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BEHAVIOURAL AND COGNITIVE CHANGES INDUCED BY TOXOPLASMA GONDII AND ITS PATHOLOGICAL DEVELOPMENT IN WISTAR RATS

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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July 2017

Chair : Assoc. Prof. Malina Osman, MD., M. Community Health
Faculty : Medicine and Health Sciences

Objective: This study aimed to identify the behavioural and cognitive changes induced by *Toxoplasma gondii* and its pathological development in Wistar rats. **Methodology:** Study involved the *in vivo* test that was conducted by using *Toxoplasma gondii* RH strain on rats to perform the behavioral tests. Locomotor activity in Open-Field Test (OFT), anxiety study in Elevated-Plus Maze (EPM) and working memory study in Novel Object Discrimination test (NOD) were performed for the determination of toxoplasmosis profile on animal model. Thirty five male Wistar rats were used in the study and divided into five groups which were control group (negative control), *Toxoplasma*-infected group and three groups of positive control; Amphetamine, Diazepam, Scopolamine. All dosage administrated orally gavages to each rats in the control and *T. gondii* groups on the first day and profile behavioral test was performed after a six weeks. After the completion of behavioral study, rats were sacrificed and their brain, liver and kidney were collected to evaluate any morphological changes through histopathological examination. DNA extraction of samples (Formalin-fixed paraffin embedded) were conducted and followed by polymerase chain reaction (PCR) amplification of *T. gondii* B1 gene. **Results:** This study showed that there was a significant difference of mean score of gridline in locomotor activity; thirty minutes (counts) of crossing the gridline at the $p<0.05$ level among the three groups F (2, 15) = 10.548, p=0.001. In anxiety study, two parameters were performed on each rat; time spent in open arms and open arm entries frequency. *Toxoplasma*-infected group showed significant increases in both of open arm time spent and open arm entries frequency compared with control group. In Novel Object Discrimination test, the result revealed that there was a significant difference in exploration time (mean time spent) on the familiar object (A3) and novel object (B) in control group for exploration time, from A3 (M = 0.04, SD = 0.03) to B (M = 0.07, SD = 0.05), t (4) = -2.92, $p < 0.04$. Both discrimination index and discrimination ratio show significant difference between three groups; F (2, 12) = 5.734, p=0.018; F (2, 12) = 5.962, p=0.016. Histopathological examination revealed that the morphological of appearance of lesions on the brain was

identified in *Toxoplasma*-infected group. Meanwhile in control group no lesions were detected (normal). Through the molecular study (PCR) it was confirmed the presence of *T. gondii* in the tissue sample. **Conclusion:** *T. gondii* RH strain in Wistar rats was demonstrated to cause hyperactivity in locomotor; memory deficit in novel object discrimination and reflect the less anxiety by entering more entries and time spent in open arm. It is clear that *Toxoplasma gondii* infection reflects the behavior profiles on the host. Due to the specific strains and species of host; RH strain is virulent strain giving less potential to produced cysts in the brain and higher sensitivity and specificity of PCR yielded the desired positive outcomes of 301 base pair B1 gene of *Toxoplasma gondii*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PERUBAHAN TINGKAH LAKU DAN KOGNITIF DISEBABKAN OLEH *TOXOPLASMA GONDII* DAN PERKEMBANGAN PATOLOGI DALAM TIKUS WISTAR

