TOXOPLASMOSIS AND NEUROLOGICAL DISEASES IN CHILDREN: A SERIOUS RISK OR MERE ASSOCIATION

By
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Abstract
Tracing the etiology of paediatric neurological disorders back to toxoplasmosis was often difficult and particularly challenged, depended mainly on serology in common practice. The current study evaluated the role of toxoplasmosis and oxidative stress in Egyptian children suffering from acute neurodevelopmental disorders, by seroprevalence in relation to IFN gamma in patients with positivity for IgG & IgM. The result showed that out of 155 patients, 128 (82.6%) were positive for IgG and 10 (6.5%) were positive for both IgG & IgM. Also, the serum malondialdehyde (MDA) was significantly higher in seropositive patients than in seronegative ones. Six children suffered from acute toxoplasmosis showed high levels of serum IFN gamma.

Keywords: Toxoplasmosis, Neurological disorders, Seroprevalence, qPCR, MDA, IFN gamma

Introduction
Toxoplasma gondii (T. gondii) is a worldwide protozoan parasitic burden, causing toxoplasmosis (Flegr et al, 2014). T. gondii has a wide range of hosts including felids as definitive hosts and humans and other warm-blooded animals as intermediate hosts (Bassiony et al, 2016). Toxoplasmosis can be transmitted in several ways and is therefore precipitated by a multiplicity of risk factors. Modes of infection include ingestion of tissue cysts which might be present in undercooked meat or dairy products or ingestion of oocysts in unwashed vegetables and fruits. Organ transplantation and blood transfusion have also been reported as potential portals for parasite entry (Flegr, 2013). In addition, congenital toxoplasmosis is caused by the transplacental transmission of tachyzoites (Alharbi et al, 2017). Although 30% of exposed fetuses acquire the infection from the mother, only 15 % of the babies showed clinical signs of infection at birth, while the majority would appear clinically normal. The development of clinical disease is determined by several factors, especially the gestational age at which infection occurred, where infection acquired at an early gestational age is known to result in a more severe clinical picture as compared to infection acquired in the second and third trimesters (Hill and Dubey, 2002).

Congenital toxoplasmosis may therefore be asymptomatic, or it may present as clinically severe disease during the neonatal period. It may also take a less severe form which manifests during the early months of life. Finally, relapse of an undiagnosed infection can occur later in life, even as late as in adulthood (Remington et al, 2011). The immune status of the host was detrimental to the outcome of toxoplasmosis, where Th1 cytokines such as IFN gamma play a pivotal role in the anti-Toxoplasma immune response (Gavrilesu and Denkers, 2001). The neurotoxoplasmosis was explained by several mechanisms such as the disturbance of glutamate homeostasis that interfered with glutamate transport (David et al, 2016), immunopathological changes and neuroendocrine alterations (Tedford and McConkey, 2017). The pathogenesis of neurotoxoplasmosis was attributed to infection of neurons, microglial cells and Purkinje fibres. Bradyzoites slowly multiply inside parasitophorous vacuoles during the asymptomatic period of infection. When tissue cysts rupture, the parasite takes the tachyzoite form to either invade other cells or induce an inflammatory response by invading mononuclear cells. Activation of latent toxoplasmosis may result from various stimuli such as immunodepression or viral infection (Flegr, 2015). Toxoplasmosis neurotoxicity has been also linked to the infection-related oxidative stress generated in neuronal cells (Dincel and Atmaca, 2016). The central nervous system (CNS) is known to have a high consumption of oxygen, which makes it particularly susceptible to oxidative damage.
Toxoplasmosis diagnosis included bioassays, serological tests, molecular techniques, and microscopic examination of biopsied specimens. While serology was the main choice in clinical practice, molecular detection is preferable when accurate diagnosis is desired (Liu et al, 2015). Tailoring the diagnosis of toxoplasmosis is thus dependent on the target of investigation, whether estimation of seroprevalence or accurate diagnosis and suitable management.

The current study aimed to evaluate the role of toxoplasmosis and oxidative stress among Egyptian children suffered from neuro-developmental disorders and also, to evaluate Toxoplasma positive prevalence among acute infection in relation to IFN gamma with IgG & IgM.

Materials and Methods

Ethical considerations: The principle and aim of the study were explained to all parents and informed consent was obtained. The study was carried out according to the Faculty of Medicine, Cairo University Institutional Ethical Guidelines.

