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The role of fluoxetine on macrophage function in chronic pain (Experimental study in Balb/c mice)

Dwi Pudjonarko*, Edi Dharmana†, OS Hartanto‡

*Department of Neurology, Faculty of Medicine, Diponegoro University-Indonesia†Department of Parasitology, Faculty of Medicine, Diponegoro University-Indonesia‡Department of Neurology, Faculty of Medicine, Sebelas Maret University-Indonesia

Email: dwipudjonarko@undip.ac.id

Abstract *- Chronic pain raises stress conditions such as depression that can lower the cellular immunity. Fluoxetine is an antidepressant used as an adjuvant in pain management but no one has been linked it with the body immune system. The objectives of this research were to proof the benefits of fluoxetine in preventing degradation of macrophage function in chronic pain by measuring the macrophage phagocytic index , macrophage NO levels and the liver bacterial count in BALB/c mice infected with Listeria Monocytogenes.A Post Test - Only Control Group Design was conducted using 28 male mice strain BALB /c, age 8-10 weeks. The control group (C), mice got the same standard feed as the other groups. Chronic pain group (P), mice were injected with 20µL intraplantar CFA on day-1. Pain + fluoxetine early group (PFE) were treated with P + fluoxetine 5 mg / kg ip day-1, the 4th, the 7th and the 10th, while the Pain + fluoxetine late group (PFL) were treated with P + fluoxetine 5 mg / kg ip on day 7th and 10th. All mice were injected with 104 live Listeria monocytogenes iv on day 8th. Termination was performed on day 13th. Differences within groups were analyzed using One-way ANOVA and Kruskall Wallis, whereas the correlation of variables were analyzed using Pearson's product moment. The experimental results showed that The macrophage phagocytic index and NO macrophage level (pg/mL) in PFE group(2,24±1,013; 0,24±0,239) was higher than than P group (1,68±0,920; 0,21±0,263) and there was no different in the macrophage phagocytic index of PFE group compared to C group (p=0,583; p=0,805). In PFL group (4,32±1,459; 0,54±0,294) the macrophage phagocytic index as well as NO macrophage level (pg/mL) was higher than P group (1,68±0,920; 0,21±0,263) with p=0,002; p=0,017. P group Bacterial count (log cfu/gram) (2,30±0,849) was significantly higher than C group(1,15±0,223) (p=0,007), while PFE group bacterial count (1,96±0,653) and PFL group bacterial count (1,84±0,403) compared to C (1,15± 0,223) was not significantly different (p=0,093; p=0,220). Correlation found between macrophage phagocytic index and macrophage NO (r=0,515, p=0,005).Macrophage phagocytic index and macrophage NO showed no correlation with bacterial count (r=-0,051, p=0,798; r=-0,071, p=0,719).It can be concluded that fluoxetine significantly incerases macrophage phagocytosis index and macrophages NO level in mice with chronic pain, on the other hand fluoxetine decreases liver bacterial count . There is a positive correlation between macrophage phagocytosis index and macrophages NO level, while no correlation observed among two variables with mice liver bacterial count in chronic pain.*

Keywords—*Chronic pain, fluoxetine, depression, phagocytic index, NO, bacterial count.*

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I. INTRODUCTION

Chronic pain, a condition related to stress and depression, is the most common psychiatric problem as reported by the Survey of Chronic Pain in Europe as well as by American Chronic Pain Association.[1,2] Study report shows there is a molecular mechanism that connects depression to stress. For example, Cortisol elevation caused by stress increases serotonin uptake both in the stimulated and the unstimulated nerves, this findings is consistent with the symptoms of depression.[3] Chronic response to stress involves the Hypothalamic-Pituitary-Adrenal Axis (HPA Axis) result in huge secretion of glucocorticoid which will be bound to the immune system receptors and leads to cellular immunity suppression. Depression correlates with high resistance to molecules normally work to cut the inflammation cascade. Failure index of inflammatory regulation underlines the morbidity and mortality related to depression.[4]

