THE EFFECT OF MINOCYCLINE ON NOVEL OBJECT RECOGNITION MEMORY AND BETA AMYLOID PROTEIN EXPRESSION IN ALZHEIMER'S DISEASE RAT MODEL

by

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ABBREVIATION

A.K.A	Also known as
AD	Alzheimer's disease
ANOVA	Analysis of variance
APP	Amyloid precursor protein
Aβ	Beta amyloid
BACE	Beta site APP cleaving enzyme
BBB	Blood brain barrier
CNS	Central nervous system
CSF	Cerebrospinal fluid
DM	Diabetes Mellitus
DNMS	Delayed-Non-Matching-to- Sample
FDA	Food and Drug Administration
IDE	Insulin degrading enzyme
IL	Interleukin
iNOS	Nitric Oxide Synthase
ISI	Intersession interval
LPS	Lipopolysaccharide
MEM	Memantine
MINO	Minocycline
NEP	Neprilysin
NFT	Neurofibrillary tangles
NMDA	N-methyl D- aspartate
NMDAr	N-methy D-aspartate
NO	Nitric Oxide

NOR	Novel Object Recognition
PBS	Phospohate buffer saline
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
S.E.M	Standard error mean
SGZ	Subgranular zone
TLR-4	Toll- like receptor 4
TNF	Tumor Necrosis Factor

CHEMICAL FORMULAS

$NaH_2PO_4.H_2O$	Sodium phosphate monobasic monohydrate
$Na_2HPO_4.7H_2O$	Sodium Monohydrogen Phosphate Heptahydrate
NaOH	Sodium Hydroxide
NaCl	Sodium Chloride

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ABSTRAK

Alzeimer adalah sejenis penyakit saraf otak yang menandakan kemerosoton kognitif yang progresif. Penyakit ini menyebabkan perubahan mental dan kognitif seperti kapasiti memori dan intelek yang rendah selain gangguan personaliti bersama perubahan tingkah laku dalam kalangan populasi yang lebih tua daripada 65 tahun (Saeed Sadigh-Eteghad, 2014) (Crew and Masliah 2010). Objektif utama kajian ini adalah untuk mengkaji kesan minocycline yang dikenali sebagai perencat pengaktifan mikroglia terhadap keputusan ujian Novel Object Recognition (NOR) dan juga terhadap plak beta amyloid. Selain itu, kajian ini juga bertujuan untuk mengenalpasti kesan LPS terhadap keputusan ujian Novel Object Recognition dan pengumpulan plak beta amyloid.

Pengumpulan protin beta amyloid adalah salah satu ciri-ciri histologi penyakit Alzheimer. LPS menyebabkan pengumpulan plak protin beta amyloid dan juga kemerosotan ingatan (Frühauf *et al.*, 2015). Oleh itu, dalam kajian ini, Suntikan LPS diberi kepada 28 tikus Sprague Dawley untuk menjadikan model penyakit Alzheimer. Walaubagaimanapun, sumbangan rawatan minocycline dalam peradangan saraf disebabkan AD terutamany *in vivo* masih tidak jelas dan memerlukan siasatan selanjutnya (Biscaro *et al.*, 2012). Ujian NOR bertujuan untuk menilai keutamaan tikus terhadap objek baru untuk mengenal pasti kemerosotan ingatan tikus yang terdiri daripada empat kumpullan (Kontrol, LPS, LPS dengan minocycline (50mg/kg), LPS dengan memantine (2mg/kg)). Kumpulan 1 terdiri daripada tikus normal dan yang selebihnya disuntik LPS. Kemudian, kumpulan 3 dan 4 dirawati menggunakan minocycline dan memantine masing- masing selama tujuh hari berturut- turut. Keputusan histologi menunjukkan LPS gagal untuk menghasilkan plak protin beta amyloid dalam tisu otak tikus. Kumpulan tikus yang diberi

rawatan minocycline dijangka untuk menunjukkan indeks rekognisi yang tinggi daripada kumpulan tikus yang disuntik LPS. Selain itu, kumpulan LPS dijangka untuk menunjukkan indeks pengiktirafan lebih rendah berbanding dengan tikus normal untuk membuktikan kemerosotan ingatan itu disebabkan oleh LPS. Analisis statistik dilakukan dengan menggunakan One-Way Anova untuk menganalisis perbezaan antara indeks pengiktirafan yang dihasilkan oleh empat kumpulan. Menurut keputusan itu, kumpulan LPS yang dirawati minocycline menunjukkan nilai indeks rekognisi yang negatif antara kumpulan lain. Malah, tiada perbezaan yang ketara dalam indeks rekognisi antara kumpulan. Walau bagaimanapun, tikus LPS berjaya menghasilakn indeks rekognisi yang tinggi daripada tikus normal seperti yang diandaikan. Rawatan minocycline gagal untuk menghasilkan kesan yang dijangka dalam ujian NOR. Secara keseluruhannya, dos minocycline tidak optimum untuk membaiki status neuroinflamasi dalam kalangan tikus. Oleh itu, pengoptimuman ubat harus dijalankan terlebih dahulu untuk mendapatkan keputusan seperti dijangka. Selain itu, LPS telah Berjaya menyebabkan kemerosotan ingatan tanpa kewujudan plak protin beta amyloid. Ubat memantine pula telah berjaya meningkatkan ingatan tikus.

