

Uric acid induces liver fibrosis through activation of inflammatory mediators and proliferating hepatic stellate cell in mice

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ABSTRACT

Introduction: Uric acid is associated with cardiometabolic risk factor and severity of liver damage. The mechanism of uric acid inducing liver damage is still elusive. This study elucidates the development of liver fibrosis under hyperuricemia.

Methods and Materials: Hyperuricemia model was performed in male Swiss Webster mice. Intraperitoneally injection of uric acid (125mg/kg body weight) was done for 7 and 14 days (UA7 and UA14 groups). Meanwhile, the UAL groups were injected with uric acid and followed by the administration of allopurinol (UAL7 and UAL14 groups). On the due date, mice were sacrificed, and liver was harvested. Uric acid, SGOT, SGPT, and albumin level were measured from the serum. The mRNA expression of TLR4, MCP1, CD68, and collagen1 were assessed through RT-PCR. Liver fibrosis was quantified through Sirius red staining, while the number of hepatic stellates cells (HSCs) and TLR4 were assessed through IHC staining.

Results: Uric acid induction for 7 and 14 days stimulated an increase of both SGOT and SGPT serum levels. Followed by enhanced inflammatory mediators: Toll-like receptor-4 (TLR-4), Monocyte Chemoattractant Protein-1 (MCP-1) and Cluster of Differentiation 68 (CD68) mRNA expression in the liver ($p < 0.05$). The histological findings showed that the UA7 and UA14 groups had higher liver fibrosis scores ($p < 0.05$), collagen I mRNA expression ($p < 0.05$), and the number of HSCs ($p < 0.05$) compared to Control group. Administration of allopurinol showed amelioration of uric acid and liver enzymes levels which followed by inflammatory mediators, liver fibrosis and collagen1, and hepatic stellate cells significantly.

Conclusion: Therefore, uric acid augmented the liver fibrosis by increasing the number of hepatic stellate cells.

KEY WORDS:

Hyperuricemia, inflammation, liver fibrosis, collagen I, hepatic stellate cells

INTRODUCTION

Uric acid is associated with several cardiometabolic risk factors, such as diabetes, hypertension, kidney disease, obesity and metabolic syndrome.¹ Uric acid is formed in the liver and excreted by the kidneys (65-75%) and colon (25-35%).² Serum uric acid concentrations may indicate the balance between production and excretion of uric acid.³ The increasing level of serum uric acid in patients with non-alcoholic fatty liver disease (NAFLD) is independently associated with the severity of liver damage.⁴ The high uric acid level also induces kidney injury through inducing inflammation and fibroblast expansion. Activation of the uric acid pathway in the renal interstitial lead to myofibroblast expansion.⁵ Uric acid also induces tubular injury with activation of epithelial to mesenchymal transition.⁶ The effect of high uric acid level in liver injury and fibrosis has not been elucidated profoundly.

Uric acid is one of the Damage-Associated Molecular Patterns (DAMP) proinflammatory that is released by dying cells,⁷ which is recognised by the Toll-like receptor (TLR) and trigger an inflammatory reactions⁸ and then activate hepatic stellate cells.⁹ Hepatic stellate cells activation may lead to liver fibrosis. Liver fibrosis is characterised by liver parenchymal shrinkage and tissue rich in collagen. Collagen in the liver secreted by activated hepatic stellate cells, portal myofibroblast and myofibroblasts derived from bone marrow that is activated by fibrogenic cytokine TGF β 1, angiotensin II, and leptin.¹⁰

This study elucidates liver injury in hyperuricemia model focusing on fibrosis and hepatic inflammation. Allopurinol commonly used for lowering uric acid serum levels, and the purpose of administration of allopurinol is to compare with hyperuricemia condition. Models of ischemic reperfusion on animals showed that allopurinol may protect against liver damage by preventing the purine metabolism, thus inhibiting the formation of reactive oxygen species.¹¹

MATERIALS AND METHODS

Animal model and Experiments

Male Swiss Webster mice (3 months-old, 30-40 grams, $n=25$), divided into five groups: Control (intraperitoneal injection of

