Molecular & Biochemical Parasitology 172 (2010) 1–8

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Biochemical and immunopathological changes in experimental neurotoxocariasis

Ahmad A. Othman [a,*], Ghada A. Abdel-Aleem [b], Eman M. Saied [c], Wael W. Mayah [d], Afaf M. Elatrash [e]

[a] Department of Medical Parasitology, Faculty of Medicine, Tanta University, Tanta, Gharbia, Egypt
[b] Department of Biochemistry, Faculty of Medicine, Tanta University, Tanta, Egypt
[c] Department of Pathology, Faculty of Medicine, Tanta University, Tanta, Egypt
[d] Department of Tropical medicine, Faculty of Medicine, Tanta University, Tanta, Egypt
[e] Department of Zoology, Faculty of Science, Tanta University, Egypt

ARTICLE INFO

Article history:
Received 27 January 2010
Received in revised form 9 March 2010
Accepted 9 March 2010
Available online 17 March 2010

Keywords:
Toxocara canis
Neurotoxocariasis
CNS
Cytokines
Neurotransmitters
GFAP
iNOS

ABSTRACT

Toxocariasis is a widespread soil-transmitted parasitic disease. *Toxocara canis* larvae migrate through the tissues with a special predilection for the central nervous system. Recently, neurotoxocariasis is being diagnosed in humans with increasing frequency due to improved diagnostic tools. The present study aimed at exploring the biochemical and immunopathological alterations in the brain in experimental *T. canis* infection. For this purpose, 75 *Toxocara*-infected mice were sacrificed at 2, 5, and 16 weeks post-infection. The brains were removed and assayed for total larval count, pro-inflammatory cytokines (TNF-α, IL-6), and central neurotransmitters (γ-aminobutyric acid, glutamate, dopamine, norepinephrine, and serotonin). Brain sections were also stained for histopathological study, and for assessment of the expression of inducible nitric oxide synthase (iNOS), and glial fibrillary acidic protein (GFAP) by immunohistochemical methods. We found that larval recovery showed progressive increase over the course of infection. Furthermore, the infected mice displayed increased expression of pro-inflammatory cytokines and iNOS, as well as significant disturbances in neurotransmitter profile. Astrocytic activation, evidenced by enhanced expression of GFAP, was also manifest in infected animals. These changes were maximal in the chronic stage of infection or intensified over time. In conclusion, experimental neurotoxocariasis is associated with significant biochemical, immunological, and pathological changes.

1. Introduction

Toxocariasis is a soil-transmitted helminthozoonosis due to infection of humans by larvae of *Toxocara canis* (*T. canis*). Seroepidemiological surveys show prevalence to be 2–5% and 14–37% in urban and rural areas of Western countries respectively, while in developing countries prevalence may reach 50–80%. The principal risk factors include male gender, geophagia, contact with dogs, and residence in a rural region. After ingestion of *Toxocara* eggs, the larvae penetrate the gut to migrate through the liver and other viscera with a special predilection for the nervous system [1].

Neurotoxocariasis is being recently diagnosed with increasing frequency due to improved diagnostic tools [1]. Cases of neurological toxocariasis may present as meningoencephalitis with eosinophilic pleocytosis [2], meningoradiculitis [3], or transverse myelitis with eosinophils in CSF [4]. Moreover, many studies have suggested possible association between zoonotic *T. canis* infection and epilepsy [5]. Other possible associations include social, learning and behavioural abnormalities especially in children [6,7]. If detected and treated early, the prognosis of neurological toxocariasis is favourable [8].

Cytokines play an important role in the immunopathology of *Toxocara* infection, and various cytokines have been described in different tissues infected with *T. canis* including the lung [9], and the brain [10]. In spite of their critical role in the immunopathology of various events in the brain, pro-inflammatory cytokines, such as IL-6 and TNF-α have not been studied in *Toxocara* infection. Likewise, disturbances in neurotransmitters in neurotoxocariasis are not well investigated, although abnormalities in various neurotransmitters have been described in many CNS infections, such as African trypanosomiasis [11], toxoplasmosis [12], and trichinosis [13].

Astrocytes are one of the two primary types of macroglia. They comprise nearly 35% of the total central nervous system (CNS) cell population and, like microglia, are found in all regions of the CNS. Gliosis is a common response to CNS injury and dysfunction. A key indicator of glial reactivity is the increased synthesis of glial fibrillary acidic protein (GFAP), which is commonly used to examine the distribution of glial cells in response to neural injury [14]. Despite the apparent absence of inflammatory reaction around *T.*

* Corresponding author. Fax: +20 2 40 3407734.
E-mail address: ahmed_ali44@hotmail.com (A.A. Othman).

0166-6851/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2010.03.006

canis larvae in experimental cerebral infection, there is evidence for astrogliosis and enhanced GFAP expression in the brain [15].

The aim of this study was to investigate the biochemical and immunopathological alterations in the brains of mice infected with *T. canis*.

2. Materials and methods

2.1. Parasite

T. canis eggs, obtained by flotation from stools of puppies, were cultured in 0.1 N H_2SO_4 for embryonation and storage. Infections were made using eggs from a single culture not more than 60 days old [16].

2.2. Animals and experimental design

Laboratory-bred male Swiss albino mice (20–25 g in weight) were used in this study. Mice were housed and infected in accordance with the institutional and national guidelines. Seventy-five animals were inoculated orally with a single dose of 1000 embryonated *T. canis* eggs each. Groups of 25 infected animals were sacrificed 2, 5, and 16 weeks post-infection (p.i.), and divided as follows: 10 mice for brain larval count, 5 mice for cytokine estimation, and 10 mice in which the brains were cut longitudinally into two equal halves; one for neurotransmitter assay and the other for histological and immunohistochemical study. At each time p.i., 10 uninfected mice were used as non-infected control: 5 animals for cytokine estimation, and 5 for neurotransmitter assay and immunohistochemistry. For cytokine and neurotransmitter assay, the infected and control animals were sacrificed by cervical dislocation, and the brain was removed from each mouse following bleeding by cardiac puncture. Further, groups of infected and control mice used for neurotransmitter estimation were treated intravenously with 1.2 mmol/kg 3-mercaptopropionic acid 2 min prior to death. This treatment was found to be suitable for prevention of postmortem elevation of GABA levels [17]. The brains were removed, frozen in liquid nitrogen and stored at −80 °C until assayed. All chemicals and solvents used were of high analytical grade, products of Sigma (USA), Merck and Reidel (Germany), BDH (England), and Fluka (Switzerland) Chemical Co.

2.3. Total larval count in the brain

Infected animals (10 mice at each time p.i.) were euthanized, and the whole brain from each animal was removed in a petri dish. The two halves of the cerebrum and the cerebellum were separated and each was compressed between microscope slides and examined under the low power of microscope. The larvae were counted directly and their motility was observed [16].

