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TNF-α (*Tumor Necrosis Factor Alpha*) and iNOS (*Inducible Nitric Oxide Synthase*) Expression in Rat Brain Infected by *Mycobacterium tuberculosis* Strain H37RV

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Abstract

Tuberculosis is an infectious disease caused by the *Mycobacterium tuberculosis*. *Mycobacterium tuberculosis* will form the primary focus or Ghon focus in the lungs of infected people. The primary focus can break and get into the bloodstream and/or lymph to the entire body, including the central nervous system, especially the brain. Tuberculosis infection in the brain can cause microglia secrete inflammatory factors such as TNF-α and IL-1β is emerging as the body's immune response. The factors that can trigger microglia to secrete iNOS (Inducible Nitric Oxide Synthase) in order to protect the brain from attacking bacteria. iNOS is shown to have an important role in tuberculosis infection in the brain. TNF-α is a pro-inflammatory cytokine which is mostly produced by macrophages/microglia through several mechanisms. Therefore, to investigate how the expression of TNF-α and iNOS in the brain tissue of the mice is not infected with tuberculosis, tuberculosis infection with an incubation period of 8 weeks and 16 weeks. This study is a semiquantitative study by comparing the amount of expression of TNF-α and iNOS and all three groups of samples with treatment as has been mentioned. The expressions observation of TNF-α and iNOS in brain cell tissue of mice was conducted using immunohistochemical staining, and was seen in a microscope with a magnification of ×100. Brain cells that express TNF-α and iNOS are brown core, cytoplasm and cell walls. The results were obtained by the longer exposure to infection of the higher expression of TNF-α ($r > 0688$) and the expression of iNOS decreased (−0.993).

Keywords

TNF-α, iNOS, Immunohistochemistry, *Mycobacterium tuberculosis*, The Brain

1. Preliminary

Tuberculosis (TB) is a disease with high morbidity and mortality, especially in developing countries. WHO reported in 2012, 8.6 million people were afflicted with tuberculosis and 1.3 million died from tuberculosis [1]. In the United States the incidence of tuberculosis in the central nervous system recorded 5% - 10% of all cases of extrapulmonary tuberculosis [2].

The spread of *Mycobacterium tuberculosis* infection is known through inhalation of droplet nuclei that contain a lot of bacteria and can cause the bacteria to live in the pulmonary alveoli. The entry of *Mycobacterium tuberculosis* to the alveoli of the lungs will cause the activation of macrophages. This bacterial stimuli activates the innate immunity system, summoning several cytokines to attack these bacteria. In the process our immune system will form granulomas in the lungs as a primary focus, which is one of the constituent materials, is the cytokine TNF-α. Haematogenous deployment will cause the spread of these bacteria, especially the oxygen-rich organs such as the brain. *Mycobacterium tuberculosis* has the ability to defend itself from macrophages. Because it has thick walls, these bacteria are using macrophages as proliferate media until the macrophage lysis. This process also occurs in macrophages in the brain microglia [2]. There are many risk factors that can cause tuberculosis. Because of the low immunity, then getting exposure to BTA (acid-resistant bacteria) positive for tuberculosis is through sputum droplets. Exposure smear positive impact is greater than the negative BTA which can be checked in patients' sputum. People with bad endurance will be more easily exposed, such as HIV/AIDS, or can be in a state of malnutrition/malnutrition [3].

The pathogenesis of TB SSP begins with the development of Rich Focus or small tuberculosis focus in the brain, spinal cord, or meninges. Rich Focus contains many inflammatory factors such as histamine, bradykinin, serotonin, leukotriene, prostaglandin and cytokine that cause tuberculosis in the SSP prognosis to get worse. In addition, the alleged existence of inflammatory factors such as cytokines, produced from cells of the brain for their signals to the body's defenses that TNF-α also facilitated easier ingress tuberculosis bacteria across the brain barrier and liquid seresbrospinal. Allegedly the involvement of immune cells such as NK cells, macrophages, T lymphocytes, lymphocytes T CD1-restricted, and the T-helper 1 will activate the IFN-g, and will stimulate microglia to express TNF-α [2]. According to Walgreen's [4], the primary focus of TB infection in the lungs will be about 1 - 6 months post infection to be brain TB.