0.9% NaCl for 14 days); UA7 (induction of uric acid for seven days); UA14 (induction of uric acid for 14 days); UAL7 (induction uric acid followed by an administration of allopurinol for seven days); UAL14 (induction uric acid followed by an administration of allopurinol for 14 days). Each group received a single intraperitoneal injection of uric acid (Sigma, U25-26G, 125mg/kg body weight) in 0.2M NaOH. Furthermore, UAL7 and UAL14 groups also administered with allopurinol (Sigma, A8003-25G, 50mg/kg body weight) dissolved in NaOH 0.15M with oral gavage. The end of the induction days, blood serum was taken for serum uric acid level quantification. Mice were deeply anesthetised using sodium pentobarbital, thorax and abdomen were opened, perfusion was done using the intraventricular injection of NaCl 0.9%, then liver was harvested, kept in RNA lysis solution (Ambion, AM7021) for RNA extraction and fixed in Normal Buffer Formalin solution for histological examination.

The mice were acclimatised for one week and were kept in a plastic cage 50x30 x15cm in size which contained two mice for each cage. They were fed using standard chow and water *ad libitum*.

This research was granted a license from the Ethical Committee of the Medical Research and Health of Faculty of Medicine Universitas Gadjah Mada for research involving animals. This ethical clearance was recognised by the Forum for Ethical Review Committees in Asia and Western Pacific (FERCAP) based on a statement letter of ethical expediency.

Blood Collection and Liver Function Test Measurement

Blood was collected from the retro-orbital vein using hematocrit capillary. To separate the serum, the blood was centrifuged at 10,000rpm for 10 minutes. The serum was analysed to assess uric acid, albumin, SGOT, and SGPT levels.

RNA isolation, cDNA making, and Reverse Transcription

The RNA extraction was performed using Genezol (GENEzol™, GZR100) according to the manufacture. Moreover, the cDNA synthesis was done from 3000ng of the mRNA using ReverTraAce (Toyobo, TRT-101, Osaka), then 200ng was used for PCR with conditions of 30°C for 10 minutes, 40°C for 60 minutes, and 99°C for five minutes. A master mix (Promega, M7122) was used with these following primers: collagen I (forward: 5'-ATGCCGCGACCTCAAGATG-3'; reverse: 5'-TGAGGCACAGACGGCTGAGTA-3'), Toll-like Receptor 4 (TLR4) (forward: 5'-GGGCCTAAACCCAGTCTGTTTG; reverse: 5'-GCCCCGTAAGGTCCATGCTA), Monocyte Chemoattractant Protein-1 (MCP-1) (forward: 5'-GGCATCACAGTCCGAGTCACA; reverse: 5'-CTACAGACAA CCACCTCAAGCACTTC), CD68 for macrophage marker (forward: 5'-CATCAGAGCCCCGAGTACAGTCTACC; reverse: 5'-AATTCTGCGCCATGAATGTCC) and GAPDH (forward: 5'-TTGCTGTTGAAGTCGCAGGAG; reverse: TGTGTCCGTCGT GGATCTGA).

PCR was done for 30 cycles, with conditions of 94°C for 10 seconds, 60°C for 30 seconds and 72°C for one minute and final extension phase with the condition 72°C for 10 minutes. The expression of the genes was quantified based on electrophoresis results of bands. Densitometry analysis was done to examine the expression of the genes and normalised by GAPDH expression.

Liver Fibrosis score

The paraffin block of the liver was cut in 4µm thickness, then stained with Sirius red to assess liver fibrosis. The histology of slides was assessed by two observers using a microscope with 400x magnification and analysed using ImageJ software. Fibrosis score was performed according to the semiquantitative method by Brunt et al., which were modified by Kleiner et al. (0=no fibrosis; 1=perisinusoidal or periportal/portal fibrosis; 2=perisinusoidal and periportal/portal fibrosis; 3=septal/bridging fibrosis; 4=cirrhosis).