2.4. Estimation of levels of IL-6 and TNF-α mRNA expression by semi-quantitative real-time PCR

Brain samples (5 randomly selected from the infected group and 5 randomly selected from control group at 2, 5, and 16 weeks p.i.) were taken. The samples were processed for total RNA extraction (MagNA Pure compact Nucleic Acid isolation kit I, Roche Diagnostics, GmbH, Mannheim, Germany) according to the manufacturer's recommendations. First strand cDNA was synthesized from total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) according to the manufacturer's instructions.

Interleukin-6 and TNF-α mRNA transcripts were quantified, relative to the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the Roche LightCycler® FastStart DNA Master^{PLUS}SYBR Green I kits (Roche Diagnostics, Mannheim, Germany) following manufacturer's instructions. Briefly, in a 1.5 ml reaction tube on ice, the following components were added in the same order they are mentioned: 13 μl sterile H_2O, PCR-grade, 2 μl LightCycler RT-PCR Reaction Mix SYBR Green I, 1.6 μl $MgCl_2$ stock solution (final conc. 5 mM), 2 μl Cytokine Primer Mix (final conc.0.5 μM) and 0.4 μl LightCycler RT-PCR Enzyme Mix, giving a total volume of 19 μl. The components were mixed gently then pipetted into pre-cooled LightCycler capillary. One microliter of the cytokine RNA template was added giving a final volume of 20 μl. The primer sequences for IL-6, TNF-α, and GAPDH used in the study were as follows: IL-6 (sense 5′-CAGCCACTGGTTCTGTGCCT-3′), IL-6 (antisense 5′-TCAATGAGGAGACTTGCCTGG-3′), TNF-α (sense 5′-AGGCAATAGGTTTTGAGGGCCAT-3′), TNF-α (antisense 5′-TCCTCCCTGCTCCGATTCCG-3′), GAPDH (sense 5′-GAAGGTGAAGGTCGGAGTC-3′), GAPDH (antisense 5′-GAAGATGGTGATGGGATTTC-3′). The optimal conditions for PCR amplification were: an initial incubation at 95 °C for 30 s, 45 cycles of denaturation at 95 °C, annealing at 55 °C (10 s), and extension at 72 °C (13 s). The values of the target gene under investigation and the value of the house-keeping gene (GAPDH) were calculated for each sample using a standard curve. The standard curve for each target gene and the house-keeping gene was made by threefold serial dilutions of a single cDNA sample from a non-infected control mouse, and the obtained crossing point (CP) values were plotted against an arbitrary log concentration. A ratio between the calculated values of the target gene and house-keeping gene was obtained, which indicates the amount of target normalized to the level of an endogenous reference gene within each unknown sample. The final results were automatically calculated from the CP values of the target and the reference genes by LightCycler® 480 Relative Quantification Software.

2.5. Biochemical assay of neurotransmitters in the brain

For determination of γ-aminobutyric acid (GABA) and norepinephrine (NE), parts of brain tissues of examined mice were thawed, washed with saline, blotted with filter paper, weighed and quickly dissected on a chilled dissection board. Brain samples (1 gm) were homogenized at 0 °C in 10 ml of 0.01 mol/l acetic acid solution containing $Na_2S_2O_5$ (sodium metabisulfite) and EDTA at a final concentration of 100 g/l each [18].

Estimation of GABA. GABA levels were determined by reverse-phase HPLC with fluorescence detection after derivation with o-phthaldialdehyde (OPA)/sulphite, according to the method used by Donzanti and Yamamoto [19].

Estimation of norepinephrine. NE was determined using the spectrofluorometric method of Schlumpf et al. [20], which depends on oxidation of noradrenaline with potassium ferricyanide into noradrenochrome and conversion of the later with alkaline ascorbate solution to noradrenolutine which has a yellowish green fluorescence. Fluorescence emission is at 250 nm when excited at 420 nm.

Estimation of glutamate. For determination of glutamate, brain samples were homogenized on ice (50 mg of tissue with 1 ml 400 mM $HClO_4$, 50 μM EDTA), and neutralized with 100 mM borate buffer (1:10). The homogenates were then centrifuged (14,000 rpm, 15 min, 4 °C) and filtered. Glutamate levels were determined by reverse-phase HPLC with fluorescence detection after derivation with o-phthaldialdehyde (OPA)/β-mercaptoethanol derivatives according to Donzanti and Yamamoto [19].

Measurement of dopamine and serotonin. Part of brain (50 mg) was homogenized in 0.1 ml hydrochloric acid–*n*-butanol (0.85 ml of 37% hydrochloric acid in 1 l *n*-butanol) for 1 min in a cool environment. The sample was then centrifuged for 10 min at 2000 rpm. 0.08 ml of supernatant phase was removed and added to an Eppen-

Table 1
Larval count (mean ± SD) in the brain in infected groups.

	2 weeks p.i.	5 weeks p.i.	16 weeks p.i.	*P*
Larval count	62.700 ± 8.629	95.700 ± 7.602	103.200 ± 5.371	$P_1 < 0.001^*$ $P_2 < 0.001^*$ $P_3 = 0.075$
% [a]	6.27	9.57	10.32	

P_1 comparison between infected groups at 2 and 5 weeks p.i., P_2 comparison between infected groups at 2 and16 weeks p.i., P_3 comparison between infected groups at 5 and 16 weeks p.i.

[a] Number of larvae expressed as a % of original dose.

[*] Significant.

dorf reagent tube containing 0.2 ml of heptane and 0.025 ml 0.1 M hydrochloric acid. After 10 min of vigorous shaking, the tube was centrifuged under same conditions to separate two phases. Upper organic phase was discarded and the aqueous phase was used for dopamine and serotonin estimation [20,21].

Estimation of dopamine. To the aqueous phase (0.02 ml) 0.005 ml of 0.4 M hydrochloric acid and 0.01 ml EDTA/sodium acetate buffer (pH 6.9) were added, followed by 0.01 ml iodine solution (0.1 M in ethanol) for oxidation. The reaction was stopped after 2 min by addition of 0.01 ml Na_2SO_3 in 5 M NaOH (0.5 g Na_2SO_3 in 2 ml H_2O + 18 ml 5 M NaOH). Acetic acid (0.01 ml, 10 M) was added 1.5 min later. The solution was then heated to 100 °C for 6 min. When the sample again reached room temperature, excitation and emission spectra were read in the microcuvette. The readings were taken at 330–375 nm [20].

Estimation of serotonin (*5-hydroxytryptamine*). Serotonin is extracted into an ascorbic acid solution by freezing and thawing and sonication. Ascorbic acid stabilizes the serotonin in the extract, and added ethanol enhances the final fluorescence of serotonin, which is measured in a concentrated hydrochloric acid medium (excitation at 295 nm and emission at 540 nm) according to the method of Vatassery et al. [21].