Antidepressant used as adjuvant therapy in the management of chronic pain may alleviate pain but none has correlated it with body immune system. Studies show that fluoxetine has been used and brings good efficacy in releaving pain and depression. Fluoxetine is a central selective serotonin reuptake inhibitor. Studies prove that Fluoxetine as a monotherapy or in combination is beneficial to pain management.[5] Minimal side effect compared to other anti-depressant particularly tricyclic group, low dose therapy using fluoxetine and can also be easily increase to reach the desired analgesic effect. Fluoxetine has been widely

used in the form of capsules, tablets or solutions. Study by Federal Aviation Administration (FAA) recommended that pilots receiving fluoxetine would still be allowed tofly.[6] Antipsychotic and antidepressant usage is at the top of the central nervous system 2011 drug prescription list in America, among those drugs Fluoxetine was at the fifth rank in prescription numbers of 24,5 millions.[7] Fluoxetine is also used as pain adjuvant therapy listed in the first national consensus of diagnostic and management of pain Perdossi 2011.[8]

This study explained the effect of fluoxetine on macrophage function in Chronic pain. In chronic pain conduction, subsequent stress modifies body immune system including innate and adaptive immunity. Macrophage holds important role in the observation of fluoxetine's effect to innate and adaptive immunity for its involvement in both systems. Macrophage function response observed by inducting individual infection by using Listeria monocytogenes. Macrophage function response was measured using macrophage phagocytic index and macrophage nitric oxide (NO) concentration. Interaction result of various body immunity was determined by calculating the bacterial count.

II. MATERIAL AND METHOD

A Post Test - Only Control Group Design.[9] was conducted using 28 male mice strain BALB /c, age 8-10 weeks. The control group (C), mice got the same standard feed as the other groups. 20 µL intraplantar CFA were injected in Chronic pain group (P) mice on day-1. Pain + fluoxetine early group (PFE) mice were treated with P + fluoxetine 5 mg / kg ip on day-1, the 4^{th}, the 7^{th} and the 10^{th}, while the Pain + fluoxetine late group (PFL) mice were treated with P + fluoxetine 5 mg / kg ip on day 7^{th} and 10^{th}. All mice were injected with 10^4 live Listeria monocytogenes iv on day 8^{th}. Termination performed on day 13^{th}. the macrophage phagocytic index, macrophage NO level as well as bacterial count from BALB/c mice liver were all measured.

Mice liver were obtained aseptically for the bacterial count in cfu/gram. Measurement were performed by counting the bacterial colony growth in blood agar+tellurite media after liver smear incubation for 24 hours. Liver smear were performed with grinder and staged dilution using NaCl 0,9% to be planted in the blood agar+tellurite media. Colony growth were calculated using colony counter.[10]

Mice peritoneal macrophage were isolated then incubated in the temperature of 37°C, with 5% level of CO_2 for 2 hours in plate 96 wells to measure the macrophage NO level. After medium change, the macrophage was cultured in 37 °C incubator, with 5% level of CO_2 for 24 hours. Macrophage NO level measurement were performed using Griess method and read by Elisa Microplate Reader 680 XR in 450 nm wave.[11]

The macrophage suspension was cultured for macrophage phagocytic index in 24 well microplates which had been applied with round coverslips in 5% CO_2 incubator with the temperature of 37°C for 30 minutes then added on complete medium and was incubated there for 2 hours. After being cleansed with Roswell park memorial institute medium (RPMI), complete mediums added and then was incubated for 24 hours. After being re-cleansed with RPMI, 200 µl/ well latex suspensions of 2,5 x 10^7/ml concentration were added and then incubated in 5% CO_2 incubator with the temperature of 37°C for 60 minutes. Cover slips were dried in room temperature, fixated with absolute methanol, and then dyed with Giemsa 20% for 30 minutes. After being cleansed with aquadest, cover slips were removed from culture well and dried in room temperature. The amount of latex particle phagocytized by mice peritoneal macrophage was calculated by using the light microscope.

