Pathology – Research and Practice 210 (2014) 526–532

Contents lists available at ScienceDirect

Pathology – Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original Article

Influence of toxoplasmosis on acetylcholinesterase activity, nitric oxide levels and cellular lesion on the brain of mice

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ARTICLE INFO

Article history:
Received 5 January 2014
Received in revised form 18 April 2014
Accepted 30 April 2014

Keywords:
Toxoplasmosis
RH
VEG
ME-49

ABSTRACT

The objective of this study was to investigate the activity of acetylcholinesterase (AChE), nitrite/nitrate (NO_x) levels, as well as the biomarkers of cellular damage in the brain of mice experimentally infected with *Toxoplasma gondii*. Sixty mice were divided into two experiments: in experiment I the mice were infected with *T. gondii*/RH strain, while in experiment II they were infected with *T. gondii*, strains VEG and ME-49. Our evaluations were carried out on brain homogenized samples, assessing the AChE and glutathione reductase (GR) activities, and NO_x, TBARS and AOPP levels in all the infected animals, compared with the control group. In both experiments, I and II, it was observed an increase in the activity of AChE and GR, as well as in the levels of NO_x in the brain of infected mice with *T. gondii*. TBARS levels were increased in mice infected with the three different strains, RH, ME-49, and VEG. AOPP concentration was increased only in mice infected with the RH strain. Animals infected with the strains VEG and ME-49 showed histological lesions, associated with the presence of the parasite in the brain. Therefore, the infection by *T. gondii* is able to interfere in cholinesterase activity and NO levels, in association with oxidative stress and histological lesion.

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Introduction

Toxoplasma gondii is an obligate intracellular parasite that infects humans and a wide range of mammals and birds [1]. This parasite has a complex life cycle: oocysts, tachyzoites and bradyzoites where sexual reproduction occurs only in felines and results in generation of oocysts shed to the environment by contaminated feces. The oocysts may be ingested by intermediate hosts, leading to the asexual reproduction of *T. gondii*. The acute invasion, characterized by parasitemia (tachyzoites), is a transient stage followed by chronic invasion when parasites reside within cysts in the tissues (bradyzoites) localized mainly in the central nervous system (CNS), muscle, and eye [1–3]. The intermediate hosts are the ones most affected by toxoplasmosis, with reports of reproductive disorders and abortion in small ruminants, blindness and fetal death in humans, and behavioral and neurological disorders in different species of mammals, among other clinical signs [3–5].

The infection by *T. gondii* can cause encephalitis, with the presence of the parasite in the CNS, and behavioral alterations [3,5,6]. In the acute phase, researchers have identified an increase in brain weight of infected animals and a decrease in chronic phase. The increased brain weight is probably related to the influx of inflammatory cells into the tissue, and the reduction is related to cell death, resulting from inflammation [7,8]. According to the scientific literature, it is speculated that the behavioral alterations may be related to changes in the concentrations of neurotransmitters in the brain, such as dopamine [9,10]; but this mechanism is not clearly understood. Neurotransmitters and neuromodulators behavior may be affected in animals with toxoplasmosis. In this sense, acetylcholine (ACh) and nitric oxide (NO•) are important neurotransmitters [11–14], and the adenosine is an important neuromodulator of CNS [15]. Free ACh can be hydrolyzed by cholinesterase or can be combined with muscarinic and nicotinic acetylcholine receptors (mAChR and nAChR, respectively) in order

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http://dx.doi.org/10.1016/j.prp.2014.04.025
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to promote its action [14,16]. The acetylcholinesterase (AChE) is a membrane-bound enzyme mainly found in the brain, muscles, erythrocytes, lymphocytes and cholinergic neurons, which preferentially hydrolyses ACh [16,17] and thus controls the levels of this molecule in the synaptic cleft.

NO• is involved in the learning and memorization processes, and glutamate mediates the release of NO from its receptor N-methyl-D-aspartate (NMDA). This free radical is synthesized by a group of isoenzymes known as nitric oxide synthases (NOS), through enzymatic catalysis of the amino acid L-arginine, and resulting in the formation of L-citrulline and NO• [18]. NO• are classified according to their activity in constitutive (cNOS) and induced (iNOS). The NOS are described as three isoforms based on three different genes. Isoforms are neuronal NOS (nNOS), endothelial NOS (eNOS) (both constitutive) and NOS induced or inflammatory (iNOS). According to some researchers, these isoforms are present and involved in physiological and pathological conditions in CNS [11]. However, excessive production of NO can cause neurotoxicity [13], which may lead to neurological disorders. Different methodologies can be used to measure the levels of NO, as well as indirect methods aimed at quantifying nitrate/nitrite (NO_x) [19].

Our hypothesis is that alterations in concentration of neurotransmitters (ACh and NO) may be a mechanism involved in the presentation of behavioral changes in mammals with toxoplasmosis [9,10]. Therefore, the objective of this study was to investigate the AChE activity, along with the measurement of levels of NO_x and markers of cellular damage on brain of mice experimentally infected by *T. gondii*, strains RH, VEG and ME-49.