TNF-α is a pro-inflammatory cytokine mostly produced by macrophages/

microglia through several mechanisms [5] [6] [7]. In immunology, TNF-α is produced by macrophages to the Antigen-Precenting Cell (APC). APC will then instruct the T-helper 1 to proliferate into IL-12 and IFN-γ in which IFN-γ will produce TNF-α as a anti-mikobakterium. While in biochemistry, production of TNF-α occurs when there is NF-kB transcription induced initially by the activation of receptors on the microglia that are specific to *Mycobacterium tuberculosis* that is toll like receptor (TLR2 and TLR4). TLR2 and TLR4 became active when microglia caught the bacteria *Mycobacterium tuberculosis* in the form itself or only recognized antigen mt-38—the antigen contained in the cell wall of *Mycobacterium tuberculosis*.

Nitric oxide is a reactive free radical that acts as a biological mediator [8]. There are three kinds, namely N-NOS (Neuronal Nitric Oxide Synthase), E-NOS (Endothelial Nitric Oxide), iNOS (Inducible Nitric Oxide Synthase). iNOS itself is part of the immune system of human body which is released by microglia and will participate in the activities of anti microbes and can be as anti-tumor [9]. When *Mycobacterium tuberculosis* goes into the brain through the limfogen, the microglia as a type of macrophages in the central nervous system will do its job to do phagocytosis or execute *Mycobacterium tuberculosis*. Activated microglia will produce cytokines-pro-inflammatory cytokines such as interleukin-1, tumor necrosis factor alpha and interferon gamma. In addition to producing proinflammatory cytokines, microglia also secrete iNOS. Using L-Arginine and L-Citru- linne, iNOS will release nitric oxide as an antimicrobial [10]. While the E-NOS is only limited in endothelial cell line that is served as a vasodilator and N-NOS is also limited in neural networks that is served as a communicator between cells [11].

Therefore, researchers wanted to validate the changes in the expression of iNOS and TNF-α in the brain that is infected with *Mycobacterium tuberculosis* for 8 weeks and 16 weeks.

2. Research Methods

2.1. Research Design

This study is an experimental study on mice (Mus musculus) kind of wild-type mice tocompare the treated groups of TB infection (Mycobacterium tuberculosis). Selected study in mice because they can be investigatedon the effect of sequential TB infection with an incubation period of 8 and 16 weeks. Furthermore, the mice can be histological examined of brain tissue to see the effect of TB infection on the expression of iNOS and TNF-α.

2.2. The Research Sample

The research sample is brain tissue of mice (Mus musculus) type Balb/c which are exposed to *Mycobacterium tuberculosis* by inhalation using a modified nose-only inhalation system or Middlebrook Inhalation Exposure System (Glas-Col) are selected randomly according inclusion and exclusion criteria. Brain samples are divided into a control group that is the brain tissue of mice without infec-

tion, then a group of brain tissue of mice after infection with *Mycobacterium tuberculosis* for 8 weeks, and the brain tissue of mice after infection with *Mycobacterium tuberculosis* during the 16 weeks.

2.3. Process of Immunohistochemistry

Immunohistochemical method performed using streptavidin-biotin-peroxidase labeled streptavidin-biotin (novocastra, USA). Before the dyeing process, each microscope slideis deparaffinizedwith xylene for 15 minutes and rehydrated with alcohol 100% and 70% in10 minutes for each. The slides are then cleaned twice with dH20 for 5 minutes andincubated using PBS for 5 minutes. Subsequently, the dosage slides are put into a glass box containing citrate buffer and then inserted into the autoclave for 15 minutes to optimize its antigenicity. The slides or preparations are frozen at room temperature for 1 hour, and after drying for a while, the tissue is timed using a pap pen. Preparations are washed using dH20 for 5 minutes and with PBS for 5 minutes before incubation with 0.3% hydrogen peroxidase for 15 minutes.