Immunohistochemical (IHC) staining of Hepatic Stellate Cells (GFAP) and TLR4

Immunohistochemical staining for Glial Fibrillary Acidic Protein (GFAP) was used to measure the number of hepatic stellate cell. The slides were deparaffinised using xylene and rehydrated using ethanol. Then, blocking endogenous peroxidase with hydrogen peroxidase 0.3% for five minutes. After that, the slides were incubated with primary antibodies GFAP (Abcam, ab48050, 1:400 dilution) and TLR4 (Bios, bs-1021R, 1:200 dilution) for overnight at 4°C. On the following day, the slides were incubated with secondary antibody using Star Trek Universal HRP kits at room temperature for 1-hour and streptavidin (Biocare medical, STUHRP700H-KIT) for 45 minutes. Finally, diaminobenzidine (DAB) was applied for 1 minute followed by hematoxylin counterstained for three minutes. The number of hepatic stellate cells assessed using microscope x400 magnification and analysed by ImageJ software. Calculated cells were stained brown and bluish-purple core in 8 fields of views.

Statistical Analysis

Statistical analysis performed with SPSS 22 software. Data was reported as mean±SEM. Uric acid serum levels and fibrosis score were analysed using the Kruskal Wallis test followed by the Mann Whitney test. The expression of collagen I and the number of hepatic stellate cells using One-way ANOVA followed LSD Post Hoc test. *P*-value <0.05 were considered significant.

RESULTS

Serum uric acid levels and liver function

We had reported earlier that injection of uric acid intraperitoneally for seven and 14 days significantly increased uric acid serum levels in both uric acid groups (*p*<0.05) compared to the control group.¹² Allopurinol treatment along with uric acid injection in UAL7 and UAL14 groups induced lower uric acid levels compared to uric acid groups.

Furthermore, we also quantified the liver function test that showed significant higher SGOT and SGPT level in UA7 and UA14 (Fig.1) compared to control. Meanwhile, lower SGOT and SGPT level were found in UAL7 and UAL14 groups compared to uric acid groups. However, UAL14 had higher SGPT and SGOT level compared to UAL7 group. Lower albumin level was also found in UA7 and UA14 groups compared to control (Fig. 1C). Allopurinol administration in UAL7 group enhanced albumin level compared to the UA7 group.

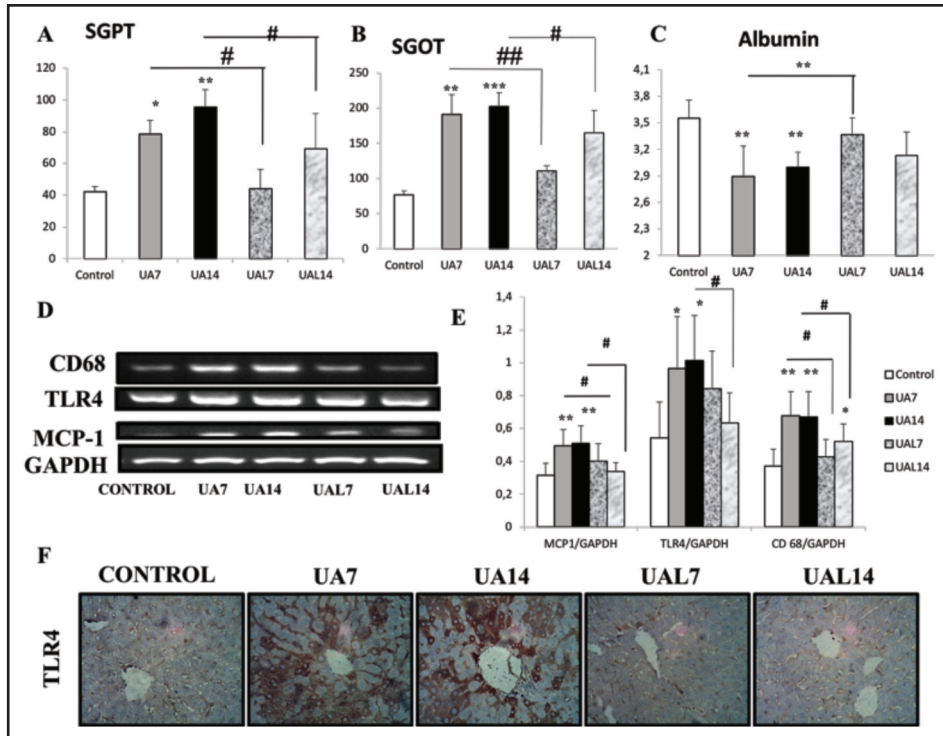


Fig. 1: A. Uric acid injection induced hyperuricemia condition after 7 and 14 days. Allopurinol reduced hyperuricemia condition. B-C. Serum SGOT and SGPT level increased hyperuricemia condition in group AU7 and AU14. Meanwhile, reduction of uric acid level reduced serum SGOT and SGPT level. D-E. Hyperuricemia condition induced inflammation mediator genes upregulation (MCP-1 and TLR4) and macrophage (CD68). F. Representative picture of TLR4 immunostaining showed upregulation of TLR4 in AU7 and AU14 groups, however AAL7 and AAL14 had lower expression compare to hyperuricemia group. * $p < 0.05$ vs Control; # $p < 0.05$.

