ORIGINAL ARTICLE

Uric Acid Induces Inflammation, Hepatocyte Apoptosis and Deterioration of Liver Function

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ABSTRACT

Introduction: Uric acid is a common cause of liver tissue damage due to its hepatotoxic effect. This study is aimed to investigate: (1) the effect of uric acid on liver damage which can be seen from the serum levels of SGOT and SGPT, (2) the inflammatory response demonstrated by *TLR-4* and *MCP-1* mRNA expression, and (3) the proportion of hepatocytes apoptosis in mice. **Methods:** A total of 25 adult male Swiss-Webster mice were divided into five groups: one control group and four uric acid groups (AU7, AU14, AU21 and AU28). The uric acid groups were administered with 125 mg/kgBW uric acid for 7, 14, 21, and 28 days. Following the treatment, mice were terminated and the liver was harvested. Blood sample was taken from retro-orbital vein to assess serum uric acid, SGOT, and SGPT levels. RT-PCR was performed to examine the mRNA expressions of *TLR-4* and *MCP-1*. TUNEL staining was used to assess the proportion of apoptotic hepatocytes. **Results:** Induction of uric acid caused hyperuricemia, increased expression of *TLR-4* and *MCP-1* mRNA significantly ($p<0.05$) which indicated an inflammatory reaction. The levels of SGOT and SGPT were elevated significantly ($p<0.05$), as well as the number of hepatocyte apoptosis ($p<0.05$). **Conclusion:** Hyperuricemia affected the inflammatory response by increasing the mRNA expression of *TLR-4* and *MCP-1*. An increased number of apoptotic hepatocytes was likely caused by the ongoing inflammatory reaction during the induction of uric acid.

Keywords: Hyperuricemia, Liver, *TLR-4*, *MCP-1*, Hepatocyte apoptosis

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INTRODUCTION

The liver is an important organ that has the ability to perform the functions of metabolism, synthesis, and detoxification of the human body. The tissue of this organ is the main target of various types of substances that can induce inflammatory response, necrosis, and fibrosis. This can result in interruption or reduction of the liver function itself (1).

Uric acid is a hepatotoxic agent that can cause tissue damage. The severity and progression of tissue damage depend on the dose and duration of exposure to the substance. Uric acid is one of the pro-inflammatory Damage-associated Molecular Patterns (DAMPs) released by dead cells, which can be recognized by the Toll-like Receptors (TLR) that can trigger inflammatory reaction and cause damage to the liver (2). Hyperuricemia is a condition characterized by excessive level of uric acid in the blood serum (3). Uric acid is an end product of purine degradation. The level of serum uric acid is affected by the synthesis of uric acid by the liver and excretion through the kidney (4).

The prevalence of hyperuricemia in each population tends to increase. In Japan, hyperuricemia was fluctuated between 25% - 30% of the adult male population and increased until a few years ago. Meta-analysis study in China reported prevalence of hyperuricemia in males reached 21.6% and in women 8.6% (5,6). In Bali, prevalence of hyperuricemia in men by 28% and 7% in women (7). A study conducted by Hwang et al. (2011) at a hospital in Korea from June 2007 to July 2009 showed that increased uric acid associated with the incidence of Non-alcoholic Fatty Liver Disease (NAFLD) (8).

Hepatocytes are the most sensitive cells, of all the liver cells, against exposure to toxic agents. Without formation of uric acid crystal, high concentration of serum uric acid level roles as damage-associated molecular pattern (DAMP) because it can stimulate cellular injury. Soluble

uric acid released by injured cells promote NLRP3 inflammasome and induces mitochondrial reactive oxygen species (ROS). The signals that is needed by inflammasome activation is mediated by Toll-like receptor (TLR) 4 to elicit immune response and increases the pro-inflammatory mediators (9-11). Hepatocyte death due to inflammation is a common cause of liver disease (12). Hepatocyte damage induces the release of Monocyte Chemo-attractant Protein-1 (*MCP-1*), which will increase the recruitment of macrophages/ Kupffer cells and lymphocytes, and the release of pro-inflammatory cytokines, such as Interleukin-1β (IL-1β), IL-1 and Tumor Necrosis Factor-α (TNF-α). Then, the cytokines will activate Nuclear Factor Kappa B (NFκB) and Mitogen-activated Protein Kinase (MAPK) and increase the production of Cyclooxygenase (COX) (9,10).