Study design: This study was done in Abu El-Reesh Paediatric University Hospital, Cairo University, from September 2018 to December 2018. Inclusion criteria were the acquisition of an informed consent from the mother and the age group between 2 & 12 years. Clinical cases involved included patients with a variety of neurological disorders. Cross-matched control children without neurological disorders were included. Children suffered from traumatic brain injuries, CNS malignancies and those on immunotherapy were excluded from the study.

Sample and data collection: Specific diagnosis of neurological diseases was performed by a specialized pediatrician based on clinical, laboratory and radiological assessment. Information obtained from patients included age, sex and residence area. Blood samples were collected from each patient in EDTA coated plastic tubes, sera were separated and stored at -20°C till use.

ELISA-IgG (Bessières et al, 2006): Anti-Toxoplasma IgG antibodies were detected using NovaLisa™Toxoplasma IgG-ELISA A Product No.TOXG0460 (Nova, TecImmunoagnostica, GmbH (D-63128 Dietzenbach, Germany).

Qualitative and quantitative measurement of T. gondii antibodies in human serum was performed according to the manufacturer’s instructions. Results were expressed as IgG International Units per milliliter (IU/ml). The resulted antibody index was considered reactive at > 35 IU/ml, equivocal at 30-35 IU/ml and non-reactive < 30 IU.

Anti-IgM antibodies (Herbrink et al, 1987): Anti-Toxoplasma IgM antibodies were detected using IgM-capture ELISA. DRG® Toxoplasma IgM (TORCH) (EIA-1799) (DRG International, Inc., USA) was used for detection of IgM antibodies against T. gondii in human sera. Results were interpreted as negative when IgM index < 0.9, equivocal when index value was 0.9 to 1.0, while positive ≥ 1.0.

Malondialdehyde (MDA) level (nmol/ mL) determination in sera (Draper and Hadley, 1990): Colorimetric measurement of serum MDA, a marker of oxidative stress, was performed using the thiobarbituric acid reaction method.

Enzyme immunoassay for avidity determination of IgG antibodies to T. gondii in human serum (Candolfi et al, 2007): IgG-ELISA NovaLisa TM Toxoplasma gondii IgG avidity test product number TOXGA 460 (Nova Tec Immundiagnostica GmbH (D-63128 Dietzenbach, Germany) was used for the determination of past and recent infection in IgM positive patients. Avidity was calculated according to the following equation:

\[
\text{Avidity %} = \frac{\text{Absorbance (sample or control) avidity reagent}}{\text{Absorbance (sample or control) washing buffer (diluted 1 +19)}} \times 100
\]

Results were interpreted as follows; avidity % > 40 indicated anti-Toxoplasma antibody with high avidity reflecting past infection, while avidity % ≤ 40 indicated anti-Toxoplasma antibody with low avidity reflecting acute or recent infection.

Molecular assay for T. gondii detection (Hierl et al, 2004): To confirm recent infec-
tion in samples which were positive for anti-
Toxoplasma IgM, extraction of genomic DNA from collected blood samples was performed using DNA-Sorb-B kit (Sacace™ DNA-Sorb-B, Sacace Biotechnologies Srl 44 Scalabrini str., 22100 Como, Italy). The eluted DNA was tested using quantitative real-time PCR (qPCR), applying SYBR® Green detection protocol & 2 primers targeting the T. gondii B1 gene: B1 sense (5'-GGAGGACTGGCAACCTGGTGTCG-3') & B1 antisense (5'-TTGTCTTCACCCGACCGT3').

Detection of serum INF gamma levels: Positive IgM samples from patients suffering from neurological disorders were tested for serum INF gamma levels using commercially available ELISA kit Bio (Cat#: ELH-IFN gamma-001) Ray Biotech, Inc. according to manufacturer’s instructions. Assessment of INF gamma level was performed by an ELISA reader at 450nm, calculated on a standard curve and expressed in pg/mL.

Statistical analysis: Microsoft excel 2013 was used for data entry and the statistical package for social science (SPSS) version 24 (SPSS, Armonk, New York: International Business Machines Corporation) was used for data analysis. Simple descriptive statistics; arithmetic mean and standard deviation (SD), summarized quantitative data and frequencies were used for qualitative data. Chi-square test, t-independent test and one-way ANOVA were used to compare normally distributed quantitative data. The level of significance was set at probability (P) value <0.05 (Dawson and Trapp, 2004).