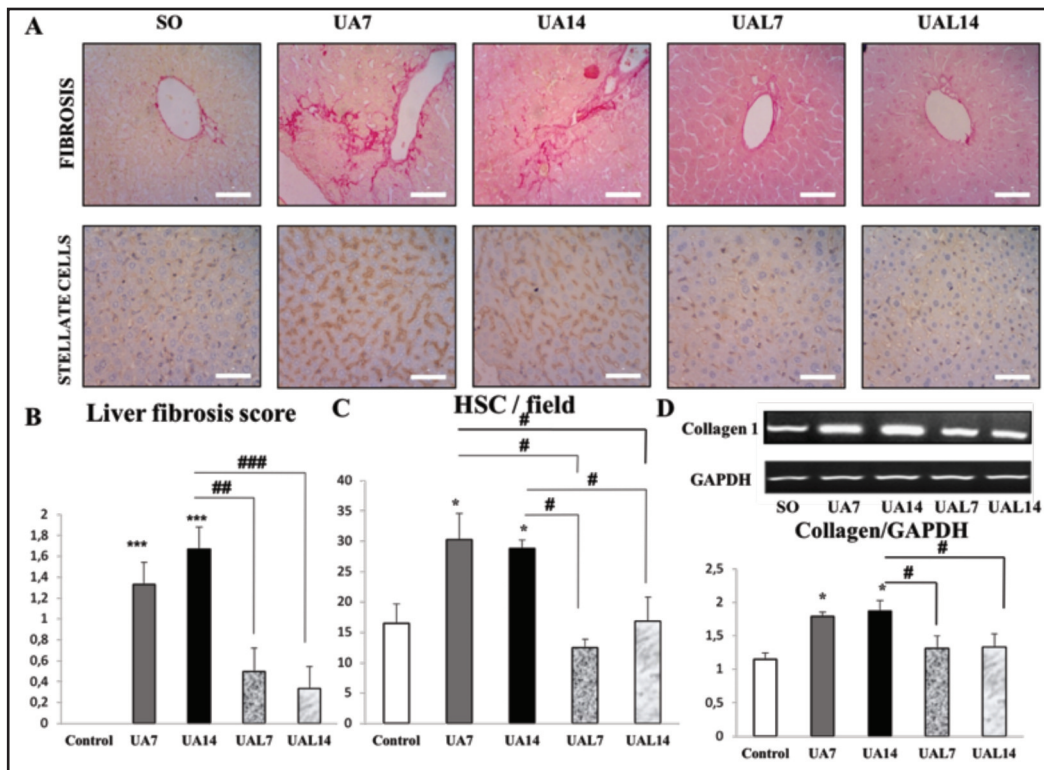


Fig. 2: A. Representative picture and quantification of interstitial fibrosis and Hepatic stellate cells (HSC) immunostaining. Hyperuricemia condition induced liver fibrosis as shown by an increase of fibrosis score in AU7 and AU14 group compare to Control. Meanwhile, Allopurinol group had lower fibrosis score and HSC cell number. D. Representative picture and densitometry analysis of Collagen 1 expression based on Reverse Transcriptase PCR (RT-PCR). * $p < 0.05$ vs Control; # $p < 0.05$.

Higher inflammation mediator expression in uric acid treated groups

We suggested the involvement of inflammatory mediator in hyperuricemia which was shown by an upregulation of TLR4, MCP-1, and CD68 mRNA expression. The uric acid groups, UA7 and UA14, showed an increase of both of that inflammatory mediator and macrophage marker compared to the control group (Fig. 1D-E). Unless there was no difference in the UA7 and UA14 groups. Allopurinol treatment groups, UAL7 and UAL14, had a significant decreased of TLR4, MCP1, and CD68 expression compared to the UA7 and UA14 group. Inflammation might occur in the hepatocyte and perisinusoidal area as shown by positive staining of TLR4 in those areas. Diminished of uric acid levels followed by the reduction of both mRNA and protein expression of TLR4 in the allopurinol groups, UAL7 and UAL14.