The result data was processed and presented in graphics. Phagocytic index shows normal distribution after normality test using Saphiro Wilk test (p>0,05), the bacterial count variable data was transformed into log10 to fulfill the normality assumption (p>0,05). Afterwards parametric analysis was performed using One Way Anova to determine the difference among 4 treatment groups. The difference between every treatment groups were analyzed using Bonferroni Post Hoc Test. The Post Hoc Test was selected because the variance fulfills the homogenity assumption (Levene's test p>0,05). For Nitric Oxide variable, macrophage did not fulfill the normality assumption (p<0,05), so Kruskall-Wallis test was performed to observe the difference between four treatment groups. The difference were all futher analyzed by Mann-Whitney posteriority test. To observe the correlation of each variables Pearson's product moment bivariate correlation test were performed. All statistical analysis were processed by computer using the SPSS. Level of Significant in this study is when the variable analyzed had p <0,05. This study acquired the ethical clearance from the Health Study Ethical Comission of Undip medical faculty and Kariadi General hospital.

III. RESULT AND DISCUSSION

The macrophage phagocytic index and NO macrophage level (pg/mL) in PFE mice (2,24±1,013; 0,24±0,239) was higher than than in P mice (1,68±0,920; 0,21±0,263). There was no different in macrophage phagocytic index and NO macrophage level (pg/mL) in PFE mice compared to C mice (p=0,583; p=0,805) while in PFL mice the macrophage phagocytic index and NO macrophage level (4,32±1,459; 0,54±0,294) was higher than in P mice (1,68±0,920; 0,21±0,263) with p=0,002; p=0,017. P mice Bacterial count (log cfu/gram) (2,30±0,849) was significantly higher than C mice (1,15±0,223) (p=0,007), while bacterial count for PFE (1,96±0,653) and PFL (1,84±0,403) compared to C (1,15± 0,223) wasnot different (p=0,093; p=0,220). Correlation found between macrophage phagocytic index and macrophage NO (r=0,515, p=0,005). Macrophage phagocytic index and macrophage NO show no correlation with bacterial count (r=-0,051, p=0,798; r=-0,071, p=0,719).

Nociceptor sensitization with Complete freund's adjuvant (CFA) produces action potential transmitted by A-δ and or C fiber. Both nerve fibers end in superficial layer of dorsal horn and continued by gelatinous substance transmission cell towards thalamus, the important part for impulse integration in brain.[12-14] Chronic pain is a stress condition which causes elimination of glucocorticoid negative feedback in the Hypothalamic-Pituitary-Adrenal Axis (HPA Axis). Chronic pain also causes diminished monoaminergic tone in monoaminergic depletion response induced by glucocorticoid, this causes inhibitory impulse to decrease. Aside from that, chronic pain causes downregulation of glucocorticoid modulation and this scheme leads to depressed mood.[15]

Both in animal and human studies, survival mechanism during chronic stress is not only by increasing corticosteroid hormon secretion maintained by negative feedback control, but

also by releasing additional corticosteroid response related to stressor. Each of adaptive responses involves HPA Axis.[16] However, continous ongoing and prolonged stress may interrupt the HPA axis, oneof which is the disruption of negative feedback mechanism. Physiologic changes in countering stressor will help individulas to fight the stressor. Chronic response to stress involves the HPA axis and symphatetic-adrenal-medullary axis (SAM axis) by chronic ongoing production of glucocorticoid and cathecolamine as final result. Chronic stress may flatten circadian rhythm of glucocorticoid production and increases ultradian frequency.[17,18] This shows involvement of biological clock in diseases related to stress. Glucocorticoid is not only resulted from circadian rhythm of adrenal gland, central and peripheral setting, but also may influence the circadian rhythm itself and interacts with other biological clock in circadian rhythm physiologically.[19]

Chronic pain effects on body immunity using several mechanisms as demonstrated in previous studies described above observed from the low level of macrophage phacoytic index (1,68±0,920) and macrophage NO 0,21±0,263 pg/mL in chronic pain group (P) (Fig 1). This result also supported by previous theories and studies that glucocorticoid reseptor expressed in various immune cells will bind cortisol alongside NF-□B function and regulates immune cells cytokine production. Adrenergic receptor binds epinephrine and norepinephrine while activating cAMP response and induce genetic transcription which code various cytokines. Genetic expression changes mediated by glucocorticoid hormone while cathecolamines may disrupt the immune system regulation.[20]