2.6. Histopathological and immunohistochemical study

All the studied specimens were fixed in 10% formalin and subsequently embedded in paraffin, then submitted to haematoxylin and eosin (H&E) staining for histopathological examination.

Immunohistochemistry was done for demonstration of activated glial cells using antibody against inducible nitric oxide synthase (iNOS), and activated astrocytes using antibody against glial fibrillary acidic protein (GFAP). Immunohistochemical staining was performed on 3–5 μm sections from randomly selected 10 paraffin blocks from the infected group and 5 blocks from the control group at 2, 5, and 16 weeks p.i., using the Ultra Vision Detection System (Anti-Polyvalent, HRP/DAB "Ready-to-Use", Cat.#TP-015-HD, Lab Vision, USA). The procedure of immunostaining was conducted according to the manufacturer's protocol. Briefly, sections were deparaffinized with xylene and rehydrated with graded alcohol series. Antigen retrieval was done by immersing the sections in 10 mmol/l citrate buffer (pH 6.0) for 10 min at 100 °C in microwave. Endogenous peroxidase activity was blocked with hydrogen peroxide block for 10 min. After thorough washing of the sections with PBS, incubation was done for 10 min with Ultra V block to prevent non-specific background staining, followed by rinsing the sections with PBS. Subsequently, an overnight incubation of the sections with the following antibodies was done at room temperature in a humidity chamber: (1) antibody against iNOS (Rabbit Polyclonal Antibody, Cat. #RB-9242-R7 "Ready-to-Use", Lab Vision, USA) and (2) antibody against GFAP (Ab-4 rabbit polyclonal antibody, Cat.#RB-087-R7 "Ready-to-Use", Lab Vision, USA). The sections were then washed with PBS and incubated with biotinylated goat anti-polyvalent (secondary antibody) for 10 min at room temperature followed by washing with PBS, then incubated with streptavidin peroxidase solution for 10 min at room temperature, then rinsed with PBS. The reaction products were visualized using 3-3′-diamino-benzidine-tetra-hydrochloride (DAB), and the sections were then counterstained with Mayer's haematoxylin, dehydrated in alcohol and mounted in Di-*n*-butyl-phthalate-polystyrene-xylene (DPX). In order to confirm the results of the staining, sections from normal mouse lung and brain were used as positive controls for iNOS and GFAP respectively. In addition, for negative controls, omission of the primary antibodies was done, and instead, normal rabbit serum was applied. Cells showing a distinct brownish cytoplasmic reaction to iNOS and GFAP were considered positive.

2.6.1. Quantification of iNOS and GFAP immunoreactivity

Within each case, a semi-quantitative score of four grades was assigned based on the number of cells showing immunopositivity for iNOS and GFAP as follows: 0 = negative staining, 1 = scattered or weakly positive staining, 2 = moderately positive staining, and 3 = strongly positive staining [22].

2.7. Statistical analysis

Data were presented as means ± standard deviation. Analysis of variance (ANOVA) test was used for comparison among different groups for quantitative data. Fisher's exact test was used for evaluating iNOS and GFAP expression. Differences were considered non-significant when ($P > 0.05$) and significant when ($P < 0.05$). Since the variations were negligible and non-significant over different times for all the measured parameters in control groups, we used the means of these values for comparison with those of infected animals. The statistical analyses were processed according to the conventional procedures using Statistical Package of Social Sciences (SPSS) software for windows, version 10.0.

3. Results

3.1. Brain larval count

Table 1 shows larval counts in the brains of infected animals. There was significant buildup of larval recovery as the infection progressed, with a peak reached at 5 weeks p.i. Larvae were more abundant in the cerebrum than in the cerebellum. Qualitative assessment showed that most larvae (more than 80%) observed in the brains of the infected animals were actively motile and there could be no doubt that they were alive. They were moving actively through the tissue, and there was no indication that larvae in the brain were trapped by an inflammatory reaction. It was also evident that the larvae were not distributed randomly throughout the brain tissue but were to a large degree clumped or showing focal distribution.

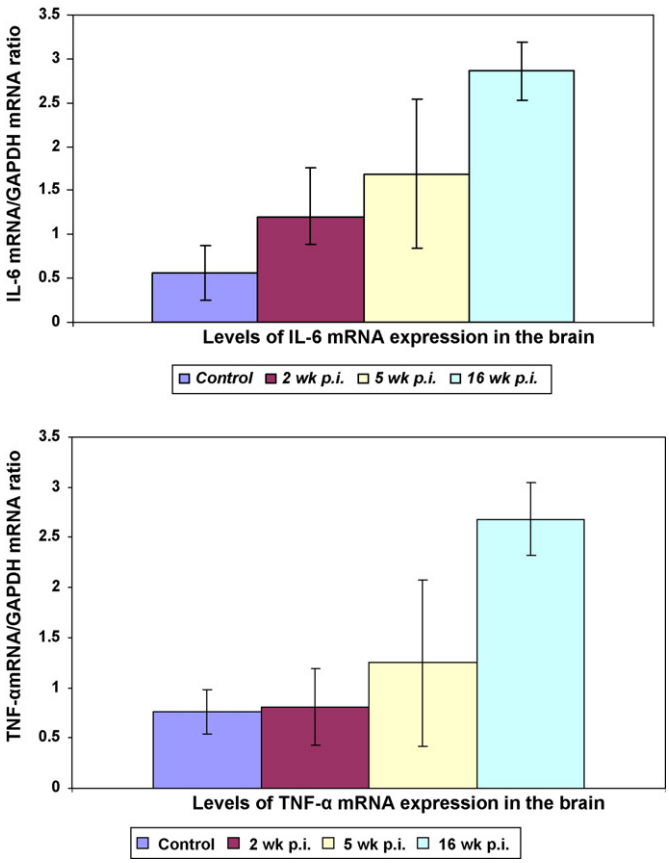

Fig. 1. Cytokine mRNA expression in the brain tissues at different time points p.i. Vertical bars represent the mean (±SD) of these results for each group ($n = 5$). Control values represent the means of those measured over different times. Significant increase in levels of IL-6 mRNA at 5 weeks and 16 weeks p.i., and TNF-α mRNA at 16 weeks p.i. was noted in infected mice compared to controls.