Hepatic apoptosis is induced by a variety of causal factors, for example, alcohol, viruses, toxic bile acids, fatty acids, drugs and immune response (13). Apoptosis is the programmed cell death which has important role in eliminating damage cells and tissues (14,15).

All things considered, this research aims to investigate the effect of uric acid on the damage of hepatic function, inflammation and apoptosis of hepatocytes in mice.

MATERIALS AND METHODS

Animal models and administration of uric acid

We conducted a quasi-experimental research using post-test only with controlled group design. The subject used were 25 adult (three-month old) 30-40 grams male Swiss-Webster mice. The mice were housed in the Laboratory of Anatomy of Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada at standard room temperature and 12-h light/dark cycle. The mice were provided with water and mice's food ad libitum. This research had obtained licence from the Medical and Health Research Ethics committee (MHREC) Faculty of Medicine Universitas Gadjah Mada-Dr. Sardjito General Hospital, which is based on statement letter of ethic expedience number KE/FK/405/EC/2016 on April 29th, 2016.

The experimental animals were divided into five groups, with five mice in each group. There were control group (control, n=5) and four groups receiving the uric acid, namely AU7 (n=5), AU14 (n=5), AU21 (n=5), and AU28 (n=5). The control group were injected with 0.9% NaCl intraperitoneally and were sacrificed after 28 days. The AU groups were inducted by 125 mg/kgBW uric acid (Sigma-Aldrich, U25-26 25G) which is dissolved into NaOH 0.15 M and injected intraperitoneally for 7, 14, 21 and 28 days. Eventually, the AU7, AU14, AU21, and AU28 groups were sacrificed at the 8th, 15th, 22nd and 29th days, respectively.

Examination of serum uric acid level, SGOT, SGPT

After performing anesthetic procedure, blood sample were taken from the retro-orbital vein of the mice. After that, the serum was accumulated in Eppendorf tube for centrifugal processing (10.000 rpm of velocities) for about 10 minutes. Serum was examined for uric acid, SGOT, and SGPT levels in the Laboratory of Clinical Pathology Faculty of Medicine Universitas Gadjah Mada according to the laboratory's standardized procedures.

Histopathology analysis of hepatocytes apoptosis

For the sacrifice process, the mice were given general anesthesia with 0.1 mL/gBW intraperitoneal injection of sodium pentobarbital. After that, the mice were dissected so that we could harvest the liver.

The liver tissue was embedded in paraffin for immunohistochemistry (IHC) studies. Paraffin-embedded liver tissue was dewaxed using standard sequential techniques, and 4 μm-thick sections were stained with TUNEL assay to determine liver cells that undergoing apoptosis. TUNEL staining was performed using an In situ Cell Death Detection Kit (ApopTaq® Peroxidase In Situ, S7100) according to the manufacturer's protocol. This step was followed by counterstaining using hematoxylin-eosin to stain the cell's nucleus. The liver tissue was histologically examined using a light microscope (Olympus CX22®) with 400x magnification for 15 fields of view, particularly on the perisinusoidal and periportal areas.

RNA extraction and cDNA synthesis

Total RNA was extracted using Genezol solution (GENEzol™, Cat.No.GZR100) based on the protocol from the manufacturer, and RNA concentration was quantified by spectrophotometry. We used 3000 ng RNA for the cDNA synthesis using Rever Tra Ace® (Toyobo, Japan, Cat. No. TRT-101) and random primer (Takara, Japan, Cat. No. 3801), with the following PCR conditions: 30°C for 10 minutes (denaturation phase), 42°C for 60 minutes (annealing phase) and 99°C for 5 minutes (extension phase).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) of *TLR-4* and *MCP-1*

RT-PCR was carried out to amplify the following specific cDNA. The primers we used for *TLR-4* were 5′-TTGGCCACGTTTTTCTCC-3′ (forward) and 5′-TGGCTGCAGAGAGGCTGT-3′ (reverse), meanwhile for *MCP-1* were 5′-ACTGAAGCTCGTACTCTC-3′(forward) and 5′-CTTGGGTTGTGGAGTGAG-3′(reverse); *GAPDH* 5′-TCACCATCTTCCAGGAGCG-3′(forward) and 5′-CTGCTTCACCACCTTCTTGA-3′ (reverse). *GAPDH* expression was used to normalize the expression.