Oleh

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Pengerusi : Prof. Madya Malina Osman, MD., M. Kesihatan Komuniti
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Objektif: Kajian ini bertujuan untuk mengenalpasti perubahan tingkah laku dan kognitif disebabkan oleh *Toxoplasma gondii* dan perkembangan patologi dalam tikus Wistar. **Kaedah kajian:** Kajian ini melibatkan kajian *in vivo* yang mengunakan baka RH *Toxoplasma gondii* ke atas tikus untuk melaksanakan ujian tingkah laku. Aktiviti lokomotor dalam *Open-Field Test* (OFT), ujian keresahan dalam *Elevated Plus Maze* (EPM), dan ujian ingatan kerja dalam *Novel Object Discrimination* (NOD) telah dijalankan bagi menentukan profil toksoplasmosis pada model haiwan. Tiga puluh lima tikus Wistar jantan telah digunakan dalam kajian ini dan dibahagikan kepada lima kumpulan iaitu kumpulan kawalan (kawalan negatif), kumpulan yang dijangkiti *Toxoplasma* dan tiga kumpulan kawalan positif; Amphetamine, Diazepam dan Scopolamine. Semua dos diberi secara oral kepada setiap tikus dalam kumpulan kawalan dan kumpulan dijangkiti *T. gondii* pada hari pertama dan ujian profile tingkah laku dilakukan selepas enam minggu. Selepas tamat ujian profile tingkah laku, tikus dikorbankan menurut prosedur dan sampel organ dalaman seperti otak, hepar dan ginjal diambil untuk menilai sebarang perubahan morfologi melalui pemeriksaan histopatologi. Pengekstrakan DNA bagi sampel (*Formalin-fixed paraffin embedded*) dijalankan dan diikuti dengan ujian *Polymerase Chain Reaction* (PCR) dengan amplifikasi gen B1 *T. gondii*. **Keputusan:** Hasil kajian menunjukkan bahawa terdapat perbezaan yang bermakna terhadap min skor garisan grid dalam aktiviti lokomotor; tiga puluh minit (kiraan) menyeberangi garisan grid pada $p < 0.05$ diantara ketiga-tiga kumpulan, $F(2, 15) = 10.548$, $p = 0.001$. Dalam ujian keresahan, dua parameter telah dinilai dalam setiap tikus; masa yang diambil berada dalam lapangan terbuka dan kemasukan ke lapangan terbuka. Kumpulan yang dijangkiti *Toxoplasma* menunjukkan peningkatan yang bermakna bagi masa yang diambil berada dalam lapangan terbuka dan kekerapan kemasukan ke lapangan terbuka berbanding dengan kumpulan kawalan. Dalam ujian *Novel Object Discrimination* (NOD), hasil kajian menunjukkan perbezaan yang bermakna dalam masa penerokaan objek (min masa) pada objek biasa (A3) dan objek berbeza (B) bagi kumpulan kawalan, dari objek biasa, A3 ($M = 0.04$, $SD = 0.03$)

ke objek berbeza, B (M = 0.07, SD = 0.05), t (4) = -2.92, p < 0.04. Pemeriksaaan histopatologi menunjukkan morfologi seperti lesi telah dikenalpasti dalam otak kumpulan tikus yang dijangkiti *Toxoplasma*. Manakala kumpulan kawalan tidak menunjukkan kehadiran lesi (normal). Melalui ujian molekular (PCR) yang dijalankan telah membuktikan bahawa terdapat *T. gondii* di dalam sampel tisu. Sample tisu seperti otak, hepar dan ginjal menunjukkan keputusan yang positif; amplikon PCR bersaiz 301bp B1 gen daripada *Toxoplasma gondii*. **Kesimpulan:** Jangkitan baka RH *T. gondii* dalam tikus Wistar menyebabkan hiperaktiviti dalam lokomotor, penurunan daya ingatan dalam *novel object discrimination* dan menunjukkan kurang keresahan dengan memasuki lebih banyak lapangan terbuka dan masa yang diambil berada dalam lapangan terbuka. Ini jelas membuktikan bahawa jangkitan *T. gondii* mencerminkan profil tingkah laku kepada faktor perumah. Disebabkan oleh baka yang khusus dan spesies perumah; baka RH yang digunakan adalah virulen dalam kajian ini menyebabkan kekurangan potensi pembentukan sista dalam tikus dan ketinggian kepekaan dan kekhususan PCR menunjukkan keputusan yang positif; 301bp B1 gen daripada *Toxoplasma gondii*.

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I certify that a Thesis Examination Committee has met on 25 July 2017 to conduct the final examination of Siti Shafiyyah binti Che Omar on her thesis entitled "Behavioural and Cognitive Changes Induced by *Toxoplasma gondii* and its Pathological Development in Wistar Rats" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

A1	Familiar object 1
A2	Familiar object 2
A3	Familiar object 3
AGE	Agarose Gel Electrophoresis
AMP	Amphetamine
ATCC	American Type Culture Collection
ANOVA	Analysis of Variance
B	Novel object
Bp	base pair
BZ	Benzodiazepines
CNS	Central nervous system
D1	Discrimination ratio
D2	Discrimination index
DAB	3,3'-Diaminobenzadine tetrahydrocloride
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DZ	Diazepam
E1	First exposure
E2	Second exposure
EIA	Enzyme immunoassay
ELISA	Enzyme Linked Immuno-Sorbent Assay
EPM	Elevated plus maze
FFPE	Formalin-fixed paraffin embedded
H&E	Hematoxylin and eosin
IACUC	Institutional Animal Care and Use Committee
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFAT	Indirect immunofluorescent antibody test
IHAT	Indirect haemangglutination test
IL	Interleukin
NBCI	National Center for Biotechnology Information
NOD	Novel object discrimination
NMDA	N-methyl-D-aspartic acid
OFT	Open field test
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
pH	Potential hydrogen
RBC	Red blood cell
RNA	Ribonucleic acid
SD	Standard deviation
S.E.M	Standard error of mean
SPSS	Statistical Package for Social Sciences
T.	*Toxoplasma*
T_m	Melting temperature
TNF-α	Tumor necrosis factor-alpha