ABSTRACT

Alzheimer's disease is a progressive neurodegenerative disorder which indicates gradual decline in cognitive impairment. It is the reason for mental and cognitive alteration such as low memory capacity, intellect and personality disorder and also behavioral changes in population older than the age of 65 (Saeed Sadigh-Eteghad, 2014) (Crew and Masliah 2010). This research study focuses mainly on the effect of minocycline on novel object recognition memory in Lipopolysaccharide (LPS) induced rats. Besides that, it is also to determine the effects of LPS administration on novel object recognition test and in Aβ protein plaque deposition in rat brain. Administration of lipopolysaccharide (LPS) triggers neuroinflammation along with memory shortfall and leads to beta amyloid protein plaque deposition (Frühauf *et al.*, 2015). Therefore, LPS- induced Sprague Dawley rat models were used as AD paradigm for this study in order to investigate the effect of minocycline on their memory impairment and beta amyloid protein plaque deposition. However the contribution of minocycline (microglial activation inhibitor) treatment in LPS- induced neuroinflammation AD model especially in in-vivo study is still unclear and need further investigation (Biscaro *et al.*, 2012). The Novel Object Recognition (NOR) test is to evaluate the rat's preference towards the novel object as to monitor its memory impairment of rats of various groups (Normal Saline, LPS, LPS with minocycline (50mg/kg), LPS with memantine (2mg/kg) treatment). 1st group consists of normal rats and the rest were injected with LPS. Then, the 3rd and 4th group were treated with minocycline and memantine respectively for 7 days consecutively. Based on histological finding, there were no beta amyloid deposition found in all of the rats. Hence, there is no data to analyze for investigating the significance. As the minocycline able to inhibit microglial activation which renders the further

cognitive impairment while improving it, the LPS induced rats under minocycline treatment are anticipated to exhibit higher object recognition index than LPS induced rats. Besides that, LPS induced rats were assumed to show lower recognition index compared to normal rats to prove the memory impairment is due to LPS. The statistical analysis was done using One-Way Anova to analyze the recognition index produced by the four groups. According to the result, LPS induced rats under minocycline treatment shows the lowest value of recognition index among the other groups. In fact, none of the comparisons between groups were significant. However, LPS induced rats showed higher recognition index than normal rats as assumed. The minocycline treatment failed to produce the expected effect on NOR test. As the conclusion, the dose of minocycline was not optimum to recover the neuroinflammatory condition in rats. The dose optimization should have done to get the expected result. LPS successfully caused memory impairment even in the absence of beta amyloid protein. The drug, memantine treatment was successful in improving the rats' memory.

Chapter 1: Introduction

1.1 Background of the Study

Lipopolysaccharide induced models are the paradigms being used in Alzheimer's disease. This is because LPS induced neuroinflammation leads to all the hallmark characteristics of Alzheimer's disease. They are deposition of beta amyloid protein plaque (Aβ plaque), hyperphosphorylation of tau protein and neurodegeneration together with neuroinflammatory responses (Murphy and LeVine, 2010; Crews and Masliah, 2010; Biscaro et al., 2012; Cai *et al.*, 2013). Beta amyloid deposition is to be focused in this study. This pathological condition triggers the microglial which acts as macrophage in the brain environment. Microglial cell is a non-resting cell that keep patrolling the brain homeostasis (ElAli and Rivest, 2015). Activated microglia clear the beta amyloid plaques. However, the overactivated microglia tend to enhance the pathogenesis of AD by secreting the proinflammatory factors instead of phagocytize and subsequently eliminate the Aβ protein plaques (Biscaro *et al.*, 2012). The neuroinflammation and memory shortfall effect were induced by LPS injection in the rats (Frühauf *et al.*, 2015). In this study, a microglial activation inhibitor, minocycline was used to treat the neuroinflammatory condition in LPS injected rats which can lead to Aβ protein plaque deposition (Cai *et al.*, 2013). The memory of LPS induced neuroinflammation in were evaluated by novel object recognition (NOR) test after being treated with minocycline. NOR test was introduced in 1988 by Ennanceur and Delacour and the aim is to assess the capability of rodents to recognize novel object in an already exposed environment to compare the preference of animals to the novel object (Moscardo *et al.*, 2012).

1.2 Significance of the study

Alzheimer's disease remains incurable and many animal models have been developed to understand the mechanisms of disease (Nazem et al., 2015). LPS-induced neuroinflamation rat is one of the animal models to be used in the AD studies. However the contribution of minocycline (microglial activation inhibitor) treatment in LPS- induced neuroinflammation AD model especially in in-vivo study is still unclear and need further investigation.

1.3 Research Questions

The main question of this study is about how significantly the administration of minocycline (microglial activation inhibitor) can affect novel objects recognition test and Aβ protein plaque deposition in LPS-induced neuroinflammation rat's brain.

1.4 Objectives of the study

Generally, the objective of this study is to determine the effects of inhibition of microglial in novel object recognition and Aβ protein plaque deposition in LPS induced neuroinflammation rats. Other than that, the specific objectives are:

1) To determine the effects of LPS administration on novel object recognition test and in Aβ protein plaque deposition in rat brain
2) To determine the effects of minocycline treatment on novel object recognition test beside Aβ protein plaque deposition in LPS-induced neuroinflammation Alzheimer's disease rat model and to compare with the effects of memantine (standard treatment for AD)

Chapter 2: Literature Review

2.1 Alzheimer's Disease

It is stated that there are an approximately 46.8 million people around the world having dementia in 2015. This number will almost elevate twice every 20 years, touching 74.7 million in 2030 and 131.5 million in 2050. The developing countries hold the most of its elevation. It's reported that 58% of dementia patients live in developing countries, and it can reach 68% by 2050. The increase in Alzheimer's disease cases is correlated with rapid inflation in the aging generation occur in China, India, south Asian and Western Pacific neighbors (Alzheimer's disease International, 2015).