Results

The study included 358 children, 155 of whom were patients suffering from a variety of neurological disorders, while 203 outpatient children free of neurological diseases were taken as control. The mean age of patients was 6+/−2.5 years, while control subjects were 6.4+/−2.7. The cases consisted of 83.9% males and 16.1% females; residence was 78.8% in urban areas and 21.3% in rural areas. Neurological disorders included 25 patients (Guillain Barre syndrome; GBS), 23 (acute encephalitis), 36 (cerebral palsy), 21 (hydrocephalus), 15 (autism), 20 (Down syndrome) and 15 (attention deficit hyperkinetic disorder ADHD).

Toxoplasmosis seroprevalence and serum MDA level: Among toxoplasmosis cases 82.6% were IgG positive (titer 211.5+/-149 IU/mL) as compared to 80.8% controls (titer 186.9+/-133.6 IU/mL), but without significant difference in IgG sero-prevalence (P >0.05) or sero-intensity (P >0.05) between patients and controls. The IgM seropositive results was significantly lower as compared to controls (P <0.05), where only 10 patients were IgM seropositive (mean index value 1.4+/−0.4) as compared to 38 controls (mean index value 1.5+/−0.3). No significant difference (P > 0.05) was detected between IgM index in seropositive patients and controls.

Evaluation of the IgG in relation to each neurological disease showed the highest percentage among patients with cerebral palsy (91.7%), and the lowest one was in children suffering from ADHD (66.7%). The IgG seropositivity in GBS, acute encephalitis, hydrocephalus, autism and Down syndrome was 80%, 73.9%, 90.5%, 73.3% & 90%, respectively. IgM seropositivity was as follows; in 4 patients suffering from acute encephalitis, 4 Down syndrome patients, and 2 cases with GBS. The MDA level in children suffering from neurological disorders was significantly higher than that found in their control counterparts. Within the cases group, the evaluation of the relation between the MDA level and sero-reactivity to anti-Toxoplasma IgG showed a significantly higher level in seropositive patients as compared to seronegative ones.

IgG avidity testing, qPCR, and serum IFN gamma in positive IgM patients: All IgM positive children suffered from neurological diseases as well. Avidity testing and qPCR were performed in these cases so as to investigate whether infection in these patients was an acute infection or a past one with persistent IgM. Acute infection was confirmed in
6 patients diagnosed with GBS (1 case), acute encephalitis (3 cases) and Down syndrome (2 cases). Quantitative genomic estimation of these positive samples in qPCR ranged from $2.5 \times 10^1$ to $9 \times 10^8$ genomic equivalents reflecting the different DNA quantities. The IgM index in these cases (mean $1.7 \pm 0.4$) was significantly higher than that of the four patients found to have a past infection (mean $1.2 \pm 0.1$) by avidity and molecular testing ($P < 0.05$). Measurement of serum IFN gamma showed significantly higher levels in confirmed acute toxoplasmosis as compared to patients with past infection.

Details were given in tables (1, 2, 3 & 4) and figures (1 & 2).

**Table 1: Sero-reactivity to anti-Toxoplasma IgG and IgM in patients and control subjects**

<table>
<thead>
<tr>
<th>IgG</th>
<th>Cases</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Positive</td>
<td>128</td>
<td>82.6</td>
<td>164</td>
</tr>
<tr>
<td>Negative</td>
<td>27</td>
<td>17.4</td>
<td>39</td>
</tr>
<tr>
<td>Total</td>
<td>155</td>
<td>100</td>
<td>203</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IgM</th>
<th>Cases</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>6.5</td>
<td>38</td>
</tr>
<tr>
<td>Negative</td>
<td>145</td>
<td>93.5</td>
<td>165</td>
</tr>
<tr>
<td>Total</td>
<td>155</td>
<td>100</td>
<td>203</td>
</tr>
</tbody>
</table>

Statistically significant; Chi-square test

**Table 2: MDA level in patients and control subjects**

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Cases (N=155)</th>
<th>Control (N=203)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/mL)</td>
<td>24.4 +/-6.8</td>
<td>17.5 +/-9.2</td>
<td>&lt; 0.05*</td>
</tr>
</tbody>
</table>

Statistically significant; one-way ANOVA

**Table 3: MDA level in patients in relation to the sero-reactivity to anti-Toxoplasma IgG**

<table>
<thead>
<tr>
<th>Study groups</th>
<th>IgG positive (N= 128)</th>
<th>IgG negative (N= 27)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/mL)</td>
<td>24.95 +/-6.7</td>
<td>22.01 +/-7.1</td>
<td>&lt; 0.05*</td>
</tr>
</tbody>
</table>

