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Pain Stimulated by Electric Foot Shock to Liver and Spleen Microscopic Immunological Response Features (BALB/c Mice Experimental Study)[#]

Dwi Pudjonarko *, M. Naharuddin Jenie *, Udadi Sadhana **

ABSTRACT

Background: *Pain affects immune system by decreasing type I-immune response that might be seen on microscopic features of liver and spleen. The objective of the study is to prove the effect of pain to immune response that confirmed by liver microabscess formation, hepatocytes destruction and spleen multinucleated giant cells of Balb/c mice suffered from pain.*

Method: *This study adapts Laboratory Experimental and Post-Test Only Control Group Design. The samples were 12 female BALB/c mice (average weight 21.88 (SD=1.75) grams and divided into two groups. The control group (C) received no other additional treatment. The Pain (P) group received pain stimulated by Electric Foot Shock (EFS) 1-3 mA at day 12th to 21st. All groups were intravenously injected with 10^4 live L. monocytogenes at day 21st and sacrificed at day 26th by chloroform anaesthesia. Then, liver microabscess formation, hepatocytes destruction and spleen multinucleated giant cells were counted. Data were analyzed by independent t-test (significant if $p<0.05$).*

Result: *There were significant differences in the liver microabscess formation, hepatocytes destruction and spleen multinucleated giant cells ($p<0.05$) between the groups. The number of liver microabscess formation and hepatocytes destruction in the P group were higher than C group. The number of spleen multinucleated giant cells in the P group were lower than C group.*

Conclusion: *Pain has an immunosuppressive effect not only on high liver microabscess formation and hepatocytes destruction, but also low spleen multinucleated giant cells.*

Keywords: *Pain, electric foot shock, macrophages, microabscess, hepatocytes, spleen multinucleated giant cells*

ABSTRAK

Pengaruh nyeri yang distimulasi electric foot shock terhadap gambaran mikroskopis respon imunologis di hepar dan lien. (Studi eksperimental pada mencit Balb/c).

Latar belakang: *Nyeri dapat mempengaruhi imunitas tubuh dengan menurunkan produksi sitokin tipe 1 yang kemungkinan akan mempengaruhi gambaran mikroskopis respon imunologis baik di hepar maupun lien. Penelitian ini bertujuan untuk membuktikan penurunan imunitas seluler yang dilihat dari mikroabses dan kerusakan hepatosit pada hepar serta sel datia lien mencit BALB/c yang mendapatkan stimulasi nyeri dengan electric foot shock (EFS).*

Metode: *Penelitian ini merupakan penelitian eksperimental laboratorik, dengan pendekatan the post test–only control group design yang menggunakan 12 ekor mencit betina strain BALB/c, umur 6-8 minggu dan rerata barat badan 21,88 (SD=1,75) gram. Sampel dibagi dalam 2 kelompok dan mendapatkan makanan standar. Pada kelompok Kontrol (K), mencit tidak mendapatkan perlakuan, sedangkan kelompok Nyeri (N), mencit mendapat sensasi nyeri menggunakan EFS mulai hari ke-12 sampai 21. Pada hari ke-21, semua mencit disuntik 10^4 listeria monocytogenes hidup iv. Dilakukan terminasi mencit pada hari ke-26 untuk dilakukan penghitungan mikroabses dan kerusakan hepatosit pada hepar serta sel datia lien. Dilakukan uji beda antar kelompok perlakuan dengan independent t-test. Perbedaan dinyatakan bermakna bila didapatkan nilai $p<0,05$.*

Hasil: *Terdapat perbedaan yang bermakna pada jumlah mikroabses dan kerusakan hepatosit pada hepar serta sel datia lien pada mencit yang distimulasi nyeri dengan EFS dibandingkan kontrol ($p<0,05$). Stimulasi nyeri dengan EFS menyebabkan mikroabses dan kerusakan hepatosit pada hepar lebih tinggi terhadap kontrol, sedangkan sel datia pada lien jumlahnya lebih rendah bila dibandingkan kontrol.*

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* *Lecturer in Departement of Neurology, Medical Faculty Diponegoro University/Dr. Kariadi General Hospital Semarang-Indonesia*

** *Lecturer in Departement of Pathology Anatomy, Medical Faculty Diponegoro University/Dr. Kariadi General Hospital Semarang-Indonesia*

Simpulan: *Stimulasi nyeri dengan electric foot shock menurunkan imunitas seluler dilihat pada mikroabses dan kerusakan hepatosit pada hepar yang tinggi serta sel datia lien yang rendah.*