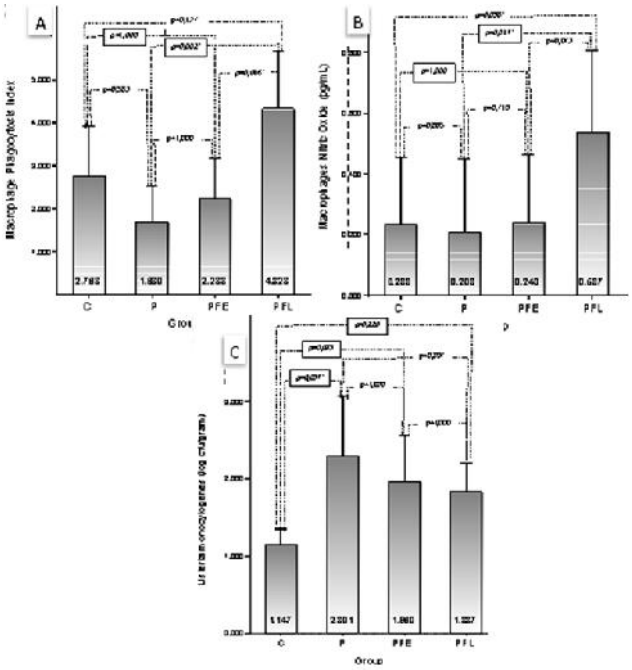

Figure 1.The figures show variability within group of study and between group differences: macrophages phagocytosis index (A), macrophages NO (B) and bacterial count in log cfu/gram (C).

Lymphocyte, monocyte or macrophage and granulocytes possess receptors for neuroendocrine products from HPA axis and SAM, like cortisol and cathecolamines which cause changes in cellular communication, proliferation, cytokine secretion, antibody production and cytolytic.[21] Marshall et al found that invitro cathecolamine administration in peripheral blood leukocytes (PBLs) may suppress IL-2 synthesis and increases IL-10 production. This can cause shifting of T helper CD4+phenotype from TH1 which has functions in cellular immunity to TH2 which plays roles in antibody production. Cytokine TH1 synthesis decrease interferon-□ (IFN-□) while cytokine TH2 increase IL-10 production. Then it is believed that stress can diminish TH1 cytokine which disrupts the cellular imnunity.[22]

Monoaminergic neurons in the pons, in normal condition provides descending inhibition to the spinal cord as nociceptive transmission "brake". Ascending fibers through spinothalamic tract springs out neurons to thalamus and periaqueductal grey matter (PAG) which possesses direct or indirect interaction with the lymbic system and monoaminergic nucleus. Periaqueductal grey matter (PAG) is an important nociceptive regulator site, where cognitive and emotional sensation from thalamus or anterior cortex area meets vegetative aspect from hypothalamus.[23-24] Although PAG is not connected directly to spinal cord dorsal horn through pons and medulla,23 it is PAG which starts descending and ascending inhibition to decrease pain. Concurrent with the statement, PAG stimulation shows deep antinociceptive,[25-26]where electrolytic lesion decrease morphine analgesic effect.[27] This data shows that this area of the brain is the main site for opioids to produce analgetic effect. Dorsal raphe nuclei (DRN) anatomically or functionally connected to PAG. PAG regulates pain information by activating DRN in rostral ventromedial medulla which causes 5-HT release in dorsal horn to inhibit the sensory stimulus.[28] Monoaminergic neurons project to various brain regions and involved in pain, mood, affective dimension of pain regulation. Ascending projection dysfunction from Dorsal raphe nuclei (DRN), Locus coeruleus (LC) and Ventral tegmental area (VTA) play role in classic depression symptoms. Cortical structure activation in human can be shown in imaging study of pain response.[29-33]

Fluoxetine is a Serotonin selective reuptake inhibitors (SSRIs) which inhbits 5-HT transporter (5-HTTT). So that it increases monoamine extracellular concentration in synaps and prolongs work duration in post-synaptic level. In the presynaptic level, 5-HTT inhibits in serotoninergic cell body leading to accumulation of 5-HT around 5-HT1A autoreceptors or α2 in dorsal raphe (DR). This will increase DR 5-HT neuron dose dependent induced resulted from neuronal elements activation produced by negative feedback.[34] In the nerve terminals, 5-HT accumulation also appears to be the aswer to 5-HTTT inactivation by SSRIs and monoamine extracellular level increase observed by microdialysis of various brain regions.[35] Fluoxetine also increases endogenic opioid receptors density besides its clear effect in mice brain serotonin.[36]