After the block of peroxide endogenous, the preparations are incubated with blocking solution for 30 minutes to block avidin contained in the tissue. Then, the preparations are incubated overnight at −4˚C with primary antibody (monoclonal anti-arginase sc-20150) and monoclonal anti-iNOS neomarker RB-1605-P0, diluted 1:100. Preparations are washed again 3 times with dH20 before incubated with secondary antibody and streptavidin-HRP for 30 minutes each. For the coloring, 3.3 diamino benzidine tetrahydrocloride is used approximately 10 minutes to obtain the staining reaction that can be detected by microscopic examination. After that stained with hematoxylin again to clear the nucleus of a cell for 30 seconds and washed with running water for 5 minutes. The preparations are dehydrated using the alcohol with the concentration increased gradually from 70%, 80%, 90% to 100% for each 2 minutes. After that, the preparations are dipped into xylene for 5 minutes.

Lastly, malinol is added to the preparations before it is sealed using cover glass. The results of immunohistochemical examination will be grouped based on the number of colored cells, which is said to be positive when there is a cytoplasmic immunostaining and quantified based on techniques developed by Soini et al, 1998; Pizem and Cor, 2003.

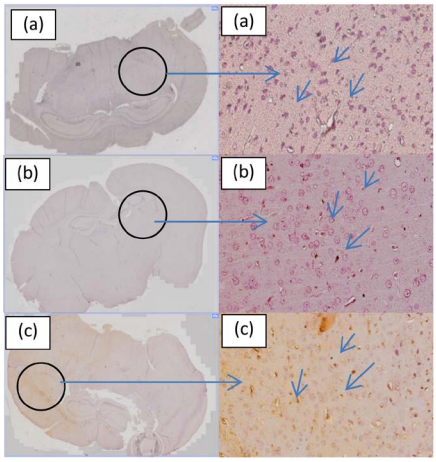
Examination of TNF-α expression is observed by immunohistochemical techniques using Novolink Polymer detection system (Leica Microsystems). Monoclonal antibodies against TNF-α provided by Santa Cruz Biotech. Visuali- zation is done with DAB and couterstaning use Hematoxilen Mayer.

Calculation Method on the results of immunohistochemical staining. This study uses brain tissue of mice. There are 27 samples in this study. Each tissue sample is made preparations slices with a thickness of 4 um, then immunohistochemistry detected the expression of TNF-α and iNOS.

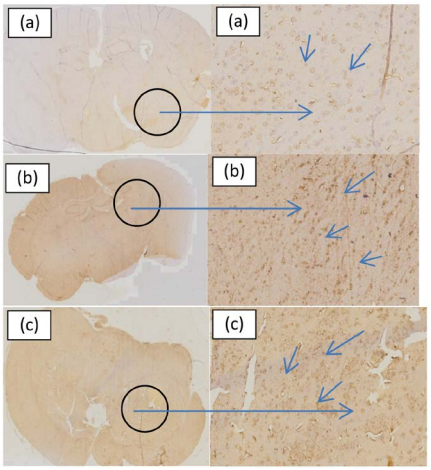
3. Research Result

In the microscope observation, the evaluation results is obtained from the slide-

sof mice brain tissue are based on an expression of TNF-*α* and iNOS after immunohistochemical staining with an antibody TNF-*α* and iNOS per 20 field of view with a magnification of ×100 tabulated as further data to be analyzed. Expression of cells containing TNF-*α* and iNOS are cells that have a brown color in the cell wall, cytoplasm and nucleus of cells of the brain's neurons (Figure 1).



