Effect of 17β-Estradiol and Progesterone on Astrocytes Infected with Toxoplasma

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Abstract | Toxoplasma gondii is an opportunistic parasite that invade microglia, astrocytes, and neurons. In guinea pigs infected with Toxoplasma, brain cysts increased significantly in males subjected to gonadectomy and treated with hexoestrol, compared with control. The effect of 17β-estradiol and progesterone on astrocytes infected with Toxoplasma is unknown. The objective of the study was to evaluate the effect of 17β-estradiol and progesterone on Toxoplasma infection in astrocytes in vitro. Astrocytes were pre-treated with 17β-estradiol or progesterone at concentrations of 10, 20, 40, 80, or 160 nM for 48 hours and infected with Toxoplasma tachyzoites. The percentage of infected astrocytes was evaluated by immunocytochemistry and parasite replication by MTT. ANOVA and a Dunnett’s T3 post-hoc test were used. Pretreatment with 17β-estradiol and 17β-estradiol + tamoxifen (1µM) resulted in a significant reduction in parasite replication at 48 hours post-infection. Infected astrocytes were gradually reduced whit 17β-estradiol plus tamoxifen. Progesterone reduced the parasite replication at 48 hours, and this effect was reversed by mifepristone. The percentage of infected astrocytes at 24 hours was reduced with progesterone at all doses. 17β-estradiol plus progesterone had a synergistic effect, increasing Toxoplasma infection in astrocytes at 24 hours however, at 48 hours the infection was reduced. The 17β-estradiol + progesterone at 160 nM, as well as PPT and DPN, reduced Toxoplasma replication and percentage of infected astrocytes. Toxoplasma infection in astrocytes was reduced by the effect of 17β-estradiol and its agonist PPT as well as progesterone. These results suggest that ERα and PRs activation diminishes Toxoplasma-infection in astrocytes.

Keywords | Toxoplasma-infection, DPN, PPT, Progesterone, 17β-estradiol, Tamoxifen, Mifepristone

INTRODUCTION

Toxoplasma gondii is the causal agent of toxoplasmosis, which infects more than a billion people worldwide (El-On, 2003). Most morbidity takes the form of mortal encephalitis in immunocompromised patients, as well as serious neurological complications or death in congenitally transmitted toxoplasmosis (Speroff et al., 1999; Carruthers and Suzuki, 2007).

Astrocytes and T. gondii Infection

T. gondii traverses the intestinal or placental epithelium by paracellular transmigration as a free parasite (Barragan and Sibley, 2002) and enters circulating cells, such as macrophages or dendritic cells (Da-Gama et al., 2004; Courret et al., 2006; Lambert et al., 2006). The parasites appear to use such cells as “Trojan Horses” to gain access to privileged sites, such as the brain. In vitro studies using mouse brain cells have demonstrated that tachyzoites invade microglia (Chao et al., 1996; Fischer et al., 1997), astrocytes (Halonen et al., 1996) and neurons (Jones et al., 1996; Lambert et al., 2006). T. gondii forms cysts within these cells (Da-Gama et al., 2004; Lambert et al., 2006). The astrocytes act as host and as activators of a protective im-
mune response in the central nervous system (Appel et al., 2001). However, more studies on the T. gondii mechanism of invasion and replication process in astrocytes are necessary. T. gondii infection in astrocytes significantly increases monocyte chemotactic protein-1 (MCP-1) secretion. This protein may contribute to the cell clustering seen during human cerebral reactivation of T. gondii (Brenier-Pinchart et al., 2004). Pro-inflammatory protein expression differs between type I and II strains and among different human nervous system cells. For example, the parasite burden declines in microglial cells and neurons over the course of infection, but remains high in endothelial cells (Mammari et al., 2014). This differential effect on early parasite multiplication may be correlated with a higher production of immune mediators by neurons and microglial cells compared with endothelial cells, and suggests that the different protein expression profiles depend on the parasitic strain and on the human nervous cell type (Contreras-Ochoa et al., 2013; Brenier-Pinchart et al., 2004).