INTRODUCTION

Animal models to determine the possible mechanism and evaluate therapeutic intervention of pain have frequently been done. These models are often used to predict the potency and efficacy of pharmacological agents that work against pain in human.[1] *Electric foot shock* (EFS) method has much been used for research, especially using animal models. The animals are placed to receive direct electric current. Both Zhang *et al* and Tsuji *et al* reported that direct contact with electric current in animal models could cause stress and pain, especially with electric current 1-3 mA.[2,3,4] Our previous study showed that pain provocated by 1-3 mA *electric foot shock* could reduce lymphocyte proliferation in BALB/c mice.[5] It also could decrease macrophage killing power, that could be seen from the low NO concentration in macrophages. This decrease of killing power was confirmed by bacterial liver count which showed larger amount of bacterias than non pain-control group.[6]

In infection induced-experiment, *listeria monocytogenes* has much been used as model to study intracellular bacterial infection. This bacteria can live inside macrophage and can survive against macrophage bactericidal mechanism. It makes macrophage as the main defense mechanism agains this organism.[7,8] Growth of *listeria monocytogenes* is affected by its ability to survive and grow inside the host macrophage. But, *in vivo* this bacteria can enter other host cells beside macrophage. Hepatocyte is also a good place for this organism to grow. That is why this bacteria should be moved out from hepatocytes before it can be destroyed by activated macrophages. The moving out process from hepatocyte is done by destruction of these cells by leucocytes that accumulate surround the infected area and do the extracellular degranulation.[9]

The macrophages inside liver are called kupffer cells, which are well-known as professional phagocytic cells, will phagocyte *listeria monocytogenes* that penetrance into liver after infection.[10] These kupffer cells have great phagocytic ability, Husadha (1996) told that these cells could clear until 99% of bacterias inside the portal vein before the blood spread through all of liver sinusoids, and also produce immunoglobulin that plays role in humoral immune system.[10] During the early stage of listeriosis, neutrophyls and macropahges migrate to spleen and liver, to form microabscess with special features. Neutophyls play important role in controlling acute phase and mediating destruction of infected hepatocytes *in vivo*. Products released by activated macrophages and neutrophyls can damage normal tissue. If the immune response is adequate, the damage is usually temporary and reversible, because the activated macrophages will also induce tissue repair by secreting *growth factor* that stimulates fibroblast proliferation, collagen synthesis, and angiogenesis.[11]

According to the mechanisms above, this experimental study was done to determine whether pain would cause any microscopical appearance change of immunological respon to pain stimulated by EFS both in liver and spleen. Immunological response in liver was observed by microabscess formation and hepatocyte destructions, whereas in spleen was observed by multinucleated giant cells count. This study is done using BALB/c mice.

METHODS

This was a laboratoric experimental study, using *the post test–only control group design* and animal models The samples were 12 female BALB/c mice, age 6-8 weeks, with average weight (*SB=1.75*) grams, obtained from Veterinaria Farma Center Surabaya. The samples were divided into 2 groups with *completely randomized design*. All mice received standar diet. The Control group (C), the mice received no treatment, whereas the Pain group (P) received *electric foot shock* 1-3 mA at day 12^{th}-21^{st}. The shock was given according to our previous study, in some sessions with 4 minutes interval. At day 12^{th} : 4 shocks-2 sessions were given, day 13^{th} : 8 shocks 2 sessions, day 14^{th} : 10 shocks 3 sessions, day 15^{th} : 12 shocks 3 sessions, day 16^{th} : 14 shocks 4 sessions, day 17^{th} : 16 shocks 4 sessions, day 18^{th} : 18 shocks 5 sessions, day 19^{th} : 20 shocks 5 sessions, day 20^{th} : 22 shocks 6 sessions, day 21^{st} : 24 shocks 6 sessions. At day 21^{st}, all mice were injected intravenously with 10^4 alived *listeria monocytogenes* that were obtained from *health laboratorium center* Semarang. All mice were sacrified using chloroform anaesthesia and neck dislocation at day 26^{th}.

Liver and spleen were aseptically taken to do histopathological examination by processing the tissue (made into paraffin block) and HE staining. The slides were observed under light microscope. The observations on liver slides included microabscesses, were counted in 10 visual fields using 100x zoom, and hepatocytes destruction, were counted every 100 cells using 400x zoom. Hepatocytes destruction was defined as smaller dead nucleus, loss of chromatin's reticular smooth fiber and became multiply folded, the nucleus became

pycnotic that could be destroyed into kariorexis and then kariolysis. Whereas observation on spleen slides was multinucleated giant cells counted in 100 visual fields using 400x zoom.