(A)



(B)

Figure 1. (A) Expression of TNF-*α* in the brains of mice. (A-a): Ekspresi TNF-*α* in brain cells of mice without treatment with a magnification of ×100. Black arrows indicate the cells that did not express TNF-*α,* are marked with a purple core, cytoplasm and cell walls; (A-b) Expression of TNF-*α* in mice treated with 8-week with a magnification of ×100. Yellow arrow indicates cells expressing TNF-*α* are marked with a brown color in the nucleus, cytoplasm and cell walls. Black arrows indicate the cells that did not express TNF-*α* are marked with a purple core, cytoplasm and cell walls; (C-c): Ekspresi TNF-*α* in mice by 16-week of treatment with a magnification of ×100. Yellow arrow indicates cells expressing TNF-*α* are marked with a brown color in the nucleus, cytoplasm and cell walls. Black arrows indicate the cells that did not express TNF-*α,* are marked with a purple core, cytoplasm and cell walls. (B) Expression of i-NOS in the brains of mice. (B-a) Expression of iNOS time 0 week (not infected) at a magnification of ×100; (B-b) Expression of iNOS time of 8-week at ×100 magnification. Black mark indicates iNOS expression in the brain; (C-c) Expression of iNOS time of 16-week at a magnification of ×100. Black mark indicates iNOS expression in the brain.

The data in this study are collected from the evaluation of the mice brain tissue preparations are based on an expression of TNF-α after staining imunohistokima by TNF-α monoclonal antibody per 20 field of view with a magnification of ×100. Inspection and calculation expression of TNF-α is observed by looking at the brown color at the core, cytoplasm and cell walls of the cells of brain neurons.

The average value and standard deviation of the first group which is 0 week or no treatment is 29.78 ± 8:42, the second group of the week 8 of treatment is 114.78 ± 8.60, and the third group at 16-week of treatment is 198.89 ± 113.40. The data are then graphed in Figure 2.

The data in this study are collected from the evaluation of the mice brain tissue preparations are based on an i-NOS expression after staining by monoclonal antibody imunohistokima i-NOS per 20 field of view with a magnification of ×100. Inspection and calculation expression i-NOS is observed by looking at the brown color at the core, cytoplasm and cell walls of the cells of brain neurons.

The average value and standard deviation of the first group which is 0 week or no treatment was 99.33 ± 3613, the second group of the 8-week of treatment is 67.44 ± 3.610, and the third group at 16-week of treatment is 32.11 ± 3928. The data are then graphed.