Fibrosis score, number of hepatic stellate cells and collagen 1 expression

Furthermore, we analysed liver fibrosis using Sirius Red staining. Fibrosis area was found in the liver interstitial area, perisinusoidal and periportal areas in both uric acid groups compared to control ($p < 0.001$ vs. control, Fig. 2A&B). Prolong uric acid treatment accentuate in UA14 group as shown by the highest fibrosis score compared to the UA7 group ($p < 0.05$). This alteration was associated with a higher number of hepatic stellate cells (HSCs) in the uric acid group. Data analysis of HSC demonstrated a significant higher of HSCs number in UA7 and UA 14 compared to control ($p < 0.05$ vs. control). We profound that most of the HSCs were found in the Disse space. The collagen1 expression was upregulated due to activation of HSCs. The RT-PCR of collagen1 was done to assess collagen1 mRNA expression. Higher collagen1 expression was represented in UA7 and UA14, which was significantly different from the control (Fig. 2D). Reduction of the uric acid level in UAL7 and UAL14 is associated with amelioration of liver fibrosis score, HSC number, and collagen1 mRNA expression.

DISCUSSION

Uric acid has been known as an inflammatory agent.¹² Here, we reported inflammation in liver due to hyperuricemia condition was associated with liver fibrosis. Intraperitoneal injection of uric acid increases uric acid serum level, while allopurinol has the ability to reduce the production of uric acid through inhibit the xanthine oxidoreductase (XOR) enzyme.¹³ Based on our previous result, uric acid induction by intraperitoneal injection at dose of 125mg/kg/day, was significantly increased serum uric acid levels at the day-7 and the day 14.^{5,6} The result was associated with induction of inflammation and Epithelial to Mesenchymal Transition (EMT) in kidney, furthermore it also deteriorated liver function as shown in this study.

Hyperuricemia induced higher expression of inflammation cascade in the liver as shown by upregulation of Toll like Receptor-4 (TLR-4), Monocyte Chemoattractant Protein-1 (MCP-1) and CD68 as a marker of macrophage (Figure 1). Cell death might be associated with inflammatory effect of uric acid.⁷ Otherwise, uric acid also functions as cell death inducer due to reactive oxygen species production. Uric acid

in human plasma can act as antioxidants and pro-oxidants. Uric acid levels play a role as a regulator of extracellular superoxide dismutase (EC-SOD) which is an antioxidant enzyme and improve the generation of nitric oxide (NO), can prevent oxidative stress,¹⁴ as well as to stabilise the activity of vitamin E and C.² Xanthine oxidoreductase is a molybdoflavoprotein enzyme contained in two forms, xanthine dehydrogenase (XD) and xanthine oxidase (XO) which has the ability to produce ROS. At the same time when XO oxidises hypoxanthine to be xanthine and xanthine to uric acid, there will be donation of electrons to oxygen molecules that will form the radical superoxide (O₂) and hydrogen peroxyda (H₂O₂).¹⁴

Superoxide that reacts with NO will produce peroxynitrite which can induce nitrosated protein, peroxidation lipid and protein, and inhibit binding of tetrahydrobiopterin (HB4) with NOS, than produce ROS^{3,15} which may induce abnormal cell function and lead to cell death.¹⁴ Uric acid stimulates the release of the chemokine MCP-1 and the synthesis of interleukin (IL) -1 β , IL-1, and tumour necrosis factors (TNF) α . Cytokines TNF α , interferon (IFN) γ , IL-1 can increase uric acid production through increased activity of XO and cells damage which is mediated by ROS.¹⁶