Independent t-test was used to analyse the difference between variables. The difference was significant if $p<0.05$. All statistical analysis were done using computer software SPSS 13.00 for windows.[12,13,14]

RESULTS

After microabscess formation, hepatocytes destruction, and multinucleated giant cells were counted, we got data presented on Table 1. Mean of microabscess formation in Pain group is (9.7±1.97), larger than control group (3.0±0.89). Variability of count can be seen on Figure 1 with significant difference (t=-7.559; *p=0.0001*) between the treatment groups.

Table 1. The result of microabscess formation, hepatocytes destruction, and multinucleated giant cells count

Variable	Group	N	$\overline{X}$	SB	t	*p*
Micro-abscess (liver)	Control (C)	6	3.0	0.89	-7.559	*0.0001*
	Pain (P)	6	9.7	1.97		
Hepatocytes destruction (liver)	Control (C)	6	26.7	4.50	-9.254	*0.0001*
	Pain (P)	6	54.7	5.89		
Multi-nucleated giant cells (spleen)	Control (C)	6	126.3	39.08	3.147	*0.020*
	Pain (P)	6	73.5	12.82		

Mean of hepatocytes destruction in Pain group was (54.7±5.89), larger than Control group (26.7±4.50). Variability of count can be seen on Figure 1 with significant difference (t=-9.254; *p=0.0001*) between the treatment groups.

Pain might induce stress, but stress might not always cause pain. Both pain and stress need evaluation and treatment. Stress is one of the factors which can modulate pain besides exercise (endorphin is produced during exercuse), acupuncture, and hypnosis.[15] Study on human has limitation to elucidate the relationship of pain that can cause stress by changing immune function and its impact on health clearly because practical and ethical problems. Animal studies, like what we have done, support the findings on human studies and might thoroughly explain the basic science of underlying mechanism. Animal studies make it possible to observe effect of various stressors pathophysiologically and administration of infectious agents into many anatomical parts. Such things of course could not be done in human. Thereby, animal studies can be done to explain comprehensive analysis on interaction between neuroendocrine-immune system in various experimental conditions.[16,17,18,19] This study used *electric foot shock* 1-3 mA.

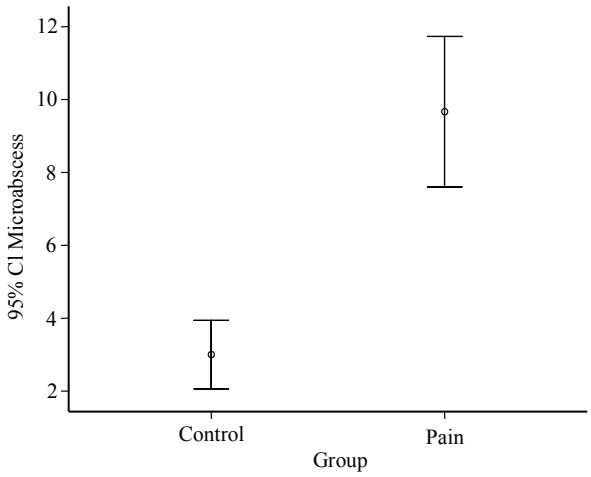

Figure 1. Graphic shows variability of microabscess count

Liver is one of lymphoid organs where many of immune responses occur, besides the lymphatic vessels, circulation, and other secondary lymphoid tissues. If there is bacterial penetration, a lot of kupffer cells in the liver will be exposured by bacterial antigen and then activate the immune responses by lymphocytes, macrophages, and cytokines. In this study, microabscess formation (Figure 2) and hepatocytes destructions (Figure 4) were highest in Pain group (P) compared to Control group (C), relevant to the theory that stress might give pressure to central nerve cells, then change immune system via endocrine pathway and autonom nervous system. This pathway is well-known as *hipothalamic pituitary adrenal-axis* (HPA-*axis*). Stress via *hypothalamic-pituitary-adrenal axis* (HPA Axis) system will cause adrenal gland to secrete cortisol hormone that will decrease immune system function by reducing Th1-cytokine synthesis, including interferon-γ (IFN-γ) which is very important in activating macrophages to fight against *listeria monocytogenes* as intracellular bacterias, and increase Th2-cytokines, including IL-10. Stress is believed to cause decrease of Th1-cytokines that will disturb the cellular immunity.[20] During the early stage of listeriosis, neutrophyls dan macrophages migrate into liver and spleen, to form microabscess with special features. Neutrophyls play important role to control acute phase and mediate infected hepatocytes destruction in vivo. Products released by macophages and neutophyls might damage normal tissues. If the immune response is adequate, the damage will be temporary and reversible, because the

activated macrophages will also induce tissue repair by secreting *growth factor* that stimulates fibroblast proliferation, collagen synthesis, and angiogenesis.[11]