17β-estradiol, Progesterone, and Astrocytes
Estradiol regulates several functions in astrocytes, such as intracellular Ca++ levels (Micevych et al., 2010), that may influence the communication between astrocytes and neurons as well as other glial cells (Perea and Araque, 2010). Furthermore, estradiol also regulates the expression of astrocyte proteins that participate in neuro-inflammation, control of extracellular glutamate levels, and neuronal homeostasis (Garcia-Segura and Melcangi, 2006). Estradiol has direct effects on astrocytes through a variety of receptors. These include estrogen receptor α (ERα) and estrogen receptor β (ERβ) (Azcocita et al., 1999; Garcia-Segura et al., 1999; Cardona-Gomez et al., 2000; Garcia-Ovejero et al., 2005; Pawlak et al., 2005; Quesada et al., 2007), which have been shown to be not only localized in the nucleus but also associated with the plasma membrane (Micevych et al., 2010; Pawlak et al., 2005; Johann and Beyer, 2013). Furthermore, G protein-coupled estrogen receptor (GPER, formerly known as GPR30) and Gαq-coupled membrane-associated receptor (Gαq-mER), which is activated by Novel Estrogen Membrane Receptor Agonist (STX), are also involved in the actions of estradiol on astrocytes (Kuo et al., 2010; Lee et al., 2013). Conditioned media from estradiol-treated astrocytes promote the survival of cortical neurons exposed to different neurodegenerative stimuli such as camptothecin, glutamate, hypoxia–ischemia, or β-amyloid (Dhandapani and Bran, 2003; Sortino et al., 2004; Carbonaro et al., 2009). The functions of 17β-estradiol (E2) in the nervous system are cell differentiation, growth, maturation, survival, and neuroprotection (Duenas et al., 1996; Arevalo et al., 2010). Increasing levels of estradiol have been associated with toxoplasmosis during pregnancy (Mauro et al., 2014; Spence and Voskuhl, 2012).

Two specific estrogen receptor agonists have been used: 2, 3-bis (4-hydroxyphenyl)-propionitrile (DPN) for ERβ, and 4, 4’,4”-(4-propyl-(1H)-pyrazole-1,3,5 triyl) trisphenol (PPT) for ERα (Garrido-Gracia et al., 2007; Arevalo et al., 2013). Astrocytes are also a target of progesterone (P4), which reduces reactive astrogliosis and neuro-inflammation (Arevalo et al., 2013). The actions of progesterone are mediated by both progesterone receptor A (PRA) and progesterone receptor B (PRB), as well as GABAA receptors (Melcangi et al., 2014). Recent studies have shown that hypothalamic astrocytes synthesize progesterone in response to estradiol (Micevych and Sinchak, 2008; Micevych et al., 2008). The synthesis of progesterone by hypothalamic astrocytes has been linked to hormonal control of reproduction. In the presence of progesterone, mifepristone acts as a competitive antagonist of PRA (Mauro et al., 2014; Gay-Andrieu et al., 2002; Jones et al., 2008).

In guinea pigs infected with the Beverley Strain of T. gondii, the number of brain cysts of T. gondii increased significantly (p<0.001) in males that were castrated and treated with hexoestrol, as well as in females, compared with control guinea pigs (Kittas and Henry, 1979). In other experiment performed on mice, the number of T. gondii brain cysts increased significantly in infected mice treated with hexoestrol (Kittas and Henry, 1980).

In infected female mice treated with pharmacological doses (0.1-10 µmoles) of 17β-estradiol, the number of T. gondii cysts (T45 strain) increased in a dose-dependent way; however, the administration of progesterone did not affect cyst formation (Pung and Luster, 1986).

The role of E2 and progesterone receptors in toxoplasmosis is currently unknown. The objective of the present study was to evaluate the effect of 17β-estradiol and progesterone on T. gondii infection in astrocytes in vitro.

MATERIALS AND METHODS
Parasites
Six-week-old male and female Swiss mice weighing 20–25 g were intraperitoneally injected with 1 x 10⁵ T. gondii tachyzoites (strain RH) and sacrificed at 7 days; the infection was maintained by injecting new mice every three
days. The mice were sacrificed in order to harvest the *T. gondii* used to infect the astrocytes.