CHAPTER 1

INTRODUCTION

1.1 Research Background

Toxoplasma gondii is a coccidian parasite and an intracellular Apicomplexan protozoan which is susceptible to all humans and animals. The infection of this parasite is significantly related to toxoplasmosis which is associated with economic, medical and veterinary importance (Berdoy, Webster & Macdonald, 2000). Thus, the term *T. gondii* has referred as protozoan parasite belonging to phylum Apicomplexa and classified into coccidian subclass (Montoya & Liesenfeld, 2004).

T. gondii is recognized globally as the most prevalent parasite that is capable of infecting all warm-blooded mammals inluding humans (Innes, 2010). Theoretically, *T. gondii* is a ubiquitous protozoan parasite that infects the definitive host (wild and domestic cats) and inhabit on a range of intermediate hosts. Not surprisingly, infection by *T. gondii* can result in severe or fatal diseases. According to Hill, Chirukandoth & Dubey (2005), it was reported that one-third of all humankind has been exposed to *T. gondii*. In addition, surveys were also stated that infection with *T. gondii* is the most prevailing in wildlife, such as bears, felids, foxes, raccoons and so forth.

Depending on the level of exposure, the occurrence of *T. gondii* infection varies in the animals. For example, it may exceed 50% in dogs, rabbits and sea otters whereas approximately 60% in rodents (mice, rats) and wild birds. Furthermore, it has also identified the infection rate of 70% occurred in bears, cats, deer and humans (Lafferty, 2006; Webster, 2007). A body of evidence have indicated that *T. gondii* has the ability of manipulating the behaviour of its host so as to facilitate its transmission to the definitive hosts (Berenreiterová, Flegr, Kubena & Nemec, 2011).

T. gondii undergoes a phase of sexual reproduction which takes place in the small intestine of the cats to culminate the formation of oocycts that are shed in cats' faeces (Hutchison, Dunachie, Siim & Work, 1969). However, the parasite undergoes asexual reproduction stage which takes place in various body tissues if it is ingested by intermediate hosts (warm-blooded animals) (Innes, 2010). The cycle of asexual reproduction begins with the rapidly dividing of tachyzoites that disseminate in the blood, followed by the slowly dividing of bradyzoites, which can enclose in a form of cyst in the brain, heart or other body tissues where they persist for lifetime (Webster, Kaushik, Bristow & McConkey, 2013). From a parasitological point of view, only the cycle initiated by the production of bradyzoites occurs in the cats. When a tissue cyst is ingested by the cats, the wall of the cyst is disintegrated by the proteolytic enzymes that are circulating in the stomach and small intestine. Bradyzoites released from the tissue

cyst invade the epithelial cells of the small intestine and initiate the development of numerous generations of *T. gondii* (Dubey, 2009).

Prevalence of *T. gondii* infections in humans may be acquired by ingesting the raw or uncooked meat that contained the tissue cysts or contaminated water with oocyst. On the other hand, they became directly infected when they accidentally ingest oocysts from the environment. However, the probability of exposed adult humans and other animals getting the disease with clinical symptoms is less percentage (Dubey & Jones, 2008). Moreover, animals were easy to get infected with *T. gondii* as they served for food such as pigs, rabbits, and sheep. Meanwhile, horses, buffaloes, and cattle were less infected than sheep and pigs. Thus, the transmission may be occurring as humans consumed the food. In addition, food animals containing *T. gondii* may survive for years in tissue cysts (Hill & Dubey, 2002).