RT-PCR was performed by mixing 4μL of cDNA, 12.5 μL of Tag master mix (GoTaq®Green Master Mix, Cat.

No. 7122), 0.6 µL of forward primer, 0.6 µL of reverse primer and 9.3 µL of PCR water. The cDNAs were amplified according to the following conditions: 94°C for 2 seconds (initial denaturation phase), 94°C for 10 seconds (denaturation phase), 60°C for 20 seconds (the annealing temperature varied for each pair of primers), 72°C for 1 minutes (extension phase) and 72°C for 10 minutes (last extension phase). The number of cycles was redetermined for each pair of primers in order to avoid the PCR plateau phase. The PCR products were analyzed on 2% agarose gel along with a 100 bp DNA ladder (Bioron, Germany, Cat. No. 306009). The expression of the genes was quantified with densitometric analysis using the ImageJ software version 1.40.

Statistical Analysis

The results are expressed as mean ± SD. Multiple comparisons among the groups were done by one-way analysis of variance (ANOVA) and followed by the post-hoc LSD test for parametric test. Kruskal-Wallis test and Mann-Whitney test were conducted for the non-parametric test. Data were significantly proved if $p<0.05$.

RESULTS

The levels of serum uric acid, SGOT, and SGPT

There was a significant difference in serum uric acid levels between the control group (0.78±0.08 mg/dL) and all of the uric acid groups. As we can see in Figure 1, The AU7 group had the highest serum uric acid level among five groups (4.03 ± 0.44 mg/dL), followed by AU14 (3.32 ± 0.51 mg/dL), AU21 (2.30 ± 0.35 mg/dL) and AU28 (1.11 ± 0.16mg/dL). The serum uric acid level of the AU28 group was higher than the control group, although this was not statistically significant. The level of SGOT and SGPT were significantly higher in all of the uric acid groups than the control group.

TLR-4 and *MCP-1* mRNA expression

To investigate the inflammatory markers in mice receiving uric acid induction, we examined the mRNA expressions of *TLR-4* and *MCP-1* (Figure 2). In this study, RT-PCR analysis revealed no significant difference between control and UA7 groups, however the mRNA expressions of *TLR-4* of the UA14, UA 21 and UA28 groups were significantly higher than the control group.

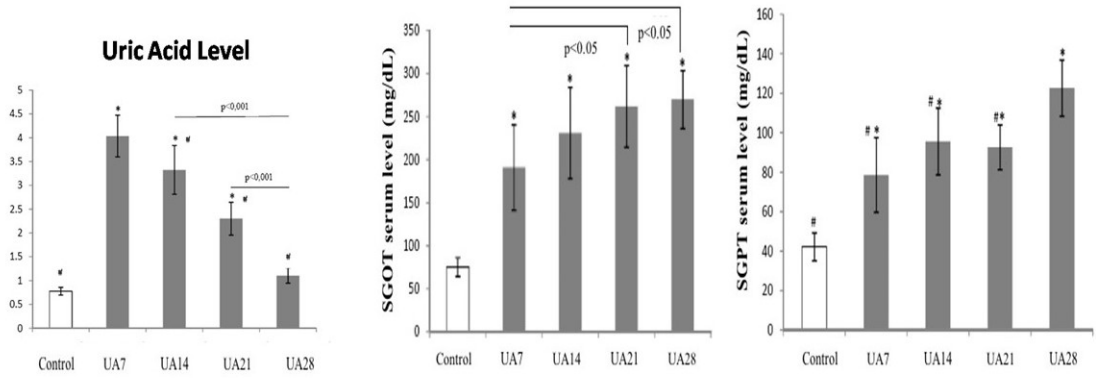

Figure 1: Effect of uric acid induction on serum uric acid, SGOT and SGPT level (mg/dl was used for unit of measurement). p<0.001 One-way ANOVA test, *p< 0.001 vs controls, #p<0.05 vs AU7 post-hoc LSD.