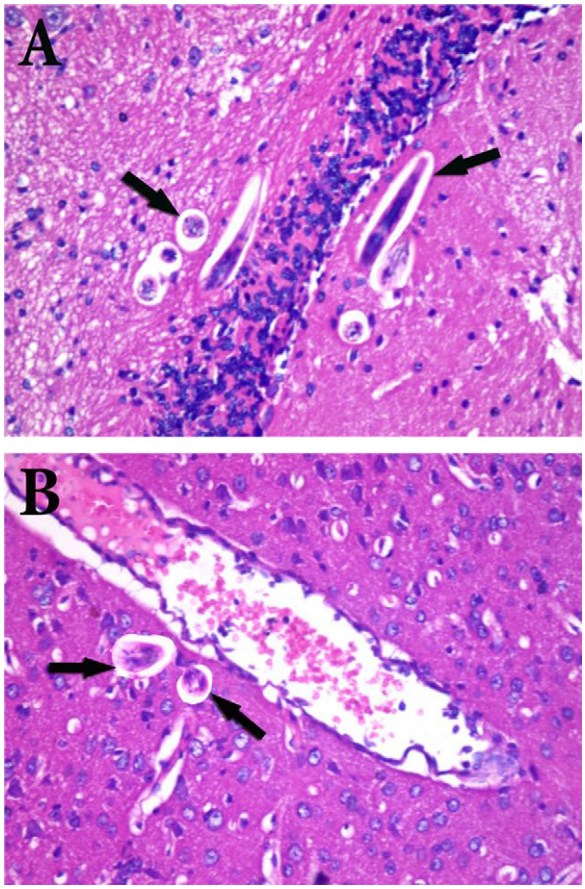

Fig. 2. Photomicrographs of brain sections in the region of diencephalon showing: (A) numerous tangential and cross-sectional profiles of *Toxocara* larvae "arrows" deposited within the cerebral tissue and (B) vascular congestion adjacent to the larvae (H&E X400).

3.2. Cytokine mRNA expression

Fig. 1 shows levels of mRNA expression of the measured cytokines. In the brain homogenates, levels of IL-6 mRNA expression were significantly increased ($P < 0.05$) in infected mice compared to controls starting from 5 weeks p.i. Meanwhile, levels of TNF-α mRNA expression were significantly increased ($P < 0.05$) in infected mice compared to controls at 16 weeks p.i.

3.3. Neurotransmitter levels in the brain

Levels of the measured neurotransmitters are shown in Table 2. As regards the central amino acids, GABA levels showed significant reduction ($P < 0.05$) in infected animals when compared to control at 5 and 16 weeks p.i., whereas glutamate levels showed the inverse pattern. Moreover, GABA levels showed significantly progressive decline with time. For monoamines, dopamine and serotonin levels significantly decreased ($P < 0.05$) in infected animals starting from 2 weeks p.i. for serotonin, and 5 weeks p.i. for dopamine. On the other hand, norepinephrine levels were significantly higher ($P < 0.05$) in infected animals compared to control at all time points p.i.

3.4. Histopathological findings

Histopathological examination revealed the presence of numerous *T. canis* larvae scattered in the parenchyma of the brain of the

Table 2
Levels (mean ± SD) of central neurotransmitters in brain homogenates.

Group	GABA (μmol/gm) wet tissue mass	Dopamine (μg/gm) wet tissue mass	Noradrenaline (μg/gm) wet tissue mass	Serotonin (μg/gm) wet tissue mass	Glutamate (μmol/gm) wet tissue mass
Control [a]	1.964 ± 0.065	0.667 ± 0.033	0.59 ± 0.1	0.605 ± 0.01	11.25 ± 0.19
2 weeks p.i.	1.854 ± 0.051	0.527 ± 0.021	0.6 ± 0.05	0.581 ± 0.04	11.91 ± 0.25
5 weeks p.i.	1.552 ± 0.096	0.405 ± 0.042	0.69 ± 0.2	0.398 ± 0.03	12.86 ±0.34
16 weeks p.i.	1.389 ± 0.040	0.321 ± 0.01	0.87 ± 0.21	0.295 ± 0.01	14.18 ± 0.14
P_1	0.049*	0.071	0.088	0.365	0.362
P_2	0.085	0.058	0.047*	0.010*	0.051
P_3	0.042*	0.030*	0.027*	0.032*	0.009*
P_4	0.038*	0.028*	0.031*	0.027*	0.006*

P_1 comparison between infected groups, P_2 comparison between control and infected group 2 weeks p.i., P_3 comparison between control and infected group 5 weeks p.i., and P_4 comparison between control and infected group 16 weeks p.i.

[a] Control values represent the means of those measured over different times for each transmitter.

* Significant.

Table 3
iNOS expression in infected groups.

Group	iNOS expression					
	Grade 1 iNOS staining		Grade 2 iNOS staining		Grade 3 iNOS staining	
	N.	%	*N.*	%	*N.*	%
2 weeks p.i. ($n = 10$)	9	90	1	10	0	0
5 weeks p.i. ($n = 10$)	5	50	4	40	1	10
16 weeks p.i. ($n = 10$)	1	10	5	50	4	40
P value	0.101[a]		0.034[*,b]		0.019[*,c]	

[a,b,c] Comparison between infected groups for grade 1, 2, and 3 iNOS expression respectively.
[*] Significant.