This study proves that early fluoxetine administration in chronic pain condition (PFA) increases macrophage phagocytic index 2,24±1,013 and macrophage NO 0,24±0,239 pg/mL. The increase of macrophage phagocytic index 4,32±1,459 and macrophage NO 0,54±0,294 pg/mL also found in late fluoxetine administration (PFL). Increasing macrophages response such as macrophage phagocytic index and macrophage NO of PFA is comparable to control (C) (p=1,000; p=1,000). Onthe other hand the increasing macrophages response inPFL is significantlyhigher compared to chronic pain group (P) (p=0,002; p=0,017)(Fig 1). This fact shows that fluoxetine may influence macrophage response. This result is supported by previous study that Serotonin transporter (SERT) inactivation related to 5-HT

extracellular level increase is a relevant strategy to alleviate pain, this is supported by observation that analgesics induced by morphine was strengthened in deficient 5-HTT mice.[37] Although spontaneous pain sensitivity in this mutant mice was not change compared to non-mutants,[37] proof from various sources show 5-HTT blockade by SSRIs alleviate acute pain in hot plate and tail flick test in rats[38-49] and mice.[50-54] Fluoxetine administration in chronic pain will instantly increase 5-HT level needed in pain modulation in Periaqueductal gray (PAG) as the meeting point of cognitive sensation and emotion from thalamus or anterior cortex area with vegetative aspect from hypothalamus.[24,25] Elimination of negative glucocorticoid feedback in HPA axis causes positive regulationto axis and downeregulationof glucocorticoid receptors in brain and peripher caused by chronic pain immediately repaired by late fluoxetine administration so that macrophage function response increases significantly compared to P group. This data shows that fluoxetine can function as macrophage activity stimulator if administered slowly in chronic pain. Early fluoxetine administration in chronic pain will immediately increase 5-HT so that it prevents the elimination of negative glucocorticoid feedback in HPA axis. Immune response in early fluoxetine resembles immune response in normal condition because the body immunity has not been disrupted as late fluoxetine administration, so that the early fluoxetine functions as an macrophage activity regulator.

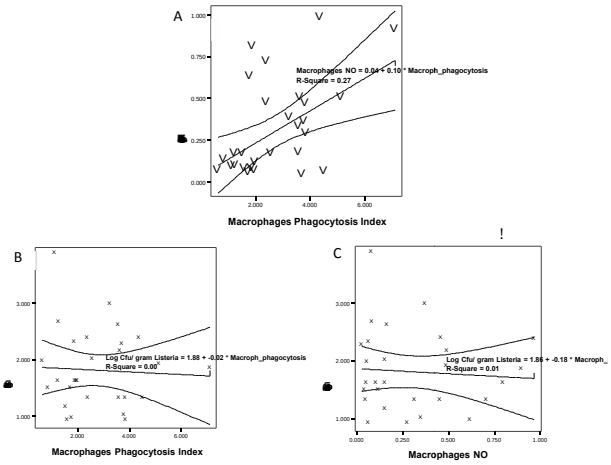

Figure 2. The figure shows positive correlation between macrophages phagocytosis index and macrophages NO (A), negative correlation between macrophages phagocytosis index and bacterial count (log cfu/gram) (B) also macrophages NO and bacterial count (log cfu/gram) (C).

Ability to eliminate bacteria is an interaction of various body immunity in countering foreign pathogen. Listeria monocytogenesis was used in this study with liver and spleen as the main infection site. Figure 3 shows collonies of Listeria monocytogenes in blood-tellurite agar).Listeria monocytogenes bacterial count is highest in the chronic pain group (P) i.e 2,30±0,849 log cfu/ gram, on the contrary the lowest bacterial count was found in the control group (C) 1,15±0,223 log cfu/ gram with significant difference (p=0,007). Highest bacterial count in chronic pain shows body immune system failure to eliminate bacterias. Fluoxetine administration in chronic pain is proven both in group receiving early fluoxetine (PFE) and in late fluoxetine (PFL) compared to chronic pain (P) group. Bacterial count in PFE and PFL are equal to control group (C) (p=0,093 and p=0,220)(Fig 1). This study shows that both early and late fluoxetine administration in chronic pain may suppress bacterial growth to the same level as mice with no chronic pain. This proves that Fluoxetine administration influences mice liver bacterial count decrease in chronic pain.