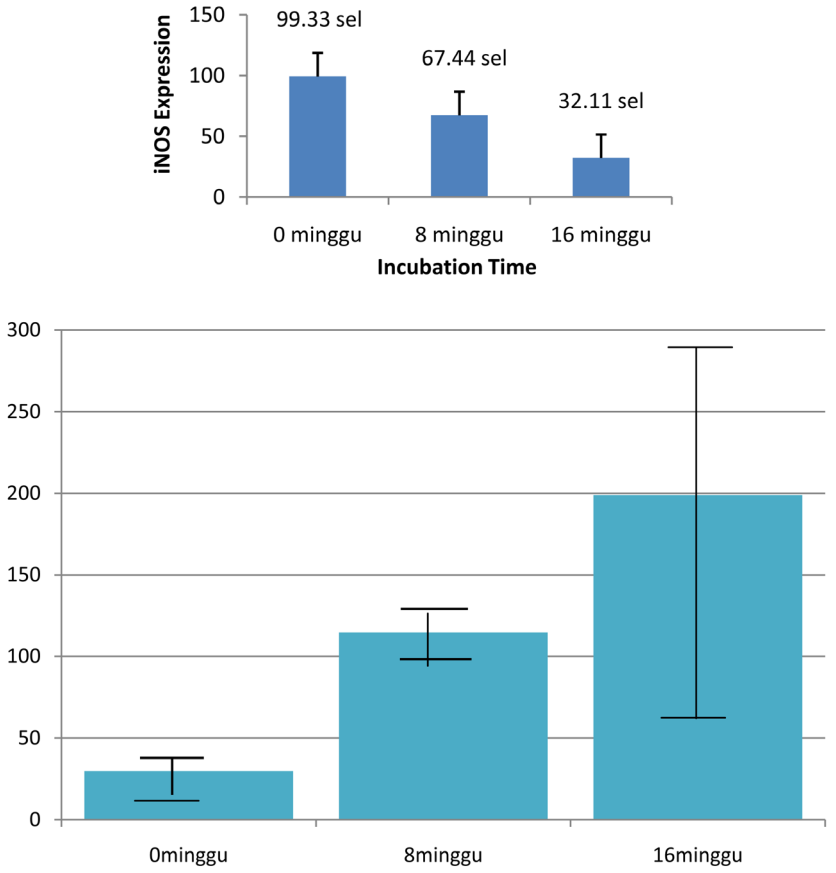


Figure 2. The graphic of TNF-α Average expression.

4. Discussion

Walgreen [4] points out that starting TB disease course of their infection/ primary focus formed 3 - 8 weeks post infection. 1 - 6 the next month can occur miliary TB and TB of the brain. The next 1 - 12 months, will spread to the pleura. Lastly, 3 - 6 years post infesi will spread into the bone TB, including spinal TB although primary complex and focus has faded away. Thus, from this reference could be concluded that the study result is approaching the existing theory thatat the 1 - 6 first months post infection will be TB brain (of course if you do not do therapy), whereas in this study the time used is 8 weeks or 2 months and 16 weeks or 4 months.

Pathogenesis Expression of TNF-*α* in brain cells Mus musculus infected with *Mycobacterium tuberculosis*

Mycobacterium tuberculosis strains H37Rv and CDC 151 has the ability to reconstruct the shape of the cytoskeleton that makes the cells become not round again, blood-brain barrier also changed shape so that the bacteria are able to enter alone or jointly penetrate macrophages. Besides previous studies using brain has also been proven that there is no change in the expression of Vascular Endothelial Chaderin on adherence junction, which means the blood brain barrier is still intact, nothing broken. So it can conclude that turns leukocytes/ macrophages are able to penetrate the blood brain barrier without damaging its structure, namely by diapedesis.

The process that occurs make the activation of tissue macrophages and monocytes infiltration. If the inflammatory process that occurs in the brain, the microglia are activated. In this study, the primary focus of *M. tuberculosis* which are formed in the lungs and then broke out and spread through the blood and lymph circulation that reached the brain as the location of a secondary infection of *M. tuberculosis* is the most common. The entry of *M. tuberculosis* into the subarachnoid space of encouraging local production of T $CD4^+$ so as to differentiate into Th1 (T-helper 1) and Th2 (T-helper 2). Th1 will work to produce IL-12 (interleukin 12) and IFN-g (Interferon g) which will initially provide immunoprotective response against *M. tuberculosis*. However, Th2 produces IL-4 (interleukin 4) and IL-5 (interleukin 5) which serves as a neuroprotective by pressing the excessive Th1 activation and induce more intense inflammatory process. Based on the basic immunology, microglia activation requires the support of certain cytokines, particularly IFN-*γ*. IFN-*γ* produced by NK cells (Natural Killer) and T CD1-restricted lymphocytes before adaptive immunity is activated, but after adaptive immunity is activated, the main source of IFN-*γ* is the Th1. Just as described above after *M. tuberculosis* arrested microglia, the microglia produced multiple cytokines including TNF-*α*. Meanwhile, MAPKs (mitogen-Activated Protein Kinase), ROS (Reactive Oxygen Species), NF-κB (Nuclear Factor Kappa B) is to play its role as an anti-*M. tuberculosis* as well as increase the production of cytokines such as TNF-*α* (Idris, 2007).