A German psychiatrist, Alois Alzheimer is the first one to report Alzheimer's disease (AD) in the early 1900s (Saeed Sadigh-Eteghad, 2014). Alzheimer's disease (AD) is a progressive neurodegenerative disease which is characterized by gradual decline in cognitive function. It is the reason for mental and cognitive alteration such as low memory capacity, intellect and personality disorder and also behavioral changes in population older than the age of 65 (Crews and Masliah, 2010; Sadigh-Eteghad *et al.*, 2015).

AD related problems are reduce life span, lowers life quality, cause physical challenges and subsequently lead to severe troubles in everyday life activities for instance, social and occupational events. Lately, there are some extraordinary efforts made to detect diagnostic markers which can predict the disease at early stage in order to cut down the social and economic costs besides lessening the burden of the disease on the patients and their families. For example,

neuroimaging methods such as magnetic resonance imaging and positron emission tomography were reinforced to aid researchers to diagnose AD earlier. Moreover, there are also certain important biomarkers developed to detect pathological features of AD that found in cerebrospinal fluid (CSF) that can be evaluated (Sadigh-Eteghad *et al.*, 2015).

According to the clinicians and researchers, there are six domains that normally interrupted in AD patients which are memory, executive functioning, visuospatial functioning, language and attention (Crews & Masliah 2010). However, memory impairment is the most prominent among these defects (Gold and Budson, 2008). The production and deposition of the Beta amyloid peptide (Aβ) is globally accepted to drive Alzheimer's disease pathogenesis (Murphy and LeVine, 2010). However, there are three hallmarks of AD: deposition of Amyloid β protein, hyperphosphorylation of tau protein and neurodegeneration along with signs of elevated inflammatory responses (Biscaro *et al.*, 2012; Murphy and LeVine, 2010).

2.2 Underlying Mechanism of AD

The main histological hallmarks of AD are deposition of Beta Amyloid protein that leads to an extracellular senile plaques, hyperphosphorylation of tau protein that forms intracellular neurofibrillary tangles (NFT) and neurodegeneration with synaptic loss accompanied by elevated inflammatory responses (Biscaro *et al.*, 2012; Murphy and LeVine, 2010; Sadigh-Eteghad *et al.*, 2015). These pathological changes majorly happen in the neocortex, hippocampus and other subcortical parts which are responsible for cognitive roles (Sadigh-Eteghad *et al.*, 2015). The protocols used to evaluate the pathology and categorize stages of AD have been systemized to issue a guide for clinicians. The density of neuritic amyloid plaques and hyper phosphorylation

of tau protein in affected brain regions are being referred as a standard criterion of pathology (Murphy and LeVine, 2010).

Actually, neuroinflammation is a crucial element in the neurodegenerative process of AD. The underlying causes of neuroinflammation determine its duration and severity. It has been declared that 'neuroinflammaging' refers to a condition of chronic neuroinflammation coupled with aging process plays a significant part in the mechanism of neurodegenerative disorder, AD. Studies proved that accumulation of activated microglia and inflammatory mediators in the cerebral neocortex showed an early involvement of neuroinflammation. In the process of aging which includes overweight, microglia get more active with its dysfunctional phagocytosis and recruitment of proinflammatory cytokines. This made the neurons to expose to a neurotoxic pro-inflammatory environment in the absence of neuroprotective microglia. This condition gets worse with the breakdown of the axonal cytoskeleton leading to the impairment of axonal transport, formation of axonal swellings of Amyloid Precursor Protein (APP) aggregates and eventually dystrophic neurites that cannot be removed by hyper-reactive microglia. Dystrophic of microglial are also associated with neurofibrillary degeneration in AD brain, especially in the temporal lobe. Such condition has led to the hypothesis that the initial trigger of AD neuropathology might be the senescence of microglia itself (Sadigh-Eteghad *et al.*, 2015). Microglia are basically are neuroprotective in the young brain, however, microglia cells react abnormally to stimuli in the aged brain and tends to be neurotoxic and eventually causes neurodegeneration. Aging-induced immune senescence occurs in the brain as age-associated microglia senescence, which makes microglia to behave dangerous to the CNS environment which subsequently promotes neurodegeneration (Block *et al.*, 2007). Besides that, dystrophic

microglia are also related to neurofibrillary degeneration in AD (Sadigh-Eteghad *et al.*, 2015). This is what happens in aging brain under Alzheimer's condition.

Beta amyloid protein is approximately a 4kDa of peptide that originated from APP, a precursor protein is a single-pass transmembrane protein which is metabolized in a quick and greatly complicated manner and expressed at high amount in the brain (Murphy and LeVine, 2010). Basically, the APP cleavage is mediated in two pathways. In the non- amyloidogenic pathway, α and γ secretases cleave the full- length APP. Cleavage through the β and γ may imprecise and forms many species of Aβ fragments. The main β secretase in the brain is the β-site APP- cleaving enzyme 1 (BACE1). This BACE1 produces neurotoxic forms of Aβ through the cleavage of APP. To be more detailed, BACE1 forms the soluble APPβ and C99 fragment which is then mediated by γ secretase to form Aβ (Sadigh-Eteghad *et al.*, 2015).