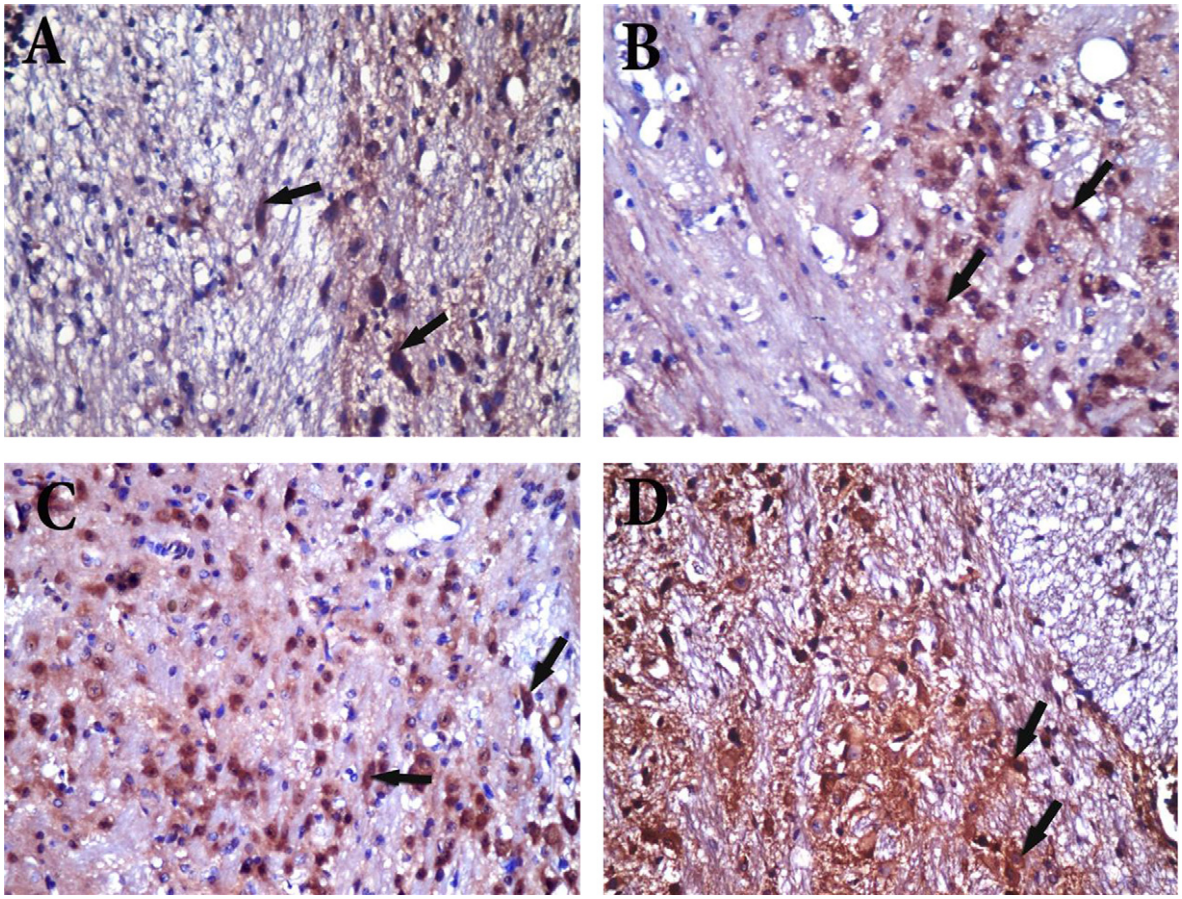

Fig. 3. iNOS staining of brain sections from the infected groups showing positive glial iNOS expression "arrows": (A) 2 weeks p.i. showing grade 1 immunoreactivity, (B) 5 weeks p.i. showing grade 2 immunoreactivity, and (C) and (D) 16 weeks p.i. showing grade 3 immunoreactivity (Immunoperoxidase X400).

infected group (Fig. 2A), especially near the choroid plexus and corpus callosum, with fewer larvae detected in the cerebellum. No visible inflammatory reaction was observed around the migrating larvae except for vascular congestion adjacent to some of them (Fig. 2B).

3.5. iNOS immunoreactivity

iNOS was expressed by glial cells of the infected group, but not in the age-matched control group of uninfected mice. Within the infected group, glial expression of iNOS was highest near the

Table 4
GFAP expression in studied groups.

Group	GFAP expression					
	Grade 1 GFAP staining		Grade 2 GFAP staining		Grade 3 GFAP staining	
	N.	%	*N.*	%	*N.*	%
Control ($n = 5$)	5	100	0	0	0	0
2 weeks p.i. ($n = 10$)	8	80	2	20	0	0
5 weeks p.i. ($n = 10$)	4	40	4	40	2	20
16 weeks p.i. ($n = 10$)	1	10	4	40	5	50
P value	0.254[a]		0.027[*,b]		0.049[*,c]	

[a,b,c] Comparison between studied groups for grade 1, 2, and 3 GFAP expression respectively.
[*] Significant.

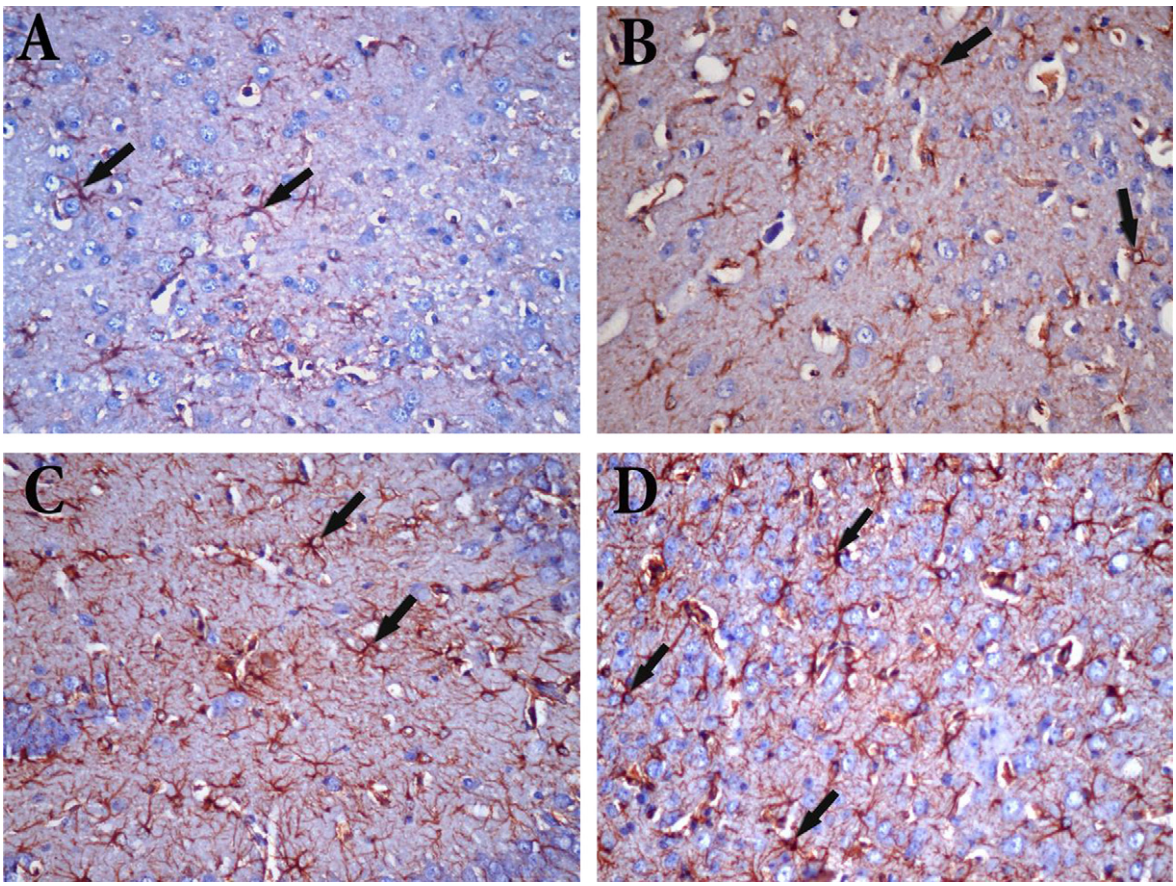

Fig. 4. GFAP staining of brain sections showing positive astrocytic GFAP expression "arrows" in: (A) uninfected control group showing few scattered GFAP-positive astrocytes, (B) infected group 2 weeks p.i. showing grade 1 astrocytic GFAP immunoreactivity, (C) infected group 5 weeks p.i. showing grade 2 astrocytic GFAP immunoreactivity, and (D) infected group 16 weeks p.i. showing grade 3 astrocytic GFAP immunoreactivity (Immunoperoxidase X400).

choroid plexus and corpus callosum, and it was detected as early as the 2nd week p.i. Moreover, there was a significant increase in its expression with increasing duration of infection, as the highest expression was observed in the 16th week p.i. These results are shown in Table 3 and Fig. 3.

