in choroids area with mononuclear inflammatory cells surrounding the parasite (IF); RPE detached from its layer, indicating cell migration into choroids area (*) and edema (star)

Fig. 6. Retina in E20 from experimental group, with mononuclear inflammatory cells (IF) in choroids area, with gaps between RPE cells

Results

Histology

As expected, the embryo eye at E7 and E20 presented the already described morphology [21, 22]. The control group at E7 presented three distinct stratus: nerve fiber layer (NF), an intense nuclear layer (NL), and a thin retinal pigmented epithelium layer (RPE) (Fig. 1). The same result was found in the sham group (data not shown). In E20 eyes, well-differentiated layers from outer to inner layers were seen as: RPE, outer segment of photoreceptors (PH), outer nuclear (ON), outer plexiform (OP), outer limiting membrane (OLM), inner nuclear (IN), inner plexiform (IP), ganglion cells (GC), NF, and inner limiting membranes that are well defined (Fig. 2). Experimental group showed an intense projection of NF and NL with detachment of RPE with difference in the disposition of this layer (Fig. 3).

The E20 eyes of experimental group showed alterations as in the disposition of the PH layer with edema in the choroid and inflammatory infiltrate, predominantly mononuclear cells surrounding the parasite cysts (Figs 4 and 5). RPE cell that migrated to the choroid was also found (Fig. 6).

Gene HSP90B1 expression

Gene expression quantified by qPCR showed no difference between control and sham groups, although the experimental group presented a lower expression (Figs 7 and 8). Expression in E20 was lower than E7 in this group. Probably, there is a tendentious inhibition of the gene *HSP90B1* expression, since the total sample did not permit a detailed statistical analysis.

Figs 7 and 8. Expression profile of *HSP90B1* gene of chicken embryo under toxoplasmosis effects compared to control and sham group

Discussion

Analysis of chicken embryo eyes in the control group showed normal layer development when E7 and E20 were compared. Basically, the ocular bulb of the E7 is still in development ending on E20 when all layers are well defined [21], eye differentiation is just completed after the embryo is born, and light stimulus causes the last differentiation [22], confirmed by the absence of protein HSP108 in 2 days postnatal and adult chickens [12, 13].

In our study, embryos that received PBS only presented unaltered layers development as the control group, demonstrating that the inoculation method used [17] did not interfere with the experiments.

Infected embryos in E7 presented cell alterations which project nuclear layers in vitreal direction, characterizing chorioretinitis [1]. Same alterations were presented in other animal infection models [11, 23, 24]. Even the absence of parasite does not discard the hypothesis of infection [25]. In E20, it was possible to visualize discontinuity of RPE, with migration of these cells to the choroid. Tedesco et al. [18] observed RPE migration to the internal layers in mice, observing that the route of infection of *T. gondii* in mammals is via the retinal blood vessels and, in the avascular avian retina, is choroidal vessels.

The RPE presents an important role during *T. gondii* infection; those cells are responsible for phagocytic external segment cells during normal homeostasis, but their role during infections has been described [26]. It was also identified edema, inflammatory infiltrate of mononuclear cells, corroborating with literature and indicating an important role during *T. gondii* infection [1, 27].

Histological findings agree with the literature about *T. gondii* eye infection. Our molecular experiment possibly suggests that there is a tendentious inhibition of *HSP90B1* gene expression in experimental groups. This implies that HSP108 protein levels regulated by these gene decrease, indicating that infection by *T. gondii* influences its expression. HSP108 protein presents a protection profile during infections [14] that is associated to the hormonal response

[28], inferring that, in cases of infection by *T. gondii*, the regulation of heat shock protein may also downregulate by negative feedback, making necessary a recruitment of inflammatory cells [18], possibly interfering in the proper development.

Shin et al. [12] had described the expression of this protein already in E5, justifying our findings and highlighting that, in E7, we found that, if the gene is expressing the RNA in begging of embryology, probably, protein is on synthesis too, disagreeing with Zhao et al. [13] who affirm that in E7 there is not this protein expressed, but already described in literature [14] having a decrease in the expression on E20 in importance for a complete development, and this decrease indicates a signaling an end of the development, regulating other signals. On the other hand, Schlesinger [15] affirms that the protein expression over temperature happens only in 43 °C to 45 °C, influencing in the development, demonstrating that, in our studies, it had not influenced, once we incubated eggs at 37 to 38 °C.

At last, embryonated eggs have been used along the years as experimental model in morphological and molecular studies and have shown excellent performance when compared to other animals as *Mesocricetus auratus* [11], rabbit [23], and *Columba* sp. [24], but do not exclude the other models, which have been necessary to consider the use and the aim of the study, inferring the facility in obtaining samples, maintenance, usability, and stereos environment of the embryo model. However, animal models do not express the real infection in humans but can guide through new discoveries.

Conclusion

Infection by *T. gondii* may cause structural alterations probably by the immune response, followed by the migration of the RPE cells. The possible inhibition of the *HSP90B1* gene expression responsible for RNA synthesis needs further work relating protein expression and external factors as hormones and immune response during infection.

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