# The effects of furosemide on kidney damage in acute kidney injury rat models

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## ABSTRACT

The most frequent cause of acute kidney injury (AKI) is ischemia reperfusion injuries that causes inflammation. Furosemide is still used in AKI's therapy. The advantages and disadvantages of furosemide in AKI remain controversial. The aim of the study was to investigate the effect of furosemide on kidney damage in AKI rat models. Twenty-five male (2-3 months old) Sprague-Dawley rats were divided into 5 groups; sham operation (SO, n=5), ischemic-reperfusion (IR, n=5), IR+furosemide 3.6 mg/kgBW (IR+F1, n=5), IR+furosemide 7.2 mg/kgBW (IR+F2, n=5), and IR+furosemide 14.4 mg/kgBW (IR+F3, n=5). Abdominal surgery was performed under ketamine anesthesia to produce ischemic reperfusion (IR) by mean of renal artery clamping for 45 min. Urine output, serum creatinine level, tubular injury score, and TLR4 gene expression were examined to investigate kidney damage. Periodic acid-schiff (PAS) staining was measured to examine kidney tubular injury. Data were analyzed using One-Way ANOVA and Kruskal-Wallis test with significance level of $p<0.05$. AKI rat models which were given 3.6 and 7.2 mg/kgBW of furosemide (0.014±0.001 mL/min; and 0.012±0.007) showed higher ($p>0.05$) creatinine clearance compared to IR (0.009±0.003) while administration of 14.4 mg/kgBW furosemide (0.009±0.004) denoted equal creatinine clearance to IR ($p>0,05$). Kidney tubular injury score of 3.6 mg/kgBW furosemide (2.89±0.13) was lower ($p>0.05$) than IR (3.26±0.19) whereas 7.2 mg/kgBW and 14.4 mg/kgBW furosemide (3.55±0.26; 3.83±0.19) were higher ($p<0.05$) than IR. Administration of 3.6 mg/kgBW furosemide (0.99±0.08) indicated lower ($p<0.05$) TLR4 gene expression than IR (1.20±0.08) whilst 7.2 mg/kgBW furosemide (1.23±0.13) was not-significantly higher ($p>0.05$) and 14.4 mg/kgBW furosemide (1.63±0.12) was significantly higher ($p<0.05$) than IR. In conclusion, administration of 3.6 mg/kgBW furosemide reduces kidney damage in AKI rat models while higher dosages (7.2 mg/kgBW and 14.4 mg/kgBW) increase kidney damage.

## ABSTRAK

Penyebab *acute kidney injury* (AKI) yang paling sering adalah cedera iskemia-reperfusi sehingga menyebabkan timbulnya inflamasi. Pada penatalaksanaan AKI masih banyak digunakan furosemid. Keuntungan serta kerugian penggunaan furosemid pada AKI masih menjadi kontroversi. Penelitian ini bertujuan untuk mengetahui pengaruh pemberian furosemid terhadap kerusakan ginjal pada model tikus AKI. Sebanyak 25 ekor tikus jantan umur 2-3 bulan galur *Sprague Dawley* dikelompokkan menjadi 5 kelompok, yaitu *Sham Operation* (SO, n=5), *Ischemia reperfusion* (IR, n=5), IR+furosemid 3,6 mg/kgBB (IR+F1, n=5), IR+furosemid 7,2 mg/kgBB (IR+F2, n=5), dan IR+furosemid 14,4 mg/kgBB (IR+F3, n=5). Luaran urin, kadar kreatinin serum, skor cedera tubulus, dan ekspresi gen TLR4 diperiksa untuk mengetahui adanya kerusakan ginjal. Dilakukan pewarnaan *Periodic Acid-Schiff* (PAS) pada sediaan histopatologi untuk menilai skor cedera tubulus