3.6. GFAP immunoreactivity

Immunohistochemical assessment showed a significant increase in GFAP expression by activated astrocytes in the infected group, localized in the cerebral parenchyma especially near the choroid plexus and corpus callosum. However, weak GFAP expression was also detected in the age-matched control group of uninfected mice. Increased GFAP expression was detected as early as the 2nd week p.i., and it was also increased significantly in an ascending pattern through the course of infection. These findings are shown in Table 4 and Fig. 4.

4. Discussion

Recent research has indicated that experimental toxocariasis is associated with a wide range of behavioural changes in infected animals compared to controls. *Toxocara*-infected mice are less exploratory, less aggressive, and show some impairment in learning and memory. These changes are correlated to the intensity of parasite-induced pathology rather than an adaptive mechanism of parasite-altered host behaviour [23,24]. In humans, however, the situation is less clear. Lower academic performance was recorded in *Toxocara*-infected children [6], as well as neuropsychological disturbances [25]. Taylor et al. reported high rates of behaviour and sleep disturbances in children with raised *Toxocara* titres [7]. Moreover, a role of *T. canis* infection in the etiology of some cases of idiopathic epilepsy in children has been proposed [5,26]. Thus, the present study aimed at exploring some of the underlying immunopathogenetic and biochemical mechanisms of neurotoxocariasis in an experimental model.

In the present work, *T. canis* larvae appear to accumulate in the brain over the course of infection; the larval count increased progressively, peaked at 5 weeks p.i., then a plateau was reached. These findings are consistent with other previous studies that described progressive larval accumulation in the brain over time [16]. Given the fact of progressive larval migration to the brain, it is not surprising that the biochemical and immunopathological changes, detected in our study, were most pronounced at the chronic stage of infection. Moreover, larval distribution in the brain does not seem to occur at random, but rather following a pattern. In animals, Good et al. found that larvae of *T. canis* showed a characteristic distribution in the murine brain with significant concentration in the telencephalon [27]. On the other hand, larvae, in humans, were reportedly detected in the thalamus [28], pons, frontal lobe, and cerebellum [29]. The pattern of distribution is presumably one factor influencing the manifestations of a symptomatic infection.

In the present work, we found significant increase in expression of TNF-α mRNA in the chronic stage of infection. TNF-α is pleiotropic pro-inflammatory cytokine secreted by many cell types such as glial cells, and its receptors are found everywhere within the CNS [30]. Typically, TNF-α is up-regulated in many CNS disorders, such as brain trauma and infections. For example, TNF-α has been particularly studied in cerebral malaria and human African trypansomiasis and has been shown to be a major component in

the pathogenesis of both diseases [31,32]. Moreover, TNF-α disturbances have been found to be associated with sleep and cognitive impairment [33].

Interleukin-6 is a plurifunctional and primarily pro-inflammatory cytokine. CNS cells that produce IL-6, include astrocytes, macrophages, microglia, neurons, and brain endothelial cells. IL-6 has both neuroprotective and neurotrophic functions. In addition, it is important in the activation of astrocytes and microglia, as well as in glial proliferation with subsequent gliosis [34]. In trauma and certain pathological conditions, IL-6 is up-regulated in the brain tissue [35]. For example, IL-6 mRNA expression is up-regulated in experimental herpes simplex encephalitis [36]. In our study, levels of IL-6 mRNA increased significantly in *Toxocara*-infected mice compared to control at 5 and 16 weeks p.i. These data point to a possible role of IL-6 in the course of neurotoxocariasis especially in the chronic stage.

Our study demonstrates for the first time, to the best of our knowledge, significant increase in gene expression of pro-inflammatory cytokines in the brains of *Toxocara*-infected mice especially in the chronic stage. Pro-inflammatory cytokines can be neuroprotective, but with sustained or overstimulated increase, they will have deleterious effects on the neurons [33]. Both TNF-α and IL-6 influence cognition, learning and memory. Further, the over expression of these stimulatory cytokines can lead to poor performance on cognitive tasks, and is proposed to be involved in neuropsychiatric disorders such as depression [37]. Thus, our findings may help explain the cognitive impairment observed in *Toxocara* infection in mice and perhaps in humans. Numerous anecdotal reports of cognitive dysfunction with toxocariasis in man are found, e.g. Scheid et al. [28]. Interestingly, IL-10 is also up-regulated in the brains of *Toxocara*-infected mice [10]. IL-10 is an anti-inflammatory cytokine that antagonizes the effects of TNF-α and IL-6. Therefore, the final impact of this cytokine–cytokine interaction will apparently be dependent on the relative levels of the secreted cytokines.

Three enzymes produce nitric oxide (NO), including inducible "iNOS", endothelial "eNOS" and neuronal "nNOS" nitric oxide synthase isotypes. iNOS is not normally expressed in the brain, but is induced in astrocytes and microglia by pathogen components and pro-inflammatory cytokines such as TNF-α, IL-1, and interferon-γ. Other cytokines (IL-4, IL-5, IL-8, and IL-10), some growth factors and glucocorticoids down-regulate this inducible enzyme [22]. In the present work, iNOS showed significantly progressive increase in its expression by activated glial cells in the brains of infected animals throughout the course of infection. Our data support previous studies that showed increased iNOS expression in tissues infected with *Toxocara*, such as the brain [10], the liver [38], and the muscles [39]. In fact, iNOS has been found to have detrimental effects on the brain during infection with *T. canis*, since mice treated with iNOS inhibitors displayed significantly less cerebral pathology than control mice [40].

Notably, our data provide indirect evidence for increased production of nitric oxide (NO) in the brains of *Toxocara*-infected mice. NO is a reactive gas that can exist in several redox states, each having different biological properties. Characteristically, NO is generated in the brain areas that are responsible for long-term behaviour and memory [41]. It has been found that cytokine-induced release of NO disturbs normal synaptic function and brings about neuronal dysfunction. Typically, lower concentrations cause behavioural changes while higher amounts result in coma [42]. Moreover, NO has been shown to induce seizures in experimental animals [42]. Thus, high levels of NO in the brain in *T. canis* infection may contribute to some of the neuropsychiatric manifestations encountered in this infection.

Reactive gliosis is a common phenomenon following nervous system damage induced by a variety of insults, and is indicated by increased production of GFAP "an intermediate filament protein" [43]. Astrocytes are key cells in gliosis, and they respond to damage stimuli by up-regulating specific proteins, releasing cytokines, becoming hypertrophic and ramified, and forming a dense meshwork of processes [44]. Gliosis is a double-edged sword: it involves the generation of neurotrophic factors (neuronal growth factor, basic fibroblast growth factor), and protection from toxins, but may also allow for the secretion of neurotoxins such as NO, quinolinic acid, and cytokines e.g. TNF-α. The detrimental effects of gliosis may also be attributed to the increased volume of astrocytes, with subsequent reduction of the extracellular space, and consequently alteration of the concentration of some neuroactive substances, so that neuronal excitability is affected. Furthermore, deficiencies in astrocytic glutamate uptake from the extracellular space lead to increased excitotoxicity [14,43].