**Ethical Aspects**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The project was approved by our Institutional Research, Ethics, and Biosecurity Committee of the University Centre of Health Sciences, University of Guadalajara, and recorded under registration number CI 053-2013. The care and use of experimental animals were performed in accordance with the Official Mexican Norm NOM-062-Z00-1999. All efforts were made to minimize suffering.

**Mixed Cortical Cells**

Cortical cell isolation for astrocyte culture can be performed using 1 to 3 newborn rats, not more than 4 days old. Three newborn Wistar rats were sacrificed by decapitation. Brain tissue was obtained by craniotomy and mechanically dissociated in OPTI-MEM 51985 medium (Invitrogen, Carlsbad, CA) supplemented with 10% horse serum (HS) and a 1% solution of penicillin streptomycin. Cells were counted using a 0.3% trypsin blue solution for determining survival percentage. The cells were plated (50,000) on 0.1% poly-L-lysine pre-treated cover slips placed in 24-well plates and maintained at 37°C in a 95% air and 5% CO2 atmosphere. The medium was changed every two days until 90% confluence was obtained.

**Astrocyte Separation**

After 7 or 8 days of culture, 90% confluent cells were shaken at 180 rpm for 30 min on an orbital shaker to remove microglia. The supernatant containing the microglia was discarded. The plate was shaken again at 240 rpm for 6 hr to eliminate oligodendrocyte precursor cells (OPC). The resulting astrocytes were obtained with 95% purity.

**Experimental Treatments in Astrocytes in Vitro**

On the fifteenth day of culture, the astrocytes (90% confluence) were hormonally pre-treated for 48 hours with 10, 20, 40, 80, or 160 nM/mL of E2 or P4, or the combination of E2 and P4 at a concentration of 160 nM/mL. Tamoxifen was used at a concentration of 1 µM/mL plus E2 10, 20, 40, 80, or 160 nM/mL. PPT or DPN was added to the cultured astrocytes at a concentration of 1 nM/mL, mifepristone at a dose of 1 µM/mL plus P4 at doses of 10, 20, 40, 80, and 160 nM/mL; then, tachyzoites of *T. gondii* were added (4 x 10³ parasites per well) and allowed to infect the astrocytes for a period of 24 and 48 hours.

Each hormone dose was evaluated independently in three replicates, repeating the experiment three times. For the immunocytochemical experiment, infected and uninfected cells were fixed with 3.7% paraformaldehyde for 5 minutes at room temperature and stored at 4°C.

**Immunocytochemical Method**

*T. gondii* infection in cultured astrocytes was detected by the immunocytochemical method, in consecutive order, as follows. To identify astrocytes, cells were washed twice with phosphate buffer solution (0.1 M PBS) for 5 minutes and permeabilized with 0.2% Triton X-100 in PBS (TPBS) for 1 hour. Cells were incubated with 10% goat serum in PBS containing 0.01% sodium azide (blocking solution) for 2 hours at 4°C. After washing, the astrocytes were incubated overnight in mouse anti-α-glial fibrillary acidic protein (GFAP) antibody (1:500, Dako Corporation, Carpinteria, CA) and rabbit anti-*Toxoplasma* antibody (1:1500, Gen Way Biotech, San Diego, CA) diluted in a blocking solution and incubated at 4°C for 16 hours in a humidity chamber. After washing with TPBS, the cells were incubated in 10% pre-immune goat serum in a PBS buffer for 1 hour at room temperature and then were incubated with Alexa Fluor 594 antibody-labelled anti-mouse IgG (1:1000, Abcam 150116) and Alexa Fluor 488 antibody-labelled anti-rabbit IgG (1:1000, Abcam 150073) for 1 hour at room temperature in darkness in a humidity chamber.

Finally, the cells were washed two times with PBS and incubated 5 minutes with Nucleic Acid Stain (DAPI, Invitrogen), diluted 1:3000 in PBS.

**Microscopic Analysis**

Astrocytes were evaluated a total of nine times for each hormone dose as well as for their agonists and antagonists. One hundred cells were analysed for each dose: The number of infected astrocytes was determined using digital images from an Olympus IX71 microscope (40x magnification), using Image Pro-Plus software 6.0 of Media Cybernetics 2.6 to merge the images.