APP is an initially isolated principal substance of amyloid accumulation in the brain and cerebrovasculature of Down's syndrome and AD patients. A wide research has advanced our knowledge of how the Aβ peptide is generated and the way it is subsequently degraded within the brain or carried to the periphery, even though the role of APP has not been clearly determined. The end amount of Aβ that deposits as amyloid plaques within the brain is caused by the interaction of these factors (Murphy and LeVine, 2010).

Both of the β- and γ-secretase have crucial role in the production of Aβ, hence they are considered as the primary targets for the development of anti-AD pharmaceuticals. The modulation in γ-secretase activity contributes to the generation of longer forms of Aβ which are the main genetic factor of early onset, familial AD even when the level of γ-secretase mechanism does not seem to elevate in AD. There is a heterogeneity in the resulting peptides upon an imprecise cleavage by γ-secretase. Therefore, many varieties of Aβ species present, those are Aβ

40 (most abundant about 80 to 90%) and Aβ 42 (approximately 5 to 10%). Aβ 42 which is longer forms of Aβ is the primary component that deposits in the brain (Murphy and LeVine, 2010). For the degradation mechanism, the enzymes responsible are neprilysin (NEP) and insulin degrading enzyme (also known as insulysin; IDE). NEP plays role for the extracellular degradation of many kind of peptides whereas for both intra and extracellular degradation, IDE is the responsible metalloprotease (Murphy and LeVine, 2010).

Both the NEP and IDE decline during aging and in pathologically altered area in the case of AD. Moreover, at the early stage of AD, the amount of NEP lessens in the CSF. Besides these two enzymes, there was a significant proof identifies that an enzyme called cathepsin B which mediates lysosomal degradation for Aβ clearance. However, there was still a notable level of Aβ stays in the brain without being catabolized. Together with other metabolites, mechanism presents to carry Aβ across the blood brain barrier (BBB) in and out to blood circulation. Disruption of this mechanism causes the amount of Aβ in the brain to rise up which then subsequentially leads to its maximum deposition. There are two principle mechanisms behind the exchange of soluble Aβ across the BBB, therefore, this causes net efflux of Aβ across the membrane which determines the amount of cerebral amyloid burden. The presence of bidirectional mechanism for the beta amyloid transport remains as an ambiguous as it might be a prominent physiological function that is not related to AD. Nevertheless, it is practically can be true that any alteration of these mechanism, together with other unrelated pathological changes in the AD brain be the factors for the growth of amyloid deposition (Murphy and LeVine, 2010).

Neuroinflammation plays a prominent role in the mechanism of beta amyloid deposition. The resulting inflammatory cytokines (IL and TNF) upon neuroinflammation increase APP expression and beta amyloid deposition. Besides that, cytokines also able to transcriptionally

elevate the β-secretase mRNA, protein and enzymatic activity. β-secretase is a key rate-limiting enzyme that starts the Aβ formation. Aβ synthesis is considerably lessened in the absence of β-secretase (Lee *et al.*, 2008). Neurodegeneration is a part of neuroinflammation. This is because activated microglia tends to be dangerous to the brain microenvironment and allows beta amyloid to deposit and eventually form plaques in hippocampus and neurite dendrites. Besides that, microglia also releases reactive oxygen species that makes the condition worse by elevating neurotoxicity. At this point, neurotoxicity due to microglial activation and beta amyloid deposition causes neuronal loss. This neuronal loss and neurodegeneration leads to memory impairment which is one of the main hallmark characteristics of Alzheimer's disease (Biscaro *et al.*, 2012; Block *et al.*, 2007; ElAli and Rivest, 2015).

2.3 Lipopolysaccharide (LPS) injection

It is proven that LPS intraperitoneal injection causes neuroinflammation that lead to cognitive impairment, learning deficits, hippocampal cell loss and even β-amyloid plaque formation in the hippocampus (Qin *et al.*, 2007). LPS injection triggers neuronal damage and subsequent cognitive impairment which are further contributed by those neuroinflammatory mediators. Besides that, LPS administration also alters contextual fear conditioning, avoidance learning and spatial memory in rodents such as mice and rats. To be more specific, in novel object recognition (NOR) test, LPS reduces the rats or mice preferences for the novel object (Frühauf *et al.*, 2015).

Besides that, the discrepancy between *in vivo* and *in vitro* concepts of microglial activation also should be taken into consideration. This is because *in vivo* microglia do not

produce IL-1 whereas *in vitro* do. Thus, most of the previous studies were not taken into account as they all are done in vitro (Streit, 2010). In *in-vivo*, the amoeboid microglia express the main plasma membrane binding sites for LPS induced cytokine which are the CD14 receptors and TLR4. A prolonged LPS exposure leads to a different kind of activated state compared to the microglial cells that are acutely exposed to LPS. However, with repetitive exposure of LPS, there is a reduction in the level of TNF-α and NO, but maintaining production of prostaglandin E_2 (PGE_2). Moreover, it was assumed that microglia have a degree of plasticity upon encountering LPS that greatly influence the firm concept of brain inflammation (Rock *et al.*, 2004).