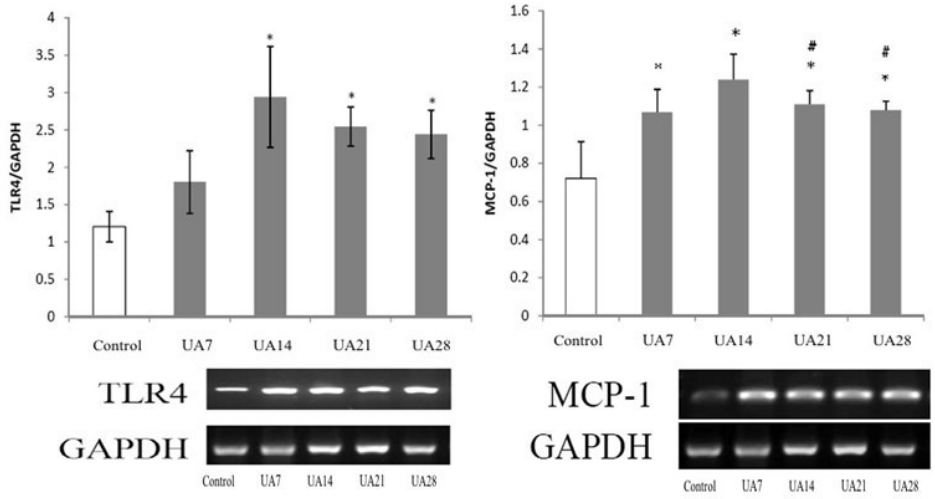

Figure 2: Representative gel electrophoresis results from RT-PCR of *TLR-4* and *MCP-1*. The results of densitometric analysis of *TLR-4* and *MCP-1* mRNA expressions was also shown. *TLR4/GAPDH* p<0.05 one-way ANOVA test, *p<0.05 vs control post-hoc LSD. *MCP-1/GAPDH* p<0.05 Kruskal-Wallis test, *p<0.05 vs control, #p<0.05 vs AU14 Mann-Whitney test.

Meanwhile, the *MCP-1* mRNA expression of uric acid groups was significantly higher than control group. The mRNA expression of *TLR4/GAPDH* in the control group was 1.20 ± 0.20, AU7 group was 1.80 ± 0.42, AU14 group was 2.95 ± 0.67, AU21 group was 2.54 ± 0.26 and AU28 group was 2.44 ± 0.32. There was a significant difference in median value expression of *MCP-1/GAPDH* based on the Kruskal-Wallis test. The mRNA expression of *MCP-1/GAPDH* in the control group was 0.72 (0.96-0.44), AU7 group 1.07 (1.29 to 1.02), AU14 group 1.24 (1.41 to 1.09), AU21 group 1.11(1.14 to 0.97) and AU28 group 1.08 (1.15 to 1.03).

Apoptosis of Hepatocytes

Based on mean±SD values proportion, the number of apoptotic hepatocytes compared to the non-apoptotic hepatocytes in the control group was 0.84±0.03, the AU7 group was 1.33±0.10, the AU14 group was1.34±0.03, the AU21 group was 1.86 ± 0.10 and the AU28 group was 2.04 ± 0.07.

The results of one-way ANOVA test showed a significant difference (p=0.000). The results of post-hoc LSD test showed that there were significant differences between the control group and all of the treatment groups (p<0.00). There were also significant differences between all treatment groups (p<0.05), except in AU7 and AU14 group (p=0.771) which showed no significant difference (Figure 3).

DISCUSSION

Mice received uric acid administration with 250 mg/kgBW/day intraperitoneal injection tends to increase serum uric acid levels significantly on the 7th day and into the 14th day (17). However, in our pre-eliminary study intraperitoneal injection of 250 mg/kg BW of uric acid increased the mice death. Therefore, induction of uric acid which was done by intraperitoneal injection using a dose of 125 mg/kgBW/day also increased serum uric acid levels significantly at day 7, 14, and 21 (p=0.000) when compared to the control group.

Meanwhile, serum uric acid level found in AU28 group was not significant compared to the control group (p=0.084). It is estimated that a decrease in serum uric acid levels in group AU28 occured due to increased activity of uricase that is able to transform uric acid into allantoin which is more soluble and can be excreted through the kidneys (18,19). Uricase is a catabolic enzyme contained uric acid in the liver. In the group AU7 serum uric acid levels were higher than the group AU14. This was allegedly due to a physiological adaptation by increasing the secretion of uric acid in the urine, causing hyperuricosuria, after induction of uric acid for 7 days (20).