**T. gondii Replication**

The MTT assay is based on the presence of the enzyme mitochondrial succinate dehydrogenase in live cells. Only viable and early apoptotic cells are capable of reducing the tetrazolium salt MTT (yellow), resulting in the formation of water-insoluble formazan crystals (purple); dead cells will therefore retain the yellow colour of the medium (Mossman, 1983).

*T. gondii* replication in infected and treated astrocytes was evaluated by the MTT assay. Using the method described above, 96-well plates were used to culture astrocytes (5 x 10⁴ cells/1.7 cm²). To began the assay, 40 µL of MTT solution (5 mg/ml, Sigma) was added for 2 h and incubated at 37°C, in 5% CO2 and 95% air for 2 h. Subsequently, 100 µL of 50% dimethyl formamide and 20% sodium dodecyl sulfate (SDS) was added for dissolving the formazan crys...
Figure 1: Representative photographs of Toxoplasma gondii infection in cultured astrocytes detected by immunocytochemistry.

A) Uninfected astrocytes, morphologically preserved; B) Untreated astrocytes infected with tachyzoites of T. gondii Rh strain (control) at 24 hours; C) Hormone treated astrocytes were morphologically preserved at 24 hours post-infection; D) Hormone treated astrocytes at 48 hours post-infection. The astrocytes have been destroyed by Toxoplasma infection, with clear membrane rupture photomicrographs were taken at 40X.

Cytotoxicity Assay
To evaluate the cytotoxicity on astrocytes, the MTT assay was used. Progesterone and 17B-estradiol, along with their antagonists or agonists, were added to the astrocytes in vitro. In one assay, cells were treated with E2 or P4, at doses of 10, 20, 40, 80, and 160 nM/mL. In another assay, the astrocytes were treated with E2 plus P4 both at 160 nM/mL or E2 at 10, 20, 40, 80, and 160 nM/mL plus 1 µM/mL tamoxifen. To test the action of progesterone, this hormone was added at doses of 10, 20, 40, 80, and 160 nM/mL plus mifepristone at a concentration of 1 µM/mL. PPT or DPN were added to the cells at a dose of 1 nM/mL. All assays were conducted at 24 and 48 hours.

Statistical Analysis
Quantitative variables were expressed as mean ± SEM.

Results
Representative T. gondii uninfected astrocytes are shown in Figure 1A. Figure 1B (control; T. gondii-infected astrocytes without hormones) shows the presence of T. gondii tachyzoites, which had replicated approximately four times at this point. Figure 1C (T. gondii-infected astrocytes treated with E2 at 160 nM) shows clear morphological preservation at 24 hours. At 48 hours post-infection, however, the astrocytes were entirely destroyed, with clearly ruptured membranes (Figure 1D).

The addition of 17β-estradiol at 160 nM significantly increased T. gondii replication at 24 hours post-infection in astrocytes in vitro compared with the untreated control (p<0.05, Figure 2A). However, at 48 hours post-infection,
Figure 2: T. gondii replication in astrocytes, as evaluated by MTT

Replication is expressed as percent of the control value. Untreated astrocytes (control), astrocytes pretreated with 17β-estradiol (E2) (blue line), and astrocytes treated with E2 + tamoxifen (orange line), were measured for T. gondii replication and infected astrocytes at 24 and 48 hours. **A** T. gondii replication increased significantly in astrocytes treated with 160 nM E2, compared with all other E2 doses and the control (p<0.05; ANOVA and post-hoc Dunnett’s T3); **B** At 48 hours, T. gondii replication decreased significantly after treatment with E2 (blue line) and E2 + tamoxifen (orange line) (*p<0.001), compared with the untreated control (green line); **C** At 24 hours post-infection, the percentage of infected astrocytes treated with E2 was significantly reduced at doses 10, 40, 80, and 160 nM with respect to the control; the combination of E2 + tamoxifen (orange line) also significantly reduced infection, at all doses, compared with the control; **D** However, at 48 hours post-infection, the percentage of infected astrocytes was significantly increased at doses of 80 nM E2, and combined E2 + tamoxifen at 80 and 160 nM showed a pattern similar to the control.