Statistically significant; one-way ANOVA

**Table 4: IFN gamma level in positive patients for IgG & IgM in relation to confirmed acute infection**

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Acute infection (N= 6)</th>
<th>Past infection (N= 4)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN gamma (pg/mL)</td>
<td>2007.17 +/-238.6</td>
<td>305 +/-112.4</td>
<td>&lt; 0.05*</td>
</tr>
</tbody>
</table>

Significant; t-test

**Discussion**

The current study showed an overall high seroprevalence of anti-*T. gondii* antibodies in the study population. The IgG seropositivity was uniformly and widely distributed in cases (82.6%) and controls (80.8%), IgM seropositivity was significantly higher in controls (18.7%) as compared to cases (6.5%). This curious data of elevated IgM in the controls agreed with Alvarado-Esquivel et al. (2017), but IgM was detected in 37.1% of controls and 14.3% of cases. Globally, reports estimated *T. gondii* seroprevalence to be up to 85% of the population, especially in the tropics of developing countries (Hernández-Cortazar et al., 2015; Nayeri et al., 2020). In Egypt, Abbas et al. (2020) stated in a review on toxoplasmosis that “the current situation is confusing” without central working group conducting national level reports in the light of an apparently high *T. gondii* seroprevalence based on various sporadic studies. Ibrahim et al. (2009) and El Deeb et al. (2012) showed a significantly high prevalence of toxoplasmosis among pregnant females of 51.49% & 67.5%, respectively.

The present study showed various degrees of IgG seropositivity among all the neurological diseases; acute encephalitis, Down syndrome, GBS, cerebral palsy, hydrocephalus, autism and ADHD. Anti-Toxoplasma IgM was used as a preliminary test for acute infection. Confirmation was achieved by IgG avidity testing and qPCR for B1 *T. gondii* gene. Cases with acute toxoplasmosis included children suffering from acute encephalitis, Down syndrome and GBS.

Eraky et al. (2016) in Benha University Hospitals found that anti-*T. gondii* IgG was significantly higher in children with cryptogenic epilepsy (20%) in comparison to children with non-cryptogenic epilepsy (0%) and healthy controls (10%). Also, Hamed et al (2018) showed a significantly high prevalence of anti-*T. gondii* IgG in mentally retarded children (42%) attending Cairo University hospital, when compared to the controls (17.5%). However, El-Beshbishi et al (2018)
in Mansoura University Hospital among children suffered from the various neurological disorders (microcephaly, hydrocephalus, ADHD & epilepsy), collectively grouped as non-syndromic neurological diseases, and Down syndrome, found that IgG seropositivity was significantly higher in non-syndromic neurologically disabled children (63.3%) and Down syndrome (13.3%), when compared to the controls (10%). Although the percentage of seropositive cases in the current study was higher in comparison to the previously mentioned ones, a significant association between presence of anti-Toxoplasma antibodies and neurological diseases could not be established due to the high prevalence of seropositivity also present among controls.

In general, TORCH infections (toxoplasmosis, Rubella, Cytomegalovirus, Herpes simplex virus and others such as syphilis) accounted for 5 to 10% of permanent neurological disabilities (Hermansen and Hermansen, 2006). Congenital toxoplasmosis diagnosis is easiest at birth; as serological findings later in life can be due to postnatally acquired toxoplasmosis, thus the differentiation from reactivation of congenital infection becomes challenging.

Toxoplasmic encephalitis was a serious cause of morbidity in immunocompromised individuals, seldom seen in immunocompetent patients (Habek et al, 2009). Though toxoplasmosis may not sound like a probable cause of Down syndrome, the possibility that it might play a role in the future development of psychiatric disease in Down syndrome patients must be considered. Dykens et al (2015) reported a significantly higher incidence for the development of psychotic disorders and slowing of motor and language functions in patients with Down syndrome. Reports on the causal relationship between toxoplasmosis and GBS were mainly available as individual case reports (Bossi et al, 1998; González et al, 2000; Calabuig et al, 2009). The importance of determining the implication of toxoplasmosis in the etiopathogenesis of GBS is highlighted by the fact that patients receiving anti-toxoplasmosis therapy shown improvement of the disease symptoms (Bossi et al, 1998; Calabuig et al, 2009).