T. gondii, the cause of toxoplasmosis and it was categorized as a parasite could exploit the proximate mechanism that modulates social behaviours in vertebrates. The absolute behavioural effect of *T. gondii* infection from infected rodents is they are more attracted to the odour of cat urine, and thus enhanced the transmission of parasite (Adamo, 2013; Webster, Lamberton, Donnelly & Torrey, 2006). Recent evidence suggests that latent toxoplasmosis is related with motor performance disturbance, cognitive deficits, decreased in anxiety, fearless to cat predator, higher movement activity and impaired of sensory attention (Berenreiterová et al. 2011). The previous study on experiment upon laboratory rats by Witting showed that learning capacity and memory of rats was also reduced as a result of *Toxoplasma* infection (Witting, 1979).

An ideal mechanism may represent by neuromodulation showed *T. gondii* may modified the host behaviour. Representative of anxiety studies showed that *T. gondii*–infected rats mostly preferred cat stimuli in the experimentally testing causes reduced in neophobia. This is due to the changes of neuromodulators by blocking anxiogenic N-methyl-D-aspartic acid receptors in the amygdala, or provision of serotonin (5-HT) antagonists. Particularly, the levels of homovanillic acid, norepinephrine, and dopamine also have been identified among *T. gondii*–infected and uninfected mice (Berdoy et al. 2000; Webster et al. 2006; Webster, 2007).

1.2 Problem Statement

The behavioural changes of animal can be known as well as "host behavioural manipulation" resulting in longer survival of the parasite which means to complete its life cycle (Henriquez, Brett, Alexander, Pratt & Roberts, 2009). Meanwhile, in human infection, *T. gondii* may reduce the function of the brain and can have devastating effects on the infected infants by developing intellectual disability (da Silva & Langoni, 2009). Through most of the reports, *T. gondii* was observed to cause the mood disorders likes schizophrenia, cause impairment on memory and learning performances in human. In human, behavioural changes are also can be seen in those who are infected which higher predisposition to accidents, or associated with other diseases that affect the central nervous system. *T. gondii* can be found worldwide and has the ability of 'modify' their hosts' behaviour and profile (da Silva & Langoni, 2009). Thus, in the local context, there were not many studies conducted on the *Toxoplasma gondii* Type I; RH strain on the behavioural analysis due to more rapidly dividing pathogenic strain compared to avirulent strain of Type II and III. As expected, infection with Type II parasites of *T. gondii* on mice lead to both acute and chronic infection. Moreover, surviving mice has an inclination to have higher chances of infection correlate with the progress of the complication called "sickness" behaviour characterized in part by lower activity levels (Ingram et al., 2013). Because of the assortment of summed up pathology and since itemized investigations of Type II infection as it identifies with mouse behaviour exist somewhere else, we did not proceed continue experiment with Type II parasites anymore. Since the behavioural changes study of *T. gondii* cannot be performed in humans, most of our experiment on the interaction of *T. gondii* with brain organ derives from experimental rodent models. New studies are needed to modify the critical point in the pathogenesis of *T. gondii* in order to understand the behavioural changes in animal model linked with human disorders.

1.3 Study Justification

Brain disorder is the fastest increasing rate in developing and developed country. The brain is the most functional organs which could lead as a potential factor of mortality in the general population. Questions have been raised about the study on the development of toxoplasmosis in animal models such as mice and rodent is related to pathological development in mood disorder in the humans. This study may help us to predict the behavioural changes that can be observed amongst infected humans, from mild to severe.

The development of Toxoplasmosis in the animal model, for example mice, is related to the pathological development in mood disorders in the humans. Therefore, we believe that it is high time for us to study this emerging parasite that may enhance the contribution of prevention of brain/psychiatric disorder related to toxoplasmosis and considered as a study for establishing upcoming psychiatric research using the animal model.

The mood disorders noted as a challenging disease in this century. It has been noted by medical underlining that several factors may contribute to mood disorders. One of them is genetic and environment factors. However, the mood disorders are common disorders characterised by emerging parasite of *Toxoplasma gondii* which can cause toxoplasmosis infection. Considering *Toxoplasma gondii* as newly emerging opportunistic pathogens in human with mood disorders mostly schizophrenia noted as public health problem and leading to 1% of disease burden (Torrey & Yolken, 2003).

1.4 Objective

1.4.1 General Objective

To study the behavioural and cognitive changes induced by *Toxoplasma gondii* and its pathological development in Wistar rats.

1.4.2 Specific Objective

1. To determine significant difference of mean scores of behavioral testing; locomotor activity, anxiety level and on working memory in infected rats and control.

2. To determine significant difference of mean time spent in pre and post of familiar and novel object on working memory in infected rats and control.