When an activation of microglia in the brain *M. tuberculosis* began to be recognized by a TLR (Toll Like Receptor) the originator of inflammatory genes

expressed by microglia cells and other glial cells when infected with *M. tuberculosis*. Conclusions of TLR will activate MyD88-dependent pathway (Myeloid Differentiation Protein 88) and IRAK (IL-1 R-Associated Kinase), activates the transcription factor NF-κB and trigger the production of proinflammatory cytokines, such as IL-12 and TNF-*α*. TNF-*α* will exert more inflammatory cells to get to the site of infection. There are several types of IRAK subfamily, but which plays a major role in the increase of cytokines are IRAK-1 and IRAK-4. Both will phosphorylated and bind with TRAF6 (TNF Receptor Associated Factor 6) through TRAF receptor (TNF Receptor Associated Factor). TRAF6 is separated from the receptor and binds to TAK1, TAB1, and TAB2 in the cell membrane, it will produce NF-κB. The Increasing production of TNF-*α* is also believed will increase the permeability of the blood brain barrier so it is more easily penetrated by macrophages (Sheffield, 1994).

The location and type cells expressing TNF-*α* in the Brain Mus musculus

The location of the dominant expressing TNF-*α* is an area of the brain parenchyma. Why are most common in the brain parenchyma can not be known with certainty why. Another study using rabbits subject also found that expression of the most common in parts of the brain parenchyma but also could not explain the reason [12]. Reason while supposedly because TNF-*α* is produced by microglia and several other glial cells, where these cells are distributed in many parts of the brain parenchyma. So that the expression of TNF-*α* is most prevalent in this area [13]. In this study, the morphology of cells expressing TNF-*α* do not seem like microglia. Morphology microglia cell nucleus is small and round surrounded by many small dendrites. Cells that are visible in the microscope in this study are round or oval and not readily apparent small dendrites. Suspected cell lines are the blood vessels, the arterioles and venules. In this study seems a lot of cell morphology arecolored expressing TNF-*α*. In addition, cytoplasm is a part that should be stained or colored, because TNF-*α* is produced in the cytoplasm, but in this study, the cell nucleus is also brown.

The time takes for Expression of TNF-*α* to appear

There is a clear difference appears in terms of time, if the animal was infected directly on the brain with a secondary infection. Several other studies with animal testing-rabbit showed different reactions to the infection with *M. tuberculosis*. Five days after infection, the expression of TNF-*α* are already visible in the brain parenchyma and TNF-*α* levels are also very high in the cerebrospinal fluid [12]. While in this study, the expression of TNF-*α* appears emerged at week 8 and very high at week 16. Previously, the brain used in this study has been tested immunohistochemistry for protein expression observed mt-38, which is owned by the antigen of *Mycobacterium tuberculosis* , The results are positive and there is an increasing expression of antigens mt-38 from week 8 and week 16. This may happen due to species/strains of *M. tuberculosis* that may be different from other studies. *M. tuberculosis* H37Rv and CDC 1151 types are the most common type attack humans. Because of this type has the

ability invasion and is able to change the structure of the complex of endothelial cells in the human brain. This occurs because of the bumps microvilli at the entrance, which encourages cells to the actin cytoskeleton to rearrange. Besides the gene Rv0986 and Rv0987 of *M. tuberculosis* species has the potential to increase the virulence and adhesion to the host cell. In other studies not mentioned species/strains of *M. tuberculosis* are used. The prevalence of secondary infection of the brain that occurs on the new man in the first year after primary infection occurs [2]. This can be explained by the theory of immunology. It also occurs in human infections of the lungs initially and then get into the bloodstream and lymphatic vessels leading to other organs. This process takes time and depends on the immune status and treatment received. While in animal testing, rabbits, do research Tsenova [12], performed directly infection of the CNS (Central Nervous System), which substantially explains why the expression of TNF-α are quickly emerging.