The percentage of infected astrocytes treated with E2 was reduced compared with the control at all doses from 20 to 160 nM (p<0.001, Figure 2C). This effect was reversed by the action of tamoxifen, mainly at 10, 20, 40, and 160 nM doses at 24 hours post-infection (Figure 2C). The percentage of infected astrocytes was also reduced when they were treated with E2 at doses from 10 to 40 nM for 48 hours. However, an 80 nM dose resulted in a significant increase in the number of infected astrocytes (p<0.001, Figure 2D). In contrast, the combination of E2 + tamoxifen reversed these effects, resulting in a significant reduction in the percentage of infected astrocytes at 10, 40, and 80 nM doses (p>0.05), but an increase at 20 nM, compared with E2 alone (Figure 2D).

The percentage of infected astrocytes 24 hours after treatment with P4 was significantly reduced, compared with untreated controls (p<0.001, Figure 3C). Mifepristone exerted an antagonistic effect on progesterone at doses from 20 to 160 nM, resulting in significantly fewer infected astrocytes (p<0.001) compared with the control (Figure 3C). A significant reduction in infected astrocytes treated with P4 was observed at doses of 10, 20, 80, and 160 nM, compared with untreated controls (p<0.001), at 48 hours (Figure 3D). Mifepristone exhibited an antagonistic effect on P4, significantly increasing the percentage of
Figure 3: T. gondii replication in astrocytes, as evaluated by MTT. Replication is expressed as percent of the control value. Untreated astrocytes (control), astrocytes pretreated with P4 (purple line), and astrocytes treated with P4+mifepristone (red line), were measured for T. gondii replication and percentage of infected astrocytes at 24 and 48 hours. At 24 hours (A) the percentage of T. gondii replication treated with all doses of progesterone (P4) (purple line) and P4 + mifepristone (red line), exhibited no statistical differences compared with the control (green line); (B) Percentage of T. gondii replication was significantly reduced in all doses of P4 and P4 + mifepristone (p<0.05). The percentage of infected astrocytes was reduced by P4 and P4 + mifepristone at all doses, compared with the control, at 24 hours (C) and 48 hours (D), with the exception of P4-40nM and P4+Mif-10nM, post-infection (p<0.001). Mifepristone reversed the effect of P4 at 20 and 40 nM, at 24 hours p.i.

infected cells (p<0.001) at doses of 10, 80, and 160 nM at 48 hours p.i.

At 24 hours, T. gondii replication in astrocytes was not affected by PPT or DPN, nor was there any difference between combined E2+P4 and controls (Figure 4A and 5A). However, at 48 hours, the replication of T. gondii was significantly reduced by PPT and DPN compared with untreated controls p<0.001 (Figure 4B). The percentage of infected astrocytes was reduced by the effect of PPT and DPN with a significant difference (p<0.001) versus controls at 24 hours (Figure 4C). However, at 48 hours, DPN induced a moderate reduction of infected astrocytes compared with the control (Figure 4D).

At 48 hours, combined E2 + P4 significantly reduced T.gondii replication (p<0.001) compared with controls (Figure 5A). The percentage of infected astrocytes was also significantly increased with E2+P4 treatment (p<0.001) vs. control, at both 24 and 48 hours post-infection (Figure 5B).

DISCUSSION

In vitro studies using mouse brain cells have demonstrated that tachyzoites invade microglia (Gay-Andrieu et al., 2002; Fischer, 1997), astrocytes (Halonen, 1998; Jones, 1996), and neurons (Barragan and Sybley, 2002; Courret et al., 2006). In studies using a primary culture from human fetal brain, T. gondii tachyzoites replicated in human astrocytes, and these cells support more replication of T. gondii than neurons in vitro (Contreras-Ochoa et al., 2013; Halonen et al., 1996).

Intracellular tachyzoites are also known to manipulate a variety of signal transduction pathways related to apoptosis, antimicrobial effector mechanisms, and immune cell maturation (Fagard et al., 1999; Li et al., 2010). In the present study, most astrocytes died at 48 hours post-infection, possibly as a result of high Toxoplasma replication or a low number of E2 or P4 receptors. However, the number of parasites in the remaining infected astrocytes increased.
Figure 4: *T. gondii* replication (expressed as % of control) at (A) 24 hours and (B) 48 hours. C, D) *T. gondii* infected astrocytes at 24 (C) and 48 (D) hours after treatment (expressed as % of control) with 4, 4', 4''-(4-propyl-(1H)-pyrazole-1, 3, 5 triyl) trisphenol (PPT, brown bar) or hydroxy-phenyl-propionitrile (DPN, beige bar).