Figure 3. The figure shows collonies of Listeria monocytogenes in blood-tellurite agar.

This study shows strong positive correlation between macrophage phagocytic index with macrophage NO level (r=0,515; p=0,005)(Fig 2), so it is well demonstrated that besides functioning as antidepressant and anti-nociceptive, fluoxetine also functions as macrophage activity modulator in chronic pain. Macrophage function has weak negative correlation towards liver bacterial count both for macrophage phagocytic index (r=-0,051; p=0,798)and macrophage NO (r=-0,071; p=0,719)(Fig 2).

Previous studies showed that Listeria monocytogenes main site of infection is in liver and spleen. Bacterias found in the Antigen presenting cell (APC) cytosol and hepatocyte. Listeria monocytogenes may spread from cell to cell without leaving the intracellular compartment, this has been the reason behind the need of T CD8+cells to cleanse the bacteria and improve immunologic protection.[55]Early bacterial growth in first day after infection was controlled by natural immunity cell like neutrophils, Natural killer (NK) cells and macrophages.[56-58] One of the main target of Listeria monocytogenes infection is monocytes which is an integral part of natural immunity also adaptive immunity effector. T cell interaction with monocytes is very important for host survival against Listeria monocytogenes. T cells don't eliminate Listeria monocytogenes, instead only lysis infected cells[59] in the process of releasing the living bacteria.[60] The main function of T cells in the defense mechanism againts Listeria monocytogenes is to help increasing monocyte response. Previous studies in the late decades had clearly identified susceptibility to primary infection of Listeria monocytogeneshad a correlation with monocyte function differentiation based on age.[61,62]

Negative correlation of macrophage phagocytic index to bacterial count in this study is relevant to previous studies which indicated not to give up to Listeria monocytogenes infection, as phagocyte cells like monocytes/ macrophages has to trap and eliminate captured bacterias immediately.[60-61,63-64] Response arises since Listeria monocytogenes was in contact with monocytes in a process to eliminate the bacteria.[65]Listeria monocytogenes enter monocytes/ macrophages through phagocytosis process. The process has been started after Listeria bound to complement alongside internalin listeria protein B and lipoteichoic lipid, functioning

as ligand for complement receptors recognize by scavenger receptor in phagocytosis process.[66]The ability of macrophage phagocytosis called latex phagocytoted by macrophages showed in Figure 4.

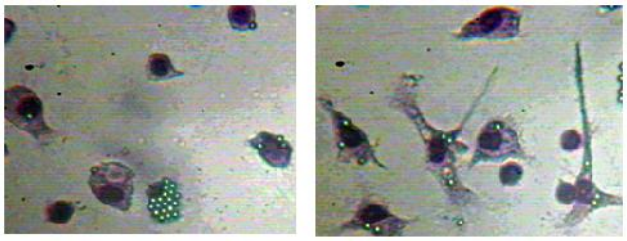

Figure 4. The figures show latex phagocytoted by macrophages NO.

Negative correlation between macrophage NO level towards bacterial count in this study is supported by previous study showing that after tightly bond to scavenger receptors or complement receptors, bacteria will be inserted into phagosome. Phagosome will undergo serial transformation through a series of interaction with endocytosis pathway sub compartment which ends at phagolisosome maturity. During the process, consumed bacterias are subject to microbisidal effector dependent to pH including ROS and RNS (produces NO), iron scavenger dan exporter,lactoferrin dan natural resistance-associated macrophage protein1 (NRAMP1), anctimicrobial peptides and proteins (such asdefensins, cathelicidins, lisozyme, carbohydrate hydrolase, fosfolipase and various protease dan peptidase) which perforate and destroy the bacteria.[67]