2.4 Cognitive Impairment in Lipopolysaccharide (LPS) induced AD

According to numerous studies done on neurodegenerative diseases such as Alzheimer's disease, LPS induced neuroinflammation rats are the one being used as the AD paradigms (Crews and Masliah, 2010; Kitazawa *et al.*, 2005) The reason for using LPS is that, it is a cell wall component of gram negative bacteria which can induce neuroinflammation, hippocampal apoptosis, cognitive impairment, learning deficits and even Amyloid-β plaques generation in the hippocampus (Qin et al., 2007). Fundamentally, activated TLR-4 by LPS causes cognitive impairment through microglial overactivation and excitotoxicty at NMDA receptor (Block *et al.*, 2007; Gold and Budson, 2008; Newcomer *et al.*, 2000).

The activated TLR-4 causes excitotoxicity at N-methyl D-Aspartate (NMDA) receptor which is responsible for controlling synaptic plasticity and memory. Excitotoxicity means overstimulation of NMDA receptor caused by glutamate which is an excitatory neurotransmitter in the CNS (Newcomer *et al.*, 2000). This excitotoxicity leads to neuronal loss which subsequently impairs the cognitive functions as the NMDA receptor is known to regulate a

substrate responsible for memory function in the brain (Frühauf *et al.*, 2015; Gold and Budson, 2008; Newcomer *et al.*, 2000).

When the LPS started to work in the body system, it triggers toll-like receptor 4 (TLR4) directly and this stimulation leads to activation of microglial cells. Activated microglia releases proinflammatory cytokines in rat's brain. The activation of glial cells upon LPS induction leads to the release of neurotoxic substances, including nitric oxide, glutamate, cytotoxic cytokines and superoxide radicals, the suppression of neurotrophic factor secretion and impaired neuroplasticity and consequently, learning and memory (Frühauf *et al.*, 2015). Previous studies show that microglia died and some became dangerous to the central nervous environment upon an overstimulation of LPS. Hence, they couldn't perform neuroprotective functions and causes neurodegeneration. Microglia even stops the adult neurogenesis during beta amyloid protein deposition (Biscaro et al., 2012). This is because LPS elevates nitric oxide (NO) secretion and reactive oxygen species (ROS) which stimulates the redox- sensitive nuclear factor kappa B, which gives rise to neuroinflammation that results in cognitive impairment (von Bernhardi et al., 2015). Neurodegeneration is one of the main hallmark characterics of AD that caused by over activated microglia in LPS induced neuroinflammation brain whereas in aging process, it happens due to dysfunctional microglia that allow deposition of beta amyloid protein that leads to neuronal loss (ElAli and Rivest, 2015; Gold and Budson, 2008; Mosher and Wyss-Coray, 2014). Neuronal loss or also considered as neurodegeneration results in cognitive impairment which includes memory deficit (ElAli and Rivest, 2015; Nazem et al., 2015; Streit, 2010).

2.5 Physiological Roles of Microglia

In 1919, microglia were discovered and a vast research carried out since then, though, the origin of this cell is still indefinable. There were certain different parentage have been put forward for this cell. One of them stated that microglia are raised from the progenitor of neuroectoderm and/ or the mesoderm, which colonize the brain from the early embryonic stage, and throughout the fetal development stage. Besides that, it was also been proposed that microglial cell was originated from circulating blood monocytes, particularly from the late gestational stage throughout the beginning of postnatal stage. Another proposal is that bone marrow was not the pioneer for microglia but they are from myeloid stem cells (a.k.a primitive macrophages) in the yolk sac. Even though, there were many proposal entangled throughout the research, majority of people agrees to the point that microglia were raised from myeloid progenitor which invade the brain during the distinct brain development levels. It is significant to say that about 95% of microglia populations are produced postnatally and more accurately after the genesis of blood-brain-barrier. Fundamentally, microglia are active cells which keep guarding the brain if there are any changes in cerebral homeostasis. In order to detect any alteration, these cells utilize their dynamic and mobile cellular processes as a security guard to patrol and scan the microenvironment within their zone. Any threat or inflammatory insult to the brain leads to the rapid activation of microglia. Once get activated, the microglia elicit a sufficient reaction to deal with the rising threat that can range from release of molecular mediators including immune and non-immune components to phagocytosis (ElAli and Rivest, 2015).

Basically, microglia are known to possess the fascinating nature whereby it maintains brain tissue integrity under both normal and pathological conditions. Unlike the other cells in the

body, microglia are able to change and adapt throughout lifespan. Its function is such as inspecting the whole brain for every two hours. In a healthy brain, microglial cell functions as neuronal function regulators. Based on several accumulating evidence, microglia mediates neuronal function through two main mechanism; phagocytosis and biochemical interactions. Initially, it was hypothesized that microglia mediated phagocytosis only occur in pathological conditions. Nonetheless, current progression showed that the cell debris that being phagocytized is actually result from apoptotic cells in the area where neurogenesis happens in an adult brain. This process is to coordinate the circuit function which is prominent for sustaining brain equilibrium and integrity of tissue. However, the ramified microglia remove the newborn neurons that undergo apoptosis at beginning stage. The phagocytosis process encompasses of three steps known as find-me, eat-me and digest me. This mechanism is mediated by signals that triggered by some signaling molecules being released, such as nucleotides, cytokines and chemokines during the recruitment and activation of microglia (ElAli and Rivest, 2015). Even though the microglia is a never resting cell, its phagocytic traits are greatly influenced by physiological aging, genetic factors and epigenetics. Nevertheless, it is very significant to state that most of studies were based on "in vitro" using simple stimulus. Amoeboid microglia are activated microglia whereas ramified cells are known as resting microglia. It is prominent to know the difference between these two kinds of microglia (Rock *et al.*, 2004).