Hydrocephalus is one of the classically described manifestation of congenital toxoplasmosis, estimated to affect about 4% of infants infected in utero. Different patterns of hydrocephalus, including those with and without anatomical obstruction were found in cases of congenital toxoplasmosis (McLeod et al, 2014; Hutson et al., 2015). As for cerebral palsy, although congenital toxoplasmosis is a well-recognized risk, further updated comprehensive epidemiological studies are needed. As to ADHD, Afsharpaniman et al (2016) detected a seroprevalence of toxoplasmosis in 4.2% of children suffering from ADHD as compared to 2.1% of controls. But, Khademvatan et al (2018) showed that 18% of children and adults suffered from ADHD were toxoplasmosis seropositive as compared to 24% of controls. Though clinical evidence for linking toxoplasmosis and ADHD is lacking, experimental infection induced hyperactivity and sensorimotor changes in laboratory animals (Carter, 2013). Concerning autism, Prandota et al (2015) found that 23.9% of autistic patients were toxoplasmosis seropositive in contrast to 4 % in the control group. Also, the seropositive patients showed significantly higher levels of nitric oxide and IFN gamma when compared to the seronegative ones.

Being an intracellular pathogen, the immune system confronts the invading T. gondii by the generation of a strong Th1- cytokine response. The principal cytokine involved in the anti-Toxoplasma immune response is IFN gamma. In the present study, the level of IFN gamma was significantly higher in patients with acute toxoplasmosis. Melzer et al (2008) reported that IFN gamma lead to the attenuation of T. gondii growth in astrocytes through disrupting the parasitophorous vacuole thus resulting in parasite egress.

IFN gamma also compromises parasite gr-
wth by the induction of inducible Nitric oxide synthase (iNOS). The consequent generation of the cytotoxic molecule nitric oxide (NO) along with other free radicals established a state of oxidative stress (Hakimi et al., 2017). The brain is particularly vulnerable to the damaging effects of reactive oxygen species due to its high oxygen consumption, weak antioxidant defense mechanism and high content of polyunsaturated fatty acids. Brain pathology may result from the DNA damage, autophagy and apoptosis (Popa-Wagner et al., 2013).

In the current study, children with neurodevelopmental disorders had significantly higher MDA levels than their healthy counterparts. Besides, seropositive patients had statistically higher MDA levels than seronegative ones. Aycicek and Iskan (2006) reported that patients suffered from cerebral palsy were found to have higher levels of lipid peroxidation and lower antioxidant capacity levels as compared to controls. Karahan et al. (2008) investigated the relation between oxidative stress and toxoplasmosis by measuring the blood levels of MDA, NO and glutathione (GSH) in seropositive cases as compared with levels in seronegative ones. Significantly higher levels of MDA & NO and lower levels of GSH were associated with seropositivity to anti-Toxoplasma IgG. Dincel and Atmaca (2016) studied the role of oxidative stress in Toxoplasma-induced neuropathology and neurodegeneration in Swiss Albino mice with toxoplasmic encephalitis. They found a markedly increased in local expression of glutathione reductase & 8-hydroxy-2’-deoxyguanosine, along with decreased activity of superoxide dismutase. This oxidative state was accompanied by neuronal apoptosis and degeneration as evidenced by increased levels of neuron specific enolase.

**Conclusion**

The high prevalence of anti-Toxoplasma antibodies per se does not prove the involvement of toxoplasmosis in the etiopathogenesis of pediatric neurological diseases, even in the presence of IgM antibodies. Furthermore, MDA and IFN gamma proved important indicators of underlying infection offering potential management modalities for neurological diseased children. The overall high seroprevalence data alerts for the development of a cost-effective screening program for toxoplasmosis together with efficient plans for prevention and control.

**Authors’ contribution:** Amin NM: Setting of study concept; Designed study plan, material supplementation carried out laboratory analysis and data recording. El-kazaz A: Data and sample collection and patient evaluation. Abdel-Shafi IR: Review & editing. El-Sebaei EH: Data analysis and statistics. Abdeltawab MSA: Interpretation of data; writing of original draft; review & editing. All authors critically revised the manuscript and approved the final manuscript.

**Conflict of Interest:** The authors declared that they neither have competing interest nor received fund.

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Explanation of figures

Fig.1: Pie chart representing the percentage of each neurological disease relative to cases (N=155). GBS= Guillain Barre syndrome, AE= acute encephalitis, CP= cerebral palsy, HP= hydrocephalus, DS= Down syndrome, ADHD= attention deficit hyperkinetic disorder

Fig. 2: Column chart representing the sero-reactivity to anti-Toxoplasma IgG in relation to each neurological disease. GBS= Guillain