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ginjal. Data dianalisis dengan *One Way* ANOVA dan Kruskal Wallis (p<0,05). *Creatinine clearance* pada model tikus AKI yang diberi furosemid dosis 3,6 mg/kgBB (0,014±0,001 mL/min) dan dosis 7,2 mg/kgBB (0,012±0,007) lebih tinggi dari IR (0,009±0,003) (p>0,05), sedangkan dosis 14,4 mg/kgBB (009±0,004) sama dengan IR (p>0,05). Skor cedera tubulus ginjal pada model tikus AKI yang diberi furosemid dosis 3,6 mg/kgBB (2,89±0,13) lebih rendah dari IR (3,26±0,19) (p<0,05), sedangkan dosis 7,2 mg/kgBB (3,55±0,26) dan 14,4 mg/kgBB (3,83±0,19) lebih tinggi dari IR (p<0,05). Ekspresi gen TLR4 pada model tikus AKI yang diberi furosemid dosis 3,6 mg/kgBB (0,99±0,08) lebih rendah dari IR (1,20±0,08) (p<0,05), sedangkan dosis 7,2 mg/kgBB (1,23±0,13) lebih tinggi dari IR (p>0,05), dan dosis 14,4 mg/kgBB (1,63±0,12) mg/kgBB juga lebih tinggi dari IR (p<0,05). Dapat disimpulkan, pemberian furosemid dosis 3,6 mg/kgBB dapat memperbaiki kerusakan ginjal pada model tikus AKI, sedangkan pada dosis lebih besar (7,2 mg/kgBB dan 14,4 mg/kgBB) memperburuk kerusakan ginjal pada model tikus AKI.

***Keywords***: acute kidney injury - ischemic-reperfusion – furosemide – creatinine – kidney tubular injury

## INTRODUCTION

Acute kidney injury (AKI) is a health problem due to increasing the incidence of AKI in both the developed and developing countries that increase mortality rate.[1–5] About 20% of AKI may progress to chronic kidney disease thereby increase the maintenance costs.[6,7] The use of furosemide in the treatment of AKI remains controversies. Furosemide improves GFR in patients with portal hypertension and ascites, as well as lower hyperkalemia, hyperchloremia, acidosis, and fluid overload on patien who are at risk of AKI.[8] Nonetheless, furosemide increase serum creatinine in cardiac surgery.[9] Furosemide is more effective than mannitol when given along with hydration fluids to prevent nephrotoxicity due cisplatin[10,11] but furosemide increase mortality in AKI patients with critical illness.[12] Furosemide in preclinical studies was known to decrease apoptosis and related gene expression in the IRI mouse model.[13] Inflammation is the main mechanism of AKI due to ischemia.[14] *Toll-like receptor4* (TLR4) activation is the major pathway of the innate imune response that started the kidney injury.[15] The study was conducted to investigate the effects of furosemide on kidney damage in AKI rat models.

## MATERIALS AND METHODS

### Animal model

Twenty-five Sprague-Dawley male 2-3 months old rats were used in the quasi-experimental study with post test only controlled group design. The rats were divided into 5 groups; sham operation (SO, n=5), ischemic-reperfusion (IR, n=5), IR+furosemide 3.6 mg/kgBW (IR+F1, n=5), IR+furosemide 7.2 mg/kgBW (body weight) (IR+F2, n=5), IR+furosemid 14.4 mg/kgBW (IR+F3, n=5). The sham operation used as control, and ischemic-reperfusion as AKI model.[16] Wheres in the group IR+F1, IR+F2, IR+F3 given furosemide 3.6, 7.2, 14.4 mg/BW, respectively, once giving after surgery. Determination of the dosage of furosemide according to Sinto and Nainggolan[17] which says that administration of furosemide can be useful in the first 12 h, and initially can be given intravenous furosemide bolus 40 mg. If the benefit is not visible the dosage can be doubled to 100-250 mg/times in 1-6 h rapidly or 10-20 mg/kgBW/d slowly with a maximum dose of 1 g/d. All animals terminated on day 3. All experimental procedures were conducted according to the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing,

Universitas Gadjah Mada, Yogyakarta. The ischemia-reperfusion injury model was performed under ketamine anesthesia 100 mg/kgBW. The AKI was induced by mean of clamping both renal pedicle using non-traumatic vascular clamp (Hammacher®) for 45 min. Then, both clamps were released and followed by reperfusion. The incision site closed using silk surgical thread 3/0 (One Med®). Blood serum was obtained from the retroorbital vein for creatinine measurement.