Hyperuricemia also results in a variety of pathological conditions, such as hepatic cirrhosis and increased Alanine Aminotransferase (ALT) and Gamma-glutamyltransferase (GGT) (21). Aminotransferase enzymes are able to catalyse transamination reactions. There are two types of serum transaminase enzymes, namely AST and ALT, that reflect the integrity of the liver cells. An increase in liver enzymes may reflect the level of liver cells's damage. The higher the levels of SGOT and SGPT enzymes, the higher the degree of liver cells damage (22).

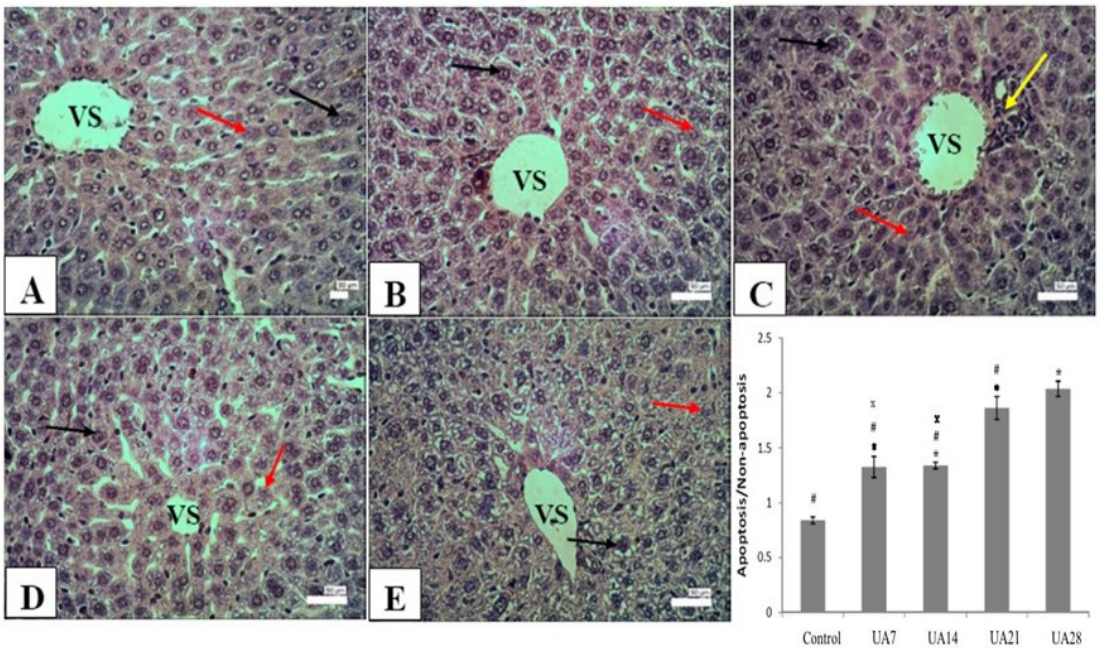

Figure 3: Histopathological analysis using TUNEL assay to examine apoptosis of the hepatocytes (VS = central vein). Bar graph summarizes a proportion of apoptotic hepatocytes and non-apoptotic hepatocytes. A represented control group, B represented UA7 group, C represented UA14 group, D represented UA21 group, and E represented UA28 group. p<0.001 one-way ANOVA test, *p<0.001 vs control, #p<0.05 vs AU28, xp<0.05 vs AU21 post-hoc LSD.

In this study, mean±SD value of SGOT and SGPT levels in serum of group AU28 is the highest. The test results of one-way ANOVA showed a significant difference for elevated levels of SGOT and SGPT between treatment groups with p=0.000. Followed by a post-hoc LSD test for SGOT, which showed that there were significant differences between the control group and all treatment groups, but no significant difference between the treatment groups except among groups AU7 with AU21 and AU7 with AU28. Followed by a post-hoc LSD test for SGPT levels, which showed the significant differences between the control group and all treatment groups, and the significant differences between group AU28 with all treatment groups.

Increased levels of SGOT and SGPT can be caused by disruption of the liver functions (23). Hepatocytes membrane damage causes aminotransferase enzyme to leave cytoplasm of the damaged cells and enter the bloodstream, so that aminotransferase level in the blood will increase. Therefore, elevation of SGOT and SGPT can be an indicator of liver damage (24).