An increase of uric acid serum level does not follow with crystal urate deposition. Some research suggested that soluble uric acid may contribute to the development of organ injury due to hyperuricemia. Soluble uric acid treatment in proximal epithelial tubules cell (PTECs) induces an increase of TLR4 mRNA expression. Then, the interaction between soluble uric acid and TLR4 promotes the production of various inflammatory chemokines such as MCP-1 through activation of the NF κ B.^{5,9,16} Therefore, we suggested that inflammation cascade pathways in the liver which including TLR-4/MCP-1 was initiated by higher uric acid level, then promoting macrophage infiltration. In addition, long-term inflammation enhances activation of the hepatic stellate cells, which are the main source of myofibroblast in the liver.¹⁷

Further, we investigated liver fibrosis induced hyperuricemia in this model. Using Sirius Red staining, we revealed fibrosis staining in the liver which occurred UA7 and UA14 groups (Fig 2). Liver fibrosis often appear in various chronic liver disease caused by the excessive accumulation of extracellular matrix, including collagens. Continuing fibrosis can lead to cirrhosis, portal hypertension and liver failures. The sources of collagens in the liver is activated hepatic stellate cells, portal myofibroblast and also myofibroblast derived from bone marrow which are activated by cytokine TGF β 1 fibrogenic, angiotensin II, and leptin.¹⁰ Uric acid might induce liver fibrosis in this study as shown by significantly increase of liver fibrosis score in UA7 ($p < 0.01$ vs. Control) and UA 14 ($p < 0.01$ vs. Control) group compared to control. Liver fibrogenesis is a complex process that involves many cells, especially hepatic stellate cells.¹⁸ The initial process liver fibrosis is the increased formed fibronectin in the space of Disse followed by an increase of collagen I, III, and IV, as well as laminin.¹⁹ There was significantly differences expression of collagen I between groups who received induction of uric acid for seven ($p < 0.05$) and 14 ($p < 0.01$) days and the control group.

To elucidate activation of HSCs in this study, immunostaining of GFAP was performed. It revealed higher HSCs number in group in uric acid treatment. Immunostaining demonstrated increased HSC activation as shown by HCC number was higher UA7 and UA14 groups compared to control. It seems that activation of inflammation might be followed by fibrogenesis in uric acid induced liver fibrosis. Kupffer cells that undergo activation will issue a paracrine signals to activate hepatic stellate cells. Activated hepatic stellate cells will generate ROS and TGF- β which can activate them self through autocrine pathway. TGF- β is a major fibrogenic cytokine that plays a role in regulating the production, degradation, and accumulation of extracellular matrix in the liver. Activated hepatic stellate cells express collagen type I, III, and IV and the mRNA that encodes collagen and laminin,²⁰ α -SMA, TGF- β 1, and Platelet-Derived Growth Factor β (PDGF β),²¹ and TIMP which would inhibit MMP, consequently collagen will prevent from degradation.²²

Allopurinol groups represented lower uric acid level, inflammation and fibrosis (Fig 1 and 2). Allopurinol plays a role in inhibiting inflammation and fibrosis caused by Bleomycin which were played by uric acid.²³ Allopurinol can inhibit production of MCP-1 and IL-6 rats vascular smooth muscle cells.²⁴ Research conducted by Kang (2002) in hyperuricemia mice showed that allopurinol can prevent smooth muscle cell proliferation and the afferent arterioles of kidney.³ Allopurinol can keep the liver from damage, induced by carbon tetrachloride (CCl₄) by inhibiting the nuclear translocation of NF- κ B, expression of TGF- β and induce MMP-13.²⁵ Elucidating another mechanism related to liver injury-induced uric acid, such as non-alcoholic fatty liver diseases (NAFLD)²⁶ may provide better underlying mechanism of high uric acid level to liver injury. It is needed in future studies to clarify lipid accumulation using Oil Red O staining or signalling pathways relate to NAFLD in high uric acid level model.

CONCLUSION

In conclusion, uric acid enhances liver fibrosis through upregulated the inflammatory mediators and the number of hepatic stellate cells.

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DISCLOSURE

The authors declare there is no conflict of interest in this research.

AUTHOR CONTRIBUTION

DC, AS and NA carried out the design of the study and

drafted manuscript: AS carried out RT-PCR analysis, and histopathological analysis. WA and MR carried out serum analysis and manuscript revision. NA and UT carried out histopathological examination and analysis. WT and DC carried out the RT-PCR examination and analysis. Approval of final manuscript: all the authors.

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