**Measurement of tubular injury**

Tubular injury score measurement was done by mean of Periodic Acid-Schiff (PAS) staining, examined with a light microscope (Olympus CX22®) and portraited with Optilab software with 400x magnification at the corticomedullary junction area as many as 15 fields per kidney. Scoring divided into 4 category, they were: 0-4 (0=normal; 1=tubular injury <25%; 2=tubular injury involve 25-50%; 3=tubular injury involve 50-75%; 4=tubular injury involve >75%). The assessment included renal tubular dilatation, loss of brush border of proximal tubules, depletion of the tubular epithelial cell and the accumulation of intraluminal cast.

**Gen expression examination**

Examination of TLR4 gene expression used RT-PCR. Total RNA was extracted using RNA Isoplus, followed by quantification of RNA concentration using sphectrophotometry. cDNA was made using Rever TraAce® (Toyobo, Japan, Cat.No.TRT-101) and random primer (Toyobo, Japan, Cat.No 3801), with PCR condition: 30°C for 10 min (denaturation), 42°C for 60 min (annealing), 99°C for 5 min (extension). Reverse transcriptase PCR was carried out to amplify the following specific cDNAs: forward rTLR4: 5' CAGGGAGCACGAGGCTTCTA-ACC-3', and reverse: 5'-CTTGTGCCCT-GTGAGGTCGTTGA-3'). PCR conditions: 94°C for 2 s (initial denaturation), 94°C for 10 s (denaturation), 60°C for 30 s (annealing) and 72°C for 1 min (extension) and 72°C for 10 min (last extension). The gene expression was quantified using ImageJ software. GAPDH was used as housekeeping gene.

**Statistical analysis**

Data were analyzed using Shapiro-Wilk test for normality and Levene test for homogeneity. Urine output, creatinine urine level, creatinine clearance, tubular injury score were analized by one-way ANOVA and followed by pos hoc LSD test. Creatinine serum level was analized by Kruskal Wallis test and followed by pos hoc Mann-Whitney test. A $p<0.05$ was used to determined the level of significance.

## RESULTS

Significantly difference in urine output between the SO group (7.8±1.79 mL) and IR group (15.00±3.08) on day 3 after surgery but non-significantly difference between IR group (15.00±3.08) and the treatment groups IR+F1 (10.30±4.66), IR+F2 (14.2±4.92), IR+F3 (14.40±2.41) were observed. Serum creatinine level of IR group [1.23 mg/dL (1.10-1.66)] was significantly lower than IR+F3 group [3.53 (3.01-4.71)] ($p<0,05$) and non-significantly higher than IR+F1 [0.76 (0.68-1.81)] ($p=0.151$) and IR+F2 [1.19 (1.09-3.01)] groups ($p=0.841$). The urinary creatinine level of IR group (0.98±0.15) was significantly lower than IR+F2 (2.16±1.31) ($p=0.049$) and IR+F3 (3.18±0.61) ($p=0.001$) groups but non-significantly higher than IR+F1 group (1.76±1.18) ($p=0.179$). There was non-significantly higher in creatinine clearance of IR+F1 (0.014±0.001) and IR+F2 (0.012±0.007) compare to IR group (0.009±0.003) and equal to IR+F3 group (0.009±0.004) ($p>0.005$).