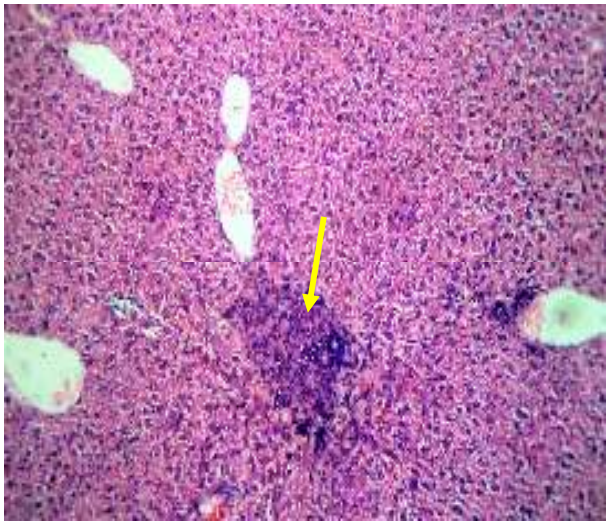

Figure 2. Microabscess (100 X zoom)

Mean of spleen multinucleated giant cells in Pain group was (73.5±12.2), lower than Control group (mean 126.3±39,08). Variability of count can be seen on Figure 5 with significant difference (t=3.147; *p=0.020*) between the treatment groups.

When *listeria monocytogenes* infects the macrophage, macrophage will release IL-12. IL-12 binds to Th1 cell that has main role to stimulate cellular immune response to fight against intracellular organism. This T-lymphocyte mediated immunity mechanism can be divided into 3 phases: recognition, activation, and effector phase. In the recognition phase, bacterias are ingested by macrophages and then processed and presented to T-helper lymphocytes. T-helper lymphocytes will be activated, and release cytokines during the effector phase, such as IFN-γ, IL-2, IL-3, and lymphotoxin. *Listeria monocytogenes* can produce enzymes that save them from phagocytosis, such as α- and β-listeriolysin. These enzymes can damage erythrocytes, thrombocytes, and macrophages, and also damage the phagosome membrane. Thus, bacterias can live inside macrophage cytoplasm and become resitant to phagocytosis. To kill the bacterias, prevent their spreading, and prevent further tissue damage, immune cells form granuloma around the infected macrophages. Extracellular matrix layer close the microenvironment of infected cells and protect tissue from destructive inflammation responses.[21]

Granuloma formation is a delayed-type hypersensitivity. Activated T-lymphocytes release lymphokine that will attract more macrophages into the lesion. At the same time, macrophages will turn into epitheloid cells, one of the macrophage differentiation stage which have main function as secretory cells.[22] The cells inside granulomas secrete lisozym, *angiotensin converting agent* and might be also proteinase which plays role in antimicrobial resistance process and tissue catabolism. Without granuloma formation, microbial infection will spread and be fatal.[22]