Recently, neuroinflammation is considered as the leading cause of AD pathology and begins early in the course of the disease even before the tau hyperphosphorylation and amyloid plaque formation (Krstic and Knuesel, 2013). Furthermore, some neuropathological studies have proved initial involvement of neuroinflammation in AD via indicating the accumulation of activated microglia and inflammatory mediators in the cerebral neocortex. In the process of

aging which includes overweight, microglia get more active with its dysfunctional phagocytosis and recruitment of proinflammatory cytokines. This made the neurons to expose to a neurotoxic pro-inflammatory environment in the absence of neuroprotective microglia (Nazem *et al.*, 2015). However, there is another statement from Streit (2010) that the microglial recruitment to the area of senile plaque is considered as an abnormal cellular reaction stimulated by the amyloid fibrils instead as an indication of an inflammation. It was also clearly stated that even an extensive accumulation of diffuse and early beta amyloid protein do no trigger any microglial alteration, but result by more advanced amyloid plaques (Streit, 2010).

2.6 Minocycline and its action

Minocycline which known for its role as microglial activation inhibitor is a tetracycline-derivative with anti- inflammatory properties. It able to cross the blood- brain barrier quickly and can ceases microglia activation within the CNS. It also restores neurogenesis which was disrupted by the LPS administration. Basically, this drug has the potential to control oxidative stress and inflammation (Cai *et al.*, 2013). Over most, minocycline is known for ceasing microglia and inflammation in many animal paradigms of neurodegenerative diseases that include AD. The reactivity of Aβ protein plaque- associated CD11b+ microglia in the hippocampus, nitric oxide synthase (iNOS) protein level and the microglia in the dentate gyrus were successfully reduced by the minocycline but it couldn't affect the reactive astrocytes.

Based on an in vitro study, the microglia were found to be dead upon over stimulation of LPS. And there are several microglia became dangerous, means no more neuroprotective to the central nervous system environment. These microglia even renders adult neurogenesis during beta amyloid deposition (Streit, 2010). These over activated microglia causes neurotoxicity by releasing reactive oxygen species (ROS) and eventually causes neurodegeneration which impairs

cognitive functions such as memory and synaptic plasticity (Block *et al.*, 2007; von Bernhardi *et al.*, 2015). Therefore, in order to inhibit this kind of microglial activation, minocycline is used in this experiment. This inhibition restores cognitive functions by reducing neurodegeneration.

Apart from that, the drug also aids to generate more new neurons in the granule cell layer without decreasing the cerebral Aβ (Biscaro *et al.*, 2012). It is proved that administration of minocycline also has upregulated the anti- inflammatory cytokines while down regulates the proinflammatory besides upgrading recognition memory in transgenic AD mice (Biscaro *et al.*, 2012). Diabetic mellitus, a metabolic disorder also exhibit the production of Aβ protein plaques and minocycline treatment can diminish beta amyloid protein in the hippocampus via inhibition of nuclear factor- kappa beta (κB) pathway activation (Biscaro *et al.*, 2012). Some evidences show that the cognitive deficits caused by diabetic metabolic disorder and ischemia have been found to improve through minocycline administration (Cai *et al.*, 2013).

2.7 Memantine is approved by Food and Drug Administration (FDA)

The US Food and Drug Administration (FDA) has approved reversible acetylcholinesterase inhibitors as first and most widely prescribed class of pharmaceuticals for treating cognitive deficits in AD. This class encompasses tacrine, rivastigmine, donepezil and galantamine. Acetylcholinestrase inhibitors are assumed to upgrade worldwide cognitive functioning by elevating the amount of cholinergic synapses at multiple regions throughout the brain. Memantine is the only FDA-approved drug therapy for cognitive impairment in AD. It is a low- affinity NMDA receptor antagonist and non-competitive. It is proclaimed that the drug works by averting excitotoxicity that can cause the death of neurons resulting from glutamate's over stimulation at NMDA receptor. However, this was not proven in human beings. Moreover, memantine able to develop cognitive performance by regulating NMDA receptor to enhance the neural signal while reducing background noise. Astonishingly, the only drug that approved for moderate and severe AD's drug therapy is Memantine (Gold and Budson, 2008).

2.8 Congo Red Staining

In 1922, the original protocol of congo red staining was firstly illustrated by Bennhold. The protocol has undergone many alteration to enhance its reliability, sensitivity and specificity. Alkaline congo red was one of the modification. This dye is well known dye utilized for evaluating amyloidosis (Linke, 2006). This histological stain is utilized for detecting amyloid and pre-amyloid protein which includes those encompass beta amyloid peptide. The β-pleated sheet conformation of amyloid basically binds to the dye. Besides congo red, thioflavin S also used for same purpose (Rajamohamedsait and Sigurdsson, 2012). To be more detailed, the structure of amyloid reveals when congo red molecules line up 90^{o} degree to the axis of amyloid α fibrila and to each other. By using polarized microscope, the amyloid proteins can be seen in apple green whereas thru light microscope, it can be seen red in colour (Linke, 2006). In this experiment, histological staining was viewed under light microscope. A 40μm of brain section was stained using Highman's congo red and counterstained with hematoxylin. Hence, the anticipated result is amyloid in red and the nuclei in blue.