Materials and methods

For this study, 80 mice were divided into two experiments: experiment I and experiment II. This division was necessary to assess the behavior of the three strains of *T. gondii*, a virulent one (RH strain), and two strains considered as cystogenics (VEG and ME-49). It is noteworthy that the VEG is considered as intermediate between the other two strains, when the evolution of the disease is compared (RH–acute; ME 49 – chronic). The infection period chosen for collection and analysis of samples was based on a pilot study.

Experiment I

Inoculum preparation

Tachyzoites of *T. gondii*, strain RH, kept in liquid nitrogen, was used to inoculate one mouse (BALB/c), intraperitoneally. Five days later, peritoneal fluid containing tachyzoites was collected and inoculated into another mouse. This procedure was repeated three times in order to reactivate parasite virulence.

Experimental design

Thirty-two male mice (BALB/c), 70 days-old, average body weight of 24.1 (±1.9 g), were kept in cages, housed in an experimental room with controlled temperature and humidity (25 °C; 70%). They were fed with commercial feed and received water, both *ad libitum*. All animals had a period of 12 days for acclimatization, and they were clinically healthy at the beginning of experiment (day 0).

All the 32 mice were divided into two groups of 16 animals each: group A (uninfected) and group B, intraperitoneally inoculated with 0.2 mL of peritoneal fluid containing 1.32×10^7 tachyzoites/mL. The number tachyzoites was estimated with a *Neubauer* chamber. Animals were checked daily for clinical signs of the disease.

It is important to emphasize, and explain, the reason why we chose the intraperitoneal (experiment I), as well as the oral via (experiment II). Our main reason is explained by a research [20] that first observed that cystic organisms (bradyzoites) were resistant to digestion by gastric juice (pepsin–HCl), whereas tachyzoites were destroyed immediately. For this reason, tachyzoites of RH strain were inoculated intraperitoneally, avoiding the stomach environment, while cysts of VEG and ME-49 strains were inoculated orally.

Sampling

Each group was divided into two subgroups, each one with eight mice in order to evaluate two periods post-inoculation (PI) (4 days PI – Group A1 and B1; 6 days PI – Group A2 and B2). Animals were anesthetized under isoflurane anesthesia and decapitated following the recommendations of the Ethics Committee, 4 and 6 days PI. Then, the brain was removed and weighed. For each group, at different times, six animals were used for biochemical analysis and two for histological analysis.

Experiment II

Inoculum preparation

Tachyzoites of *T. gondii*, strains VEG and ME-49, kept in liquid nitrogen, were used to inoculate two mice (BALB/c), respectively. Thirty days later, brain homogenate (in saline solution), containing cysts with bradyzoites, was collected and inoculated orally in other two mice for each strain. This procedure was done in order to reactivate parasite virulence. 35 days PI, two mice (strain ME 49), plus other two (VEG strain) were euthanized for collection of brain, cysts counting and separation in order to be used later for the animal inoculation in experiment II. For preparation of the inoculum the brain was homogenized in saline solution.

Experimental design

Forty-eight male mice (BALB/c), 80 days old, average body weight of 23.9 (±2.4 g), were kept in cages housed in an experimental room with controlled temperature and humidity (25 °C; 70%). They were fed with commercial feed and received water, both *ad libitum*. All animals had a period of five days for acclimatization and were clinically healthy at the beginning of experiment (day 0).

All mice were divided into groups of 16 animals each: group C (uninfected); group D orally inoculated with 0.25 mL of brain homogenate containing 50 cysts with bradyzoites of *T. gondii* (ME-49 strain); and group E orally inoculated with 0.25 mL of brain homogenate containing 50 cysts with bradyzoites of *T. gondii* (VEG strain). Animals were checked daily for clinical signs of the disease.

Sampling

Group D – ME-49 strain. Each group was divided into two subgroups, each one with eight mice in order to evaluate two periods PI (30 days PI – Group C1 and Group D1; 60 days PI – Group C2 and Group D2). Animals were anesthetized under isoflurane anesthesia, followed by decapitation. The brain was removed, divided into two hemispheres and weighed. This study used the entire left hemisphere for biochemical analysis, and the right hemisphere (only from 6 animals) was used for histopathology.

Group E – VEG strain. The animals were divided into two subgroups, each one with eight mice in order to evaluate two periods PI (30 days PI and 60 days PI). However, during the study, eight mice infected with strain VEG of *T. gondii* died before completing 60 days of research, as a result of the infection by the parasite. Then, on 30th day PI, the animals were anesthetized with isoflurane and decapitated. The brain was also removed and weighed. Unfortunately, due to the mortality of half group (8 animals survived until day 30 PI), we used the samples of six of them for biochemical analysis and the other two for histological analysis.

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