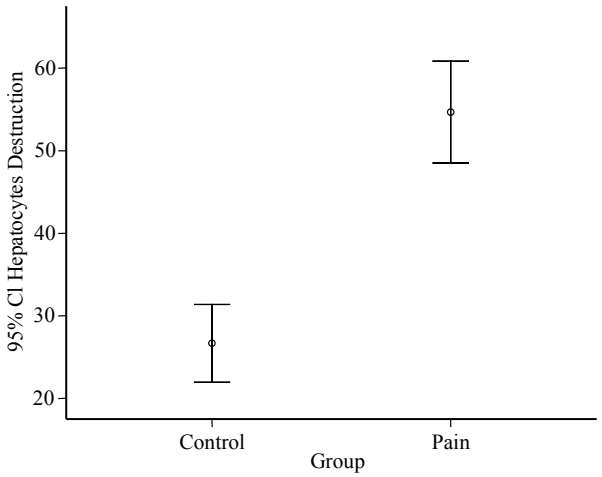

Figure 3. Graphic shows variability of hepatocytes destruction count

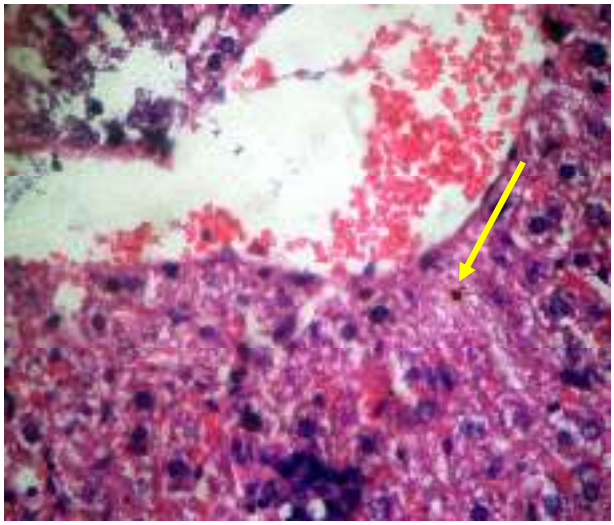

Figure 4. Hepatocyte with pycnotic nucleus (400X zoom)

Granuloma microscopically consists of macrophages accumulation that have already fused, lymphocytes, and sometimes plasm cells. Macrophages that have already fused are called multinucleated giant cells or datia cells (Figure 6).[22] The mechanism of datia cells formation is unclear. Some studies showed that macrophage fusion are induced by IFN-γ, TNF-α, indirect effect of microbial product (eg. mycobacterium) and also affected by maturation level of monocyte into macrophage.[22,23,24,25,26]

In the datia cell formation observed in alveolitis, from lung biopsy the histopathological examination showed accumulation of macrophages into lesion, increase of lymphocytes, formation of multinucleated giant cells, more apparent lesion border, and finally the granuloma formation.[27] Thus, datia cells are considered to be the beginning of granuloma formation. By datia cells formation, spreading of *listeria monocytogenes* infection can be prevented. In Control group (C), multinucleated giant cells count were relatively high. It could be interpretated as adequate celluar immune response activity, so the cells still can do fusion and prevent the spreading of infection.

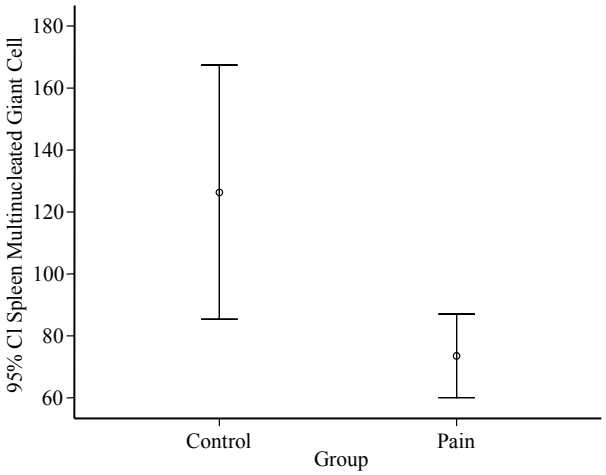

Figure 5. Graphic shows variability of multinucleated giant cells count

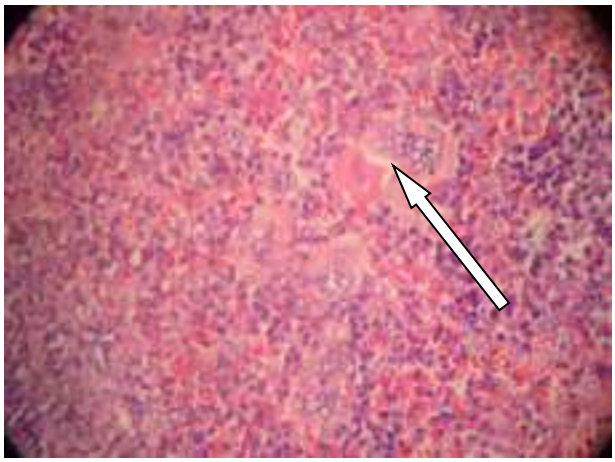

Figure 6. Multinucleated giant cell in spleen (400 X zoom)

Stress decreases cellular immune respons, that can decrease the synthesis of Th1 and also IFN-γ, whereas one of IFN-γ role in multinucleated giant cell formation is to induce macrophage fusion. Because of this mechanism, the number of multinucleated giant cells formed in Pain (P) group were relatively lower than Control group. This was supported by our previous study which proved that multinucleated giant cells formation could be inhibited by administration of antibody to IFN-γ.[25,26]

CONCLUSION

Pain stimulated by *electric foot shock* reduced cellular immunity, showed by significant higher liver microabscess formation and hepatocytes destruction and lower number of spleen multineucleated giant cells in the Pain group than the control group.

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SYNOPSIS

Pain stimulated by Electric Foot Shock has an effect not only higher liver microabscess formation and hepatocytes destruction, but also lower spleen multinucleated giant cells in the Pain group than Control group.

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