Our study provides evidence for astrocytic activation in the brains of *Toxocara*-infected mice, highlighted by increased expression of GFAP, and this activation is apparently time-dependent. Similarly, Liao et al. [15] have demonstrated that GFAP expression was enhanced in the brains of *Toxocara*-infected mice and concluded that this increase was responsible for blood–brain barrier injury detected in their study.

This study demonstrates significant changes in neurotransmitter profile of *Toxocara*-infected mice. This area has received little attention by investigators. Regarding amino acids, GABA levels decreased in infected animals compared to control starting from 5 weeks p.i. and showed significantly progressive decline with time, whereas glutamate levels showed the inverse pattern. For monoamines, dopamine and serotonin levels significantly decreased in infected animals starting from 2 weeks p.i. for serotonin, and 5 weeks p.i. for dopamine. Norepinephrine levels were higher in infected animals compared to control at all time points p.i. These findings are in partial agreement with Abdel Ghafar et al. [13] who reported significant reduction in dopamine levels in *Toxocara*-infected animals compared to control 2 weeks p.i. Meanwhile, both serotonin and norepinephrine levels were not different in infected and control groups. However, in their study, neurotransmitters were assayed only at 2 weeks p.i.

As a key CNS inhibitory transmitter, GABA levels were depressed, whereas levels of glutamate, ubiquitous CNS excitatory transmitter, were increased in infected animal groups. These data may provide explanation, at least in part, for increased incidence of seizure disorder in children with positive *Toxocara* titres. Further, catecholamines, namely dopamine and norepinephine are involved in the regulation of movement, mood, attention and visceral function, and dopamine abnormalities are implicated in the behavioural disturbances in schizophrenic patients [41]. Interestingly, high levels of cerebral norepinephrine in various stress conditions lead to increase in generation of central pro-inflammatory cytokines [45]. Similarly, serotonin is a monoamine that is considered a neuromodulator as much as a neurotransmitter. It regulates mood, emotional behaviour, and sleep [41]. In addition, it regulates the appetite [41], and disturbance of this transmitter may account for the abnormal appetite – geophagia – experienced by many *Toxocara*-infected children.

Research has indicated that any change in the neurotransmitter levels could induce changes in personality profile [46]. For example, *Toxoplasma gondii* provokes behavioural changes in animals [12], and in humans [46] probably by affecting dopamine and/or serotonin biosynthesis [46]. Taken together, the disturbances in neurotransmitters, in parallel with those of cytokines, presumably account for the behavioural, sleep, and cognitive impairment in *Toxocara*-infected animals and perhaps in humans as well. Fortunately, animal models can provide an opportunity to identify other important central players e.g. neuropeptides, histamine, prostaglandins, and reactive oxygen species.

These findings could be relevant to human hosts infected with *Toxocara*. Despite the fact that *T. canis* larvae have been identified in the brains of humans [29], no data exist on the intensity of infection or the relationship between neurological involvement and pathogenesis in humans. Two opposing situations seem to exist in humans: on the one hand, *Toxocara* infection is usually of low dose and repeated nature in humans, similar to trickle infections used in some experiments; on the other, human infections are of long duration and exhibit florid inflammatory reaction around *T. canis* larvae with glial scar formation [29]. Therefore, further human studies albeit difficult are needed to assess the true impact of this widespread parasitosis. The advances in neuroimaging e.g. functional MRI may permit the correlation between CNS lesions and changes in behaviour or cognition in man.

In conclusion, experimental *T. canis* infection induced significant biochemical and immunopathological alterations in the brains of infected animals. Increased expression of pro-inflammatory cytokines and nitric oxide was observed as well as disturbances of neurotransmitter profile in infected animals. Also, reactive astrogliosis was evident in the brains of infected mice. Remarkably, these changes were maximal in the chronic phase of infection – a situation that warrants therapeutic intervention for this infection even in the chronic stage. Finally, further studies are warranted to establish the immunopathogenetic mechanisms of neurotoxocariasis which may provide future therapeutic options in this infection and probably in other chronic CNS infections as well.