3. To identify the morphological changes (microscopic) in the brain of infected rat and control.

4. To identify 301 bp *T. gondii* B1 gene in infected rat group using Polymerase Chain Reaction.

1.5 Hypothesis

1. There is a significant difference of mean scores of behavioural testing; locomotor activity, anxiety level and on working memory in infected rats and control.

2. There is a significant difference of mean time spent in pre and post of familiar and novel object on working memory in infected rats and control.

CONCEPTUAL FRAMEWORK

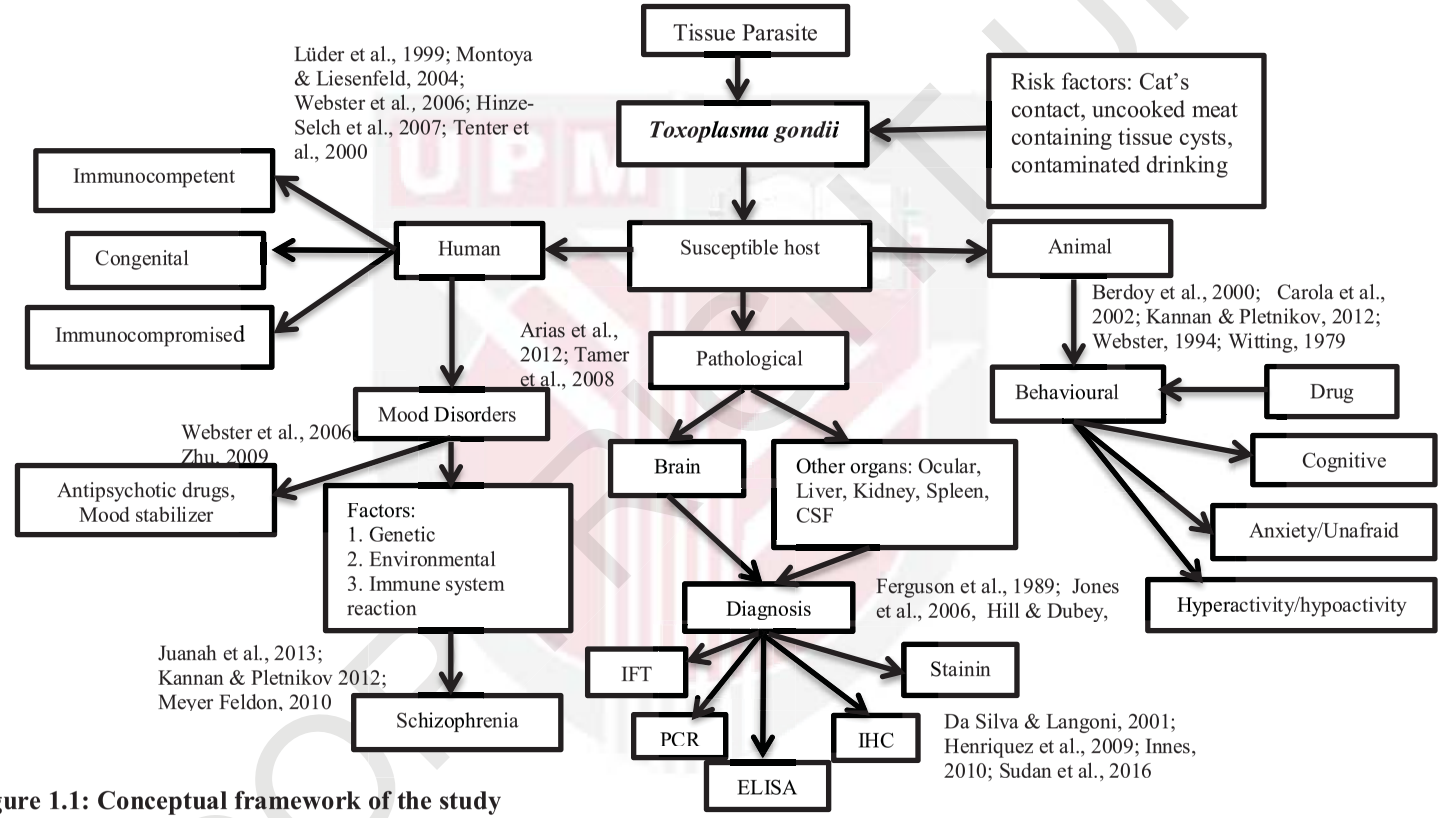

Figure 1.1: Conceptual framework of the study

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