Natural immune system effector has the ability to control infection only at limited time in mice, just as mice Severe combined immunodeficiency (SCID) upholds the infection[68] but the immune system can not perform sterilization. Natural immune sytem has to activate adaptive immunity to completely end Listeria monocytogenes. The adaptive immune response peak for Listeria monocytogenes in mice is about one week after infection. Previous studies with mice infection model had shown that T cells response is an integral part in Listeria monocytogenes elimination while humoral response only plays small part.[69-70] Antigen presentation through Major histocompatibility complex (MHC) I and MHC II pathway are mainly mediated by dendritic cells, activating T $CD8^+$ dan $CD4^+$cells specific to Listeria antigens.,T cytotoxic $CD8^+$ cell is more important than $CD4^+$in controlling listeriosis,[71] although it is still unclear and several mechanisms of protection potency related to it is still on debate. Natural cytokine IL-12p70 is important to T $CD8^+$cells expansion phase,[72] IL-12p70 arises to activate T cell to be a full effector needed to control Listeria monocytogenes infection. T $CD4^+$cell role needs IFN-γ production by these cells and involves macrophage feedback activation.[73] T $CD4^+$cell presence is important to early phase of priming T $CD8^+$cell and for long term memory.[69,74-75] T γδ murine cell also known to play part in IFN-γ production during infection.[76]

The weak correlation of macrophage phagocytic index or macrophage NO to liver bacterial count show the complexity of immune response against Listeria monocytogenes. Still There are a lot of factors contributing to killing mechanism against Listeria monocytogenes that have not been studied yet. The weakness of this study is that there are some other factors from the bacterias and other immunologic parameters in the phagocytosis process particularly for Listeria monocytogenes such as the role of neutrophil, NK cells, lymphocyt γ-interferon, lymphocyt proliferation index and T $CD8^+$.Inphagocytosis process against Listeria monocytogenes seems that NO is not the only dominant factors, although it is mentioned in the reference that RNI is more dominant in mice. One of the chance of elimination can be caused by the more influential ROS. Interaction between NO and ROS is very complex. Sometimes both molecules inactivate each other, but through other mechanism sometimes combine to form the highly reactive peroxynitrite.[77]

Macrophage activity increase marked by high macrophage phagocytic index and macrophage NO in chronic pain receiving fluoxetine has been proven in this study. This increase is strengthened by the no difference between group receiving fluoxetine compared to control furthermore chronic pain group shows bacterial count significantly higher compared to control. Fluoxetine administered early in chronic pain shows insignificanty different macrophage activity compared to control either in macrophage phagocytic index variables, or macrophage NO or bacterial count. This shows that fluoxetine functions as an macrophage activity regulator which influences body cellular immunity to return the function back to the original condition as the control group. Fluoxetine administered slowly in chronic pain also shows insignificant difference compared to control either in macrophage phagoctic index or macrophage NO. However in comparison with chronic pain group, it is clear that administration of fluoxetine result in significantly higher score of macrophage phagoctic index and macrophage NO . This shows that fluoxetine administered slowly in chronic pain functions as an macrophage activity stimulator which influences body cellular immunity by increasing its function significantly in chronic pain. Fluoxetine administration in early chronic pain functions as an macrophage activity regulator, while late administration functions as an macrophage activity stimulator. Besides functioning as antidepressant and analgetic mentioned in previous reference, this study adds fluoxetinefunction as an macrophage activity modulator in chronic pain.

IV.CONLUSIONS

Fluoxetine increases macrophage phagocytic index and macrophage nitric oxide concentration in mice with chronic pain, on the other hand fluoxetine decreases liver bacterial count . There is a positive correlation betweeen macrophage phagocytic index and macrophage NO concentration, while weak negative correlation occurs among two variables with mice liver bacterial count in chronic pain. This study suggests fluoxetine administration in early chronic pain may functions as macrophage activity regulator. The study result provides an alternative that fluoxetinein chronic pain may be administered early or late. There are still possibilities for thids study to be developed further to determine other immune system role (neutrophil, NK cells, lymphocyt γ-interferon , lymphocyt proliferation index and T CD8+ cells), while ROI can be observed to describe the killing mechanism.

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