References

[1] Magnaval JF, Glickman LT, Dorchies P, Morassin B. Highlights of human toxocariasis. Korean J Parasitol 2003;39:1–11.
[2] Vidal JE, Sztajnbok J, Seguro AC. Eosinophilic meningoencephalitis due to *Toxocara canis*. Case report and review of literature. Am J Trop Med Hyg 2003;69:341–3.
[3] Robinson A, Tannier C, Magnaval JF. *Toxocara canis* meningoradiculitis. Rev Neurol (Paris) 2002;158:351–3.
[4] Goffette S, Jeanjean AP, Bigaignon G, Sindic CJ. Eosinophilic pleocytosis and myelitis related to *Toxocara canis* infection. Eur J Neurol 2000;67:703–6.
[5] Arpino C, Gattinara GC, Piergili D, Curatolo P. *Toxocara* infection and epilepsy in children: a case-control study. Epilepsia 1990;31:33–6.
[6] Worely G, Green JA, Frothingham TE. *Toxocara canis* infection. Clinical and epidemiological association with seropositivity in Kindergarten children. J Infect Dis 1984;149:591–7.
[7] Taylor MR, Keane CT, O'Connor P, Mulvihill E, Holland C. The expanded spectrum of toxocaral disease. Lancet 1988;1:692–5.
[8] Finsterer J, Auer H. Neurotoxocarosis. Rev Inst Med Trop Sao Paulo 2007;49:279–87.
[9] Pinelli E, Brandes S, Dormans J, Fonville M, Hamilton CM, der Giessen J. *Toxocara canis*: effect of inoculum size on pulmonary pathology and cytokine expression in BALB/c mice. Exp Parasitol 2007;115:76–82.
[10] Hamilton CM, Brandes S, Holland CV, Pinelli E. Cytokine expression in the brains of *Toxocara canis*-infected mice. Parasite Immunol 2008;30:181–5.
[11] Stibb HH, Curtis DA. Neurochemical changes in experimental African trypanosomiasis in voles and mice. Ann Trop Med Parasitol 1987;81:673–9.
[12] Webster JP. The effect of *Toxoplasma gondii* on animal behavior: playing cat and mouse. Schizophr Bull 2007;33:752–6.
[13] Abdel Ghafar AE, El Kowrany SE, Salem SA, Menaisy AA, Fadel WA, Awara WM. Effect of some parasitic infections on neurotransmitters in the brain of experimentally infected mice before and after treatment. J Egypt Soc Parasitol 1996;26:497–508.
[14] Carson MJ, Walter B. The cellular response in neuroinflammation: the role of leukocytes, microglia and astrocytes in neuronal death and survival. Clinic Neurosci Res 2006;6:237–45.
[15] Liao CW, Cho WL, Kao TC, Su KE, Lin YH, Fan CK. Blood–brain barrier impairment with enhanced SP, NK-1R, GFAP and claudin-5 expressions in experimental cerebral toxocariasis. Parasite Immunol 2008;30:525–34.
[16] Kayes SG, Oaks JA. Effect of inoculum size and length of infection on the distribution of *Toxocara canis* larvae in the mouse. Am Soc Trop Med Hyg 1976;25:573–80.
[17] Erdó SL. Postmortem increase of GABA levels in peripheral rat tissues: prevention by 3-mercapto-propionic acid. J Neural Transm 1984;60:303–14.
[18] Kema IP, de Vries EG, Slooff MJ, Biesma B, Muskiet FA. Serotonin, catecholamine, histamine, and their metabolites in urine, platelets, and tumor tissue of patients with carcinoid tumors. Clin Chem 1994;40:86–95.
[19] Donzanti BA, Yamamoto BK. An improved and rapid HPLC-EC method for the isocratic separation of amino acid neurotransmitters from brain tissue and microdialysis perfusates. Life Sci 1988;43:913–22.
[20] Schlumpf M, Lichtensteiger W, Langemann H, Waser PG, Hefti F. A fluorimetric micromethod for the simultaneous determination of serotonin, noradrenaline and dopamine in milligram amounts of brain tissue. Biochem Pharmacol 1974;23:2337–46.
[21] Vatassery GT, Sheridan MA, Krezowski AM. Spectrophotofluorometry of serotonin in blood platelets. Clin Chem 1981;27:328.
[22] Maneerat Y, Viriyavejakul P, Punpoowong B, et al. Inducible nitric oxide synthase expression is increased in the brain in fatal cerebral malaria. Histopathology 2000;37:269–77.
[23] Cox DM, Holland CV. Relationship between three intensity levels of *Toxocara canis* larvae in the brain and effects on exploration, anxiety, learning and memory in the murine host. J Helminthol 2001;75:33–41.
[24] Hamilton CM, Stafford P, Pinelli E, Holland CV. A murine model for cerebral toxocariasis: characterization of host susceptibility and behaviour. Parasitology 2006;132:791–801.
[25] Marmor M, Glickman LT, Shofer F, et al. *Toxocara canis* infection of children; epidemiologic and neuropsychologic findings. Am J Public Health 1987;77:554–9.
[26] Bächli H, Minet JC, Gratzl O. Cerebral toxocariasis: a possible cause of epileptic seizure in children. Childs Nerv Syst 2004;20:468–72.
[27] Good B, Holland CV, Stafford P. The influence of inoculum size and time post-infection on the number and position of *Toxocara canis* larvae recovered from the brains of outbred CD1 mice. J Helminthol 2001;75:175–81.
[28] Scheid R, Jentzsch RT, Schroeter ML. Cognitive dysfunction, urinary retention, and a lesion in the thalamus – beware of possible toxocariasis of the central nervous system. Clin Neurol Neurosurg 2008;110:1054–7.
[29] Hill IR, Denham DA, Scholtz CL. *Toxocara canis* larvae in the brain of a British Child. Trans R Soc Trop Med Hyg 1985;79:351–4.
[30] Barone FC, Kilgore KS. Role of inflammation and cellular stress in brain injury and central nervous system diseases. Clin Neurosci Res 2006;6:329–56.
[31] Brown H, Turner G, Rogerson S, et al. Cytokine expression in the brain in human cerebral malaria. J Infect Dis 1999;180:1742–6.
[32] Gimenez F, Barraud de Lagerie S, Fernandez C, Pino P, Mazier D. Tumour necrosis factor alpha in the pathogenesis of cerebral malaria. Cell Mol Life Sci 2003;60:1623–35.
[33] Turrin NP, Plata-Salaman CR. Cytokine–cytokine interactions and the brain. Brain Res Bull 2000;51:3–9.
[34] Swartz KR, Liu F, Sewell D, et al. Interleukin-6 promotes post-traumatic healing in the central nervous system. Brain Res 2001;896:86–95.
[35] Suzuki S, Tanaka K, Onawa S, et al. Temporal profile and cellular localization of interleukin-6 protein after focal cerebral ischemia in rats. J Cereb Blood Flow Metab 1999;19:1256–62.
[36] Dvorak F, Martinez-Torres F, Sellner J, et al. Experimental herpes simplex virus encephalitis: a long-term study of interleukin-6 expression in mouse brain tissue. Neurosci Lett 2004;367:289–92.
[37] Song C, Lin A, Bonaccorso S, et al. The inflammatory response system and the availability of plasma tryptophan in patients with primary sleep disorders and major depression. J Affect Disord 1998;49:211–9.
[38] Fan CK, Lin YH, Hung CC, Chang SF, Su KE. Enhanced inducible nitric oxide synthase expression and nitrotyrosine accumulation in experimental granulomatous hepatitis caused by *Toxocara canis* in mice. Parasite Immunol 2004;26:273–81.
[39] Lin SM, Liao CW, Lin YH, Lee CC, Kao TC, Fan CK. Inducible nitric oxide synthase inhibition influenced granuloma formation with suppressed collagen expression in myositis caused by *Toxocara canis* in mice. Parasitol Res 2008;102:577–85.
[40] Gargili A, Demirci C, Kandil A, Cetinkaya H, Atukeren P, Gümüştaş MK. In vivo inhibition of inducible nitric oxide and evaluation of the brain tissue damage induced by *Toxocara canis* larvae in experimentally infected mice. Chin J Physiol 2004;47:189–96.
[41] Bloom FE. Neurotransmission and the central nervous system. In: Hardman JG, Limbind LE, Gilman AG, editors. Goodman and Gilman's the pharmacological basis of therapeutics. 10th ed. USA: McGraw-Hill (Medical Publishing division); 2001.
[42] Desarro G, Dipaola ED, Desarro A, Vidal MJ. L-arginine potentiates excitatory amino acid-induced seizures elicited in the deep prepiriform cortex. Eur J Pharm 1993;230:151–8.
[43] Jang S, Suh SH, Yoo HS, Lee YM, Oh S. Changes in iNOS, GFAP and NR1 expression in various brain regions and elevation of sphingosine-1-phosphate in serum after immobilized stress. Neurochem Res 2008;33:842–51.
[44] James P, Emma E, Jon G. Monitoring reactive gliosis in 3-dimensional astrocyte cultures; a model of spinal cord damage. In: Neuroscience 2008: 38th annual meeting of the Society for Neuroscience. 2008. p. 15–9.
[45] Johson JD, Campisi J, Sharkey CM, et al. Catecholamines mediates stress-induced increases in peripheral and central inflammatory cytokines. Neuroscience 2005;135:1295–307.
[46] Henriquez SA, Brett R, Alexander J, Pratt J, Roberts CW. Neuropsychiatric disease and *Toxoplasma gondii*. Neuro Endocrinol Lett 2005;26:480–6.