2.9 Novel Object Recognition (NOR) Test

In 1988, Ennanceur and Delacour have introduced Novel Object Recognition (NOR) test and it was considered as a spontaneous Delayed-Non-Matching-to- Sample (DNMS). It is because this test is relied on spontaneous behavior. The primary purpose of this test is to check preference of rodents towards novel object or an environment. This obvious 'unlimited preference' towards novelty has been applied in NOR test to conduct studies based on memory functions and to evaluate rodents ability to recognize a new object in an accustomed area (Moscardo *et al.*, 2012). Ordinarily, this test involves two trials (familiarization and test) separated by an intersession interval (ISI). Two exactly same objects were placed during first trial. Then, in the second trial which is the test, one of the objects was replaced with new (novel

object). Higher preference will be given to the new object if the rats remember the old objects (Frühauf *et al.*, 2015; Leger *et al.*, 2013; Moscardo *et al.*, 2012). Basically, this test is regarded as "pure" recognition memory as it does not involve any reference memory components. And this test is generally simple to be conducted and, hence, it is extensively used in pre-clinical research to evaluate mild cognitive impairment (Moscardo *et al.*, 2012).

This type of test originated from the "visual paired comparison paradigm" and generally applied in both the non-human primates and humans in which the recognition is based on the preference to the novelty. Besides that, this test is suitable for comparing interspecies. The experimental research that involves this test are on memory, for instance neurotransmission alteration due to drugs, after brain lesions or neurodegenerative diseases and in ageing studies. Generally, the have several variations in object recognition protocol. One of them is object exploration duration. Next is habituation, in which the rats are allowed to explore an empty open-field apparatus. This phase helps the rodents to lower their stress and prevent neophobic response. Consequently, this will leads to a better exploration of objects.

In the first and foremost study, the habituation session have been carried out about 2 minutes on the day before test. Ever since, a number of protocols have become known with different period of habituation phase that ranging from minutes to hours and even for days. There are three types such as long habituation, short habituation and no habituation. In long habituation, this phase is done twice a day and at each session, it was conducted about 5 minutes. This is normally done for 2 or 3 consecutive days. Meanwhile, short habituation refers to 5 minutes per day and the next is for familiarization. This habituation pattern alters the experimental design as the habituation phase is important for relaxing the rodents thus enhancing their activity.

Equipment used in this test also influence the protocol. Basically, there was no study on comparing the rodents' performance based on the shape of the open-field apparatus. However, the objects to be tested must be focused in term of colour, shape, size, brightness, odor, and texture of them. An experimenter must prioritize the texture and brightness of the objects because rodents have trouble in distinguishing colours. This is to intensify the preference over only one object as the two objects differ from each other. And it's better to choose objects that can't be climbed by rodents. Besides that, heavy objects should be chosen so that rodents won't be able to move them and if can, it is advisable to hold the objects stuck on the floor using double-sided adhesive tape. Timing of test is also another aspect to consider about. Animal's condition influences exploratory behavior. The light circle and circardian rhythm have effect on the rodents' status. Rodents always must be tested in the dark phase and placed in a light-dark cycle. Their active phase is from 8am to 8pm. The familiarization is better to conduct starts from 9 in the morning. Timing is very essential to give thought (Leger *et al.*, 2013).

Apart from that, handling animals also reduce their stress level and can achieve anticipated results in performance. They must be used to experimenter's handling method as this aspect can affect their behavior responses in the NOR test. Hence, handling must be consistent for control group and treatment groups. In order to lessen novelty induced stress, handling such as 5 minutes per day or 1 minute for twice a week may give better outcome (Aslani *et al.*, 2014; Leger *et al.*, 2013). Other than that, exploratory behavior toward objects is very crucial component in this test protocol. However, the right method for evaluating exploratory behavior is still not vivid. Nonetheless, in the first paper, pointing nose towards the object about less than or equal to 2cm refers as exploration. Besides that, many agreed that activities such as leaning on object or climbing over it are not considered as exploration unless that activity was coupled with

sniffing as mentioned earlier. Hence, sniffing and touching the objects while looking at them is strongly accepted whereas climbing onto or chewing the object are not. Anyhow, the distance of 2cm or lesser than that should be monitored by experimenter (Leger *et al.*, 2013).