INOS expression in *Mycobacterium tuberculosis* infection in the brain tissue of mice

Research result in Figure 2 shows that the decreased expression of iNOS occurs if the incubation period of tuberculosis infection of the brain is getting longer. This result can occur because of inflammatory responses in tuberculosis infection of the brain caused by TNF-α as one of the inflammatory factors. Microorganisms have developed several mechanisms to survive in their host environment. These include competition with their host cells for the acquisition of metal and endurance to become defense systems such as NO (Nitric Oxide), cytotoxic weapons produced by macrophages. In eukaryotic cells, metabolic NO produced by NOS (NO synthase) that consists of L-Arginine, O2 (Oxygen Molecular), and NADPH (Nicotinamide adenine Dinucleotide, Reduced). Inside the macrophages, the inducible NO synthase (iNOS) is produced after activation of endotoxin or cytokines and produce copious amounts of NO to execute or inhibit the growth of microorganisms that attack or neoplastic tissue. Although iNOS is identified and marked in macrophages, itcomes in many cell types including endothelial cells, fibroblasts, smooth muscle cells of blood vessels and cardiac myocytes. iNOS catalytic activity is governed by the availability of substrates such as Calm (Calmodulin), L-Arginine, and cofactor, NADPH and tetrahydrobiopterin. iNOS utilize oxygen and electrons from NADPH to oxidize substrates L-Arginine to OH-L-Arginine, which is then oxidized to NO and L-citrulline. NO and superoxide (O2) is a radical effectors of the innate immune system that can directly inhibit the replication of the pathogen [14].

Components of bacterial cell walls trigger the innate immune signals, which caused the expression of iNOS. Lipopolisakharida (LPS), which is a wall component of Gram-negative, binds LBP (LPS-Binding Protein), which gives LPS to CD14. TLR4 (Toll -like Receptor-4) in conjunction with the MD2 small extracellular protein interacts with CD14-LPS complex, and then activate a cascade of intracellular signals through an adapter that includes IRAK (Interleukin-1 Receptor-Associated Kinase) and MyD88 (Myeloid Differentiation

Primary Response Gene-88). This adapter is in turn activates downstream molecules including TRAF6 (TNF Receptor-Associated Factor-6), Tab1 (TAK1-Binding Protein-1) and p38. LPS activation of TLR4 causes phosphorylation of IKK (inhibitor of kappaB kinase), which phosphorylates I-kappaB and released the transcription factor NF-kB (Nuclear Factor-kappaB). NF-kB translocate from the cytoplasm to the nucleus, where it interacts with the elements kappa B in iNOS, triggeringiNOS transcription. Cytokines are released from the infected host cells also activates the production of NO, including TNF-α (Tumor Necrosis Factor-Alpha) and IL-1β (Interleukin-1 beta). IFN-γ (Interferon-Gamma) interacts with IFNR1 (Interferon Receptor-1) and IFNR2 complex, which activates the kinase of the JAK (Janus kinase) family and STAT (Signal Transducer and Activator from Transcription) pathway that leads to the synthesis of transcription factors IRF1 (interferon Response factor-1) and the stimulation of iNOS mRNA transcription. IFN-γ also provides a synergistic boost to LPS induction of iNOS transcription for IRF1 interacts with NF-kβ, changing the conformation of iNOS promoter. Other transcription factors, including STAT1α and HIF1 (Hypoxia Inducible Factor-1) also controls the expression of iNOS [15].

There should be an increasing of iNOS expression in the longer incubation period. Therefore, iNOS that exists in the brain could initially increase because the inflammatory process and the release of cytokines-pro-inflammatory cytokines. Then after a long time that bacteria exist in the brain, the immune system is in the bacteria makes the activation of iNOS, so the number of iNOS in the brain is decreased [16].

5. Conclusion

From the results of this study, this can be concluded that there is an increasing expression of TNF-α and decreasing expression of iNOS in the brain tissue of mice that are infected with *Mycobacterium tuberculosis* for 8 and 16 weeks.

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