A) *T. gondii* replication was similar in both groups and the control, with no statistically significant differences observed; B) The percentage of replication of *T. gondii* in astrocytes decreased significantly in both the PPT and the DPN groups, compared with the control (p<0.001); C) At 24 hours, the percentage of infected astrocytes treated with PPT and DPN was significantly reduced, compared with the control (p<0.001); D) At 48 hours, the percentage of infected astrocytes in the group treated with PPT was similar to the control. In contrast, in the DPN-treated group, infection was significantly reduced compared with the control (p<0.05).

Figure 5: *T. gondii* replication (expressed as % of control) after treatment with E2 combined with progesterone (E2+P4) (purple and blue bar) at 24 hours and 48 hours (A) and percentage of infected astrocytes (B)

A) The replication of *T. gondii* in astrocytes decreased with E2 + P4, compared with the control, at 48 hours post-infection (p<0.001); B) The percentage of infected astrocytes treated with E2+P4 increased compared with the control at 24 hours (p<0.001) and at 48 hours (p<0.05).

*T. gondii* may control the apoptotic machinery in the astrocytes, through inhibition of caspases 3/7 or PUMA gene expression at the beginning of the infection, to promote replication (Appel et al., 2001).

Li et al. (2010) observed that tachyzoites of the *T. gondii* RH strain had entered astrocytes at 1 h post-infection, and that autophagosomes, which appeared at 4 h, were pronouncedly increased. However, after 12 h, their number was considerably decreased and damage to the cells occurred 48 h later; autophagosomes disappeared and more astrocytes were destroyed. In our study, the astrocytes...
were almost totally destroyed at 48 hours, when *T. gondii* had replicated eight times. This might be due to the disappearance of the astrocyte autophagosomes; however, further studies would be required to test this hypothesis (Li et al., 2010).

Astrocytes are cells of the central nervous system that are sensitive to steroid hormones because they have receptors for 17β-estradiol and progesterone, among others. In the present study, pre-treatment of astrocytes with 17β-estradiol reduced *T. gondii* replication at 48 hours; however, this pattern changed with high doses. E2 may exert a protective action against *T. gondii* infection, because a reduction in the percentage of infected astrocytes was observed at 24 hours post-infection. E2 can regulate immune mediators (cytokines) in the infection process (Halonen et al., 1996; Fagard et al., 1999; Mammari et al., 2014).

Pung and Luster have shown that tamoxifen inhibits *T. gondii* infection susceptibility in mice (Pung and Luster, 1986). We confirmed in our results that tamoxifen reversed the effect of E2 on the susceptibility to infection at 48 hours, possibly because E2 receptors are antagonized by tamoxifen in infected astrocytes.

Progesterone at 48 hours p.i., combined with mifepristone, reduced the replication of the parasite. Mifepristone may be a progesterone receptor antagonist in infected astrocytes (Khan et al., 2013; Bouchard et al., 2011; Farina et al., 2005; Melcangi et al., 2014), possibly affected by dose used or time of exposure to mifepristone. Since the combination of P4 with mifepristone further reduced the percentage of infected astrocytes at 24 hours post-infection, this result suggests that they act at low doses modulating the infection.

The percentage of infected astrocytes treated with E2 plus P4 reduced *T. gondii* replication at 48 hours p.i., but not at 24 hours. This result is probably due to a synergistic effect, with both hormones activating the same cellular signaling pathway (De Marinis et al., 2013). However, more studies are necessary to confirm this.

*T. gondii* replication was reduced by PPT and DPN at 48 hours p.i. These results suggest that an activation of α and β estradiol receptors in astrocytes is induced. PPT and DPN reduced the number of infected astrocytes at 24 hours. Paradoxically, at 48 hours, PPT produced an increase in infected astrocytes. This effect could be due to a reduction in estradiol receptor α response; however, this must be confirmed.

DPN produced a moderate reduction in the percentage of infected astrocytes, compared with controls, at 24 hours and 48 hours p.i. These results suggest that ERβ partici-