Uric acid can act as a Pathogen-associated Molecular Patterns (PAMPs) which is a signal that can trigger an inflammatory reaction in the liver (25). Toll-like Receptors (TLRs) is a protein that plays a role in causing the activation of the innate immunity system. TLR function as Pathogen Recognition Receptors (PRRs), recognize the uric acid that acts as PAMPs (26).

TLR-4 gene expression in the liver increased after administration of uric acid and showed that AU14 was the highest in the group receiving induction for 14 days. The results of the statistical analysis of one-way ANOVA test showed significant difference between the increased mRNA expression of *TLR-4* treatment group (p=0.045). Followed by a post-hoc LSD test, there were significant differences between the control group and the treatment group AU14, AU21 and AU28, and there was also significant difference among the treatment groups. This is caused by the interaction of uric acid and *TLR-4* which induces the signal to release a wide range of cytokines, leading to the recruitment of macrophages, including Kupffer cells and other leukocytes (27). Cytokines and proinflammatory chemokines produced include TNF-α, IL-β and IL-6, IFN-γ, IL-8 and MIP-2, MCP-1, as well as adhesion molecules such as ICAM-1 and VCAM-1 so that the inflammation can be continued (28).

Continous stimulation of inflammation by the uric acid can also be seen from the production of Reactive Oxygen Species (ROS) production and inactivity of Mitogen-activated Protein Kinase Phosphatase-1 (MKP-1), which results in production of the Monocyte Chemoattractant protein-1 (MCP-1) in macrophages (26). In this study, in addition to *TLR-4*, we also carried out RT-PCR for *MCP-1*. The *MCP-1* mRNA expression in the liver also increased after induction with uric acid and AU14 was the highest of the groups which received induction for 14 days.

Kruskal-Wallis test results obtained the value of p<0.05 (p=0.009) which showed that there were significant differences in mRNA expression of *MCP-1/GAPDH* among the groups. The Mann-Whitney test showed significant difference between the control group and all treatment groups, but not significantly different between treatment groups except among groups AU14 to AU21 and AU14 to AU28. The results obtained in accordance with the *TLR4* mRNA expression was also increased. The findings of these two mRNA expression strengthened the allegations of an ongoing chronic inflammatory process.

Uric acid, which acts as PAMPs, is a signal that can trigger an inflammatory reaction in the liver that is generally associated with death or necrosis of hepatocytes (30). The damaged liver cells will undergo apoptosis process and form apoptotic bodies, which are recognized and removed by phagocytosis by macrophages or surrounding cells (31).

Through TUNEL staining method, hepatocytes were stained brown. The other cells undergo apoptosis, while hepatocytes which stain the cells with brown marking do not undergo apoptosis. Additionally, cells undergoing apoptosis can also be identified morphologically with condensed chromatin, DNA fragments, enlarged plasma membrane, cell shrinkage, and fragments of cells coated membrane (32).

Based on calculation of the number of cells, mean±SD value proportion of apoptotic/non-apoptotic hepatocytes increased along with longer duration of uric acid administration. The one-way ANOVA test showed a significant difference among the treatment groups with p=0.000. This was followed by a post-hoc LSD test that showed significant differences between the control group and all treatment groups (p=0.000). The result was also in accordance with the means of SGOT and SGPT, which were the highest in AU28 group (p=0.000). Elevation of both liver enzymes, which become markers of liver damage, followed by an increasing number of hepatocytes undergoing apoptosis corresponds to the duration of uric acid exposure.

The liver has high capacity in removal of toxic substances through biotransformation. The detoxification process exceeding capacity of the liver will force the liver to work harder. Continous exposure to high levels of serum uric acid will ultimately damage the liver cells by promoting inflammation, necrosis, and apoptosis (33).

CONCLUSION

Hyperuricemia affected the inflammatory response by increasing the mRNA expression of *TLR-4* and *MCP-1*. An increased number of apoptotic hepatocytes was

likely caused by the ongoing inflammatory reaction during the induction of uric acid.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. Mulyana, and Mrs. Wiwit Ananda Wahyu, S.Keb, M.Sc for their technical assistance during this research. This publication has been funded by the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada.

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