Chapter 3: Materials and Methodology

3.1 Materials and Apparatus

3.1.1 Reagents, Drugs and Instruments

Table 3.1.1 (a) List of drugs, reagents and other materials required in this experiment

No	Materials (Drugs, reagents and other)	Description
1	Lipopolysaccharide 5mg/ml	The powder form of LPS was mixed in normal saline to be injected into rats. The calculation for preparation is shown in 3.1.3.
2	Minocycline 50mg/ml	The powder of Minocycline was mixed in normal saline to be injected into rats. The calculation for preparation is shown in 3.1.3.
3	Memantine 2mg/ml	The powder of Memantine was mixed in normal saline to be injected into rats. The calculation for preparation is shown in 3.1.3.
4	Sodium pentobarbital	Commercial drug used for euthanasia purpose
5	Phosohate buffer saline (PBS) pH 7.4	A 1000ml (pH 7.4) is prepared by mixing 165ml of stock 1 and 35ml of stock 2. Stock 1

		contains 42.6g of Na_2HPO_4 and 238.4g of NaCl. Stock 2 contains 13.8g of $NaH_2PO_4.H_2O$ and 79.45g of NaCl. Then, distilled water was added until 1000ml. This solution was used for perfusion purpose to drain the blood out of the system to get a clear brain.
6	Phosphate buffer stock 0.2M	For 1000ml solution preparation, 5.25g of $NaH_2PO_4.H_2O$ and 23g of $Na_2HPO_4.7H_2O$ were mixed in 1000ml of distilled water. Then, it was stored in $4^{O}C$.
7	8% Paraformladehyde stock	A 300ml of distilled water was heated until it reached $60^{O}C$ and a 40g of Paraformaldehyde was mixed. This solution was stirred at least for 30 minutes. Then, a 10M Normality (N) NaOH until solution becomes clear. After filtering, distilled water was added up to 500ml and stored in $4^{O}C$.
8	4% Paraformaldehyde pH 7.4	For 4% Paraformladehyde, PB stock and PFA stock were mixed in 1:1 ratio and the pH was adjusted at 7.4. This solution is used as fixative for the rats after perfusion.

9	20% Sucrose pH 7.4	For 1000ml of distilled water, 2.6g of $NaH_2PO_4.H_2O$, 11.5 of $Na_2HPO_4.7H_2O$ and a 300g of sucrose were mixed in distilled water. It is a cryoprotectant is a partial dehydrant that prevents the formation of ice crystal artefact in frozen tissue sections.
10	Congo Red stain	It is used to stain beta amyloid in red. The preparation is stated in 3.5.3 subchapter.
11	Distilled water	Used for many purposes like preparing alcohol into different percentage, rinsing and washing purpose
12	Alkaline alcohol	Used as decolorizer to remove excess stains
13	Absolute alcohol	To deep the microscopic slides together with tissue section before mounting.
14	Haematoxylin	Used as counterstain. It stains the nucleus in blue color.
15	DPX mounting medium	Mounting medium
16	Normal Saline	It is used for washing purpose and to expand brain tissues in

		the well sometimes.
17	Tissue freezing medium for froze tissue specimens	It is used to freeze the tissue sections before sectioning

Table 3.1.1 (b) List of Apparatus and Machines

No.	Apparatus and Machines
1	Surgical Instruments: Scissors, needles, various forceps, syringe
2	1.5 ml tube
3	24 well tissue culture plates
4	Sterile container
5	Paint brushes
6	Micropipettes
7	Micropipette tips
8	Microscope slides and cover slips
9	Light Microscope
10	Cryostat (Carl Zeiss Micron)
11	Balances

3.1.2 Animals

A 28 number of Sprague-Dawley male rats about 12 weeks were used in this experiment. They had a body mass about ± 200g and received from Animal Research Centre (ARASC), Universiti Sains Malaysia (USM). Polypropylene cages were used to house the rats and maintained at room temperature about 23^{O}C (air-conditioned room) while exposed to 12 hour light- dark cycles. The food and water were ad libitum. The Research and Ethics Committee, USM approved this experimental protocol.

The rats were divided randomly into four groups, thus each group consists of 7. The groups are as follows: (i) Normal Saline, (ii) LPS induced, (iii) LPS rats treated with minocycline (LPS+ MINO), and (iv) LPS rats treated with Memantine (LPS+MEM). Each group was divided into 4 batch. Hence, in each batch, there rats of all the groups and placed in cage according to their groups. For example, first rat of control group in batch 1 was named as Control B1R1. The body mass of rats were recorded before and after injecting LPS (5mg/kg), at the end of treatment and before sacrificing. LPS injection was done on 1st day. From the 2nd day until 8th (7 days), the treatment was allocated. From 9th until 12th day, they were subjected to participate in Novel Object Recognition (NOR) test before being sacrificed. Hence, the sacrifice was on 13th day. They were euthanized and decapitated after completing the NOR test in order to collect their brains for histological staining purpose. Histological staining was done after 13th day and 6 samples were done for every day. It took almost more than 1 month.

3.1.3 LPS and Drugs Preparation and Administration

The dose of LPS is 5mg/kg. Hence, a 5mg of LPS was added into 1ml of normal saline for making the calculation easier as shown below:

$$\text{Volume for 1 rat} = \frac{5\text{mg/kg} \times \text{body weight (kg)}}{5\ \text{mg/ml}}$$

$$= \_\_\_\_\_\text{ml}$$

The same method goes for Minocycline preparation. A 50mg/kg dose of minocycline was prepared by weighing 50 mg and mixed it in 1ml of normal saline

$$\text{Volume for 1 rat} = \frac{50\text{mg/kg} \times \text{body weight (kg)}}{50\ \text{mg/ml}}$$

$$= \_\_\_\_\_\text{ml}$$

The dose for Memantine is 2mg/kg. Therefore, it was prepared by mixing 2mg of the drug in 1ml of normal saline and the formula to get volume for each rat as shown below.

$$\text{Volume for 1 rat} = \frac{2\text{mg/kg} \times \text{body weight (kg)}}{2\ \text{mg/ml}}$$

$$= \_\_\_\_\_\text{ml}$$