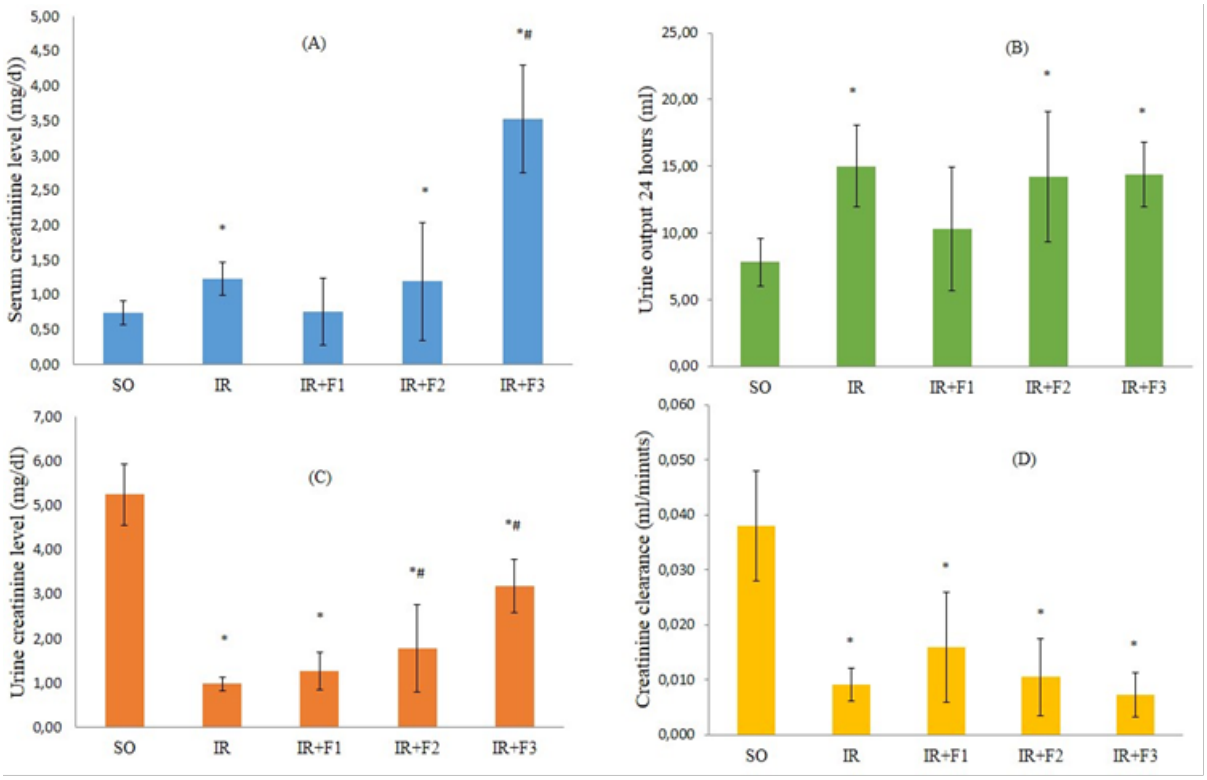


FIGURE 1. The effect of furosemide on serum creatinine level (A), urine output 24 hours (B), urine creatinine level (C), and creatinine clearance (D). * = p<0,05 vs SO, # =p<0,05 vs IR. SO (Sham Operation), IR (Ischemia-reperfusion), IR+F1 (Ischemia-reperfusion+furosemide 3.6 mg/kgBW, IR+F2 (Ischemia-reperfusion+furosemide 7.2 mg/kgBW), IR+F3 (Ischemia-reperfusion+furosemide 14.4 mg/kgBW).

The kidney tubular injury score of IR group (3.26±0.19) was significantly higher than IR+F1 group (2.89±0.13) (p=0.005) but significantly lower than IR+F2 (3.55±0.26) (p=0.024) and IR+F3 (3.83±0.19) (p=0.000) groups. Significantly lower in TLR4 gene expression of IR group (1.20±0.08) than IR+F1 group (0.99±0.08) (p=0.002) but non-significantly higher than IR+F2 (1.23±0.13) (p=0.680) and significantly higher than IR+F3 (1.63±0.12) (p=0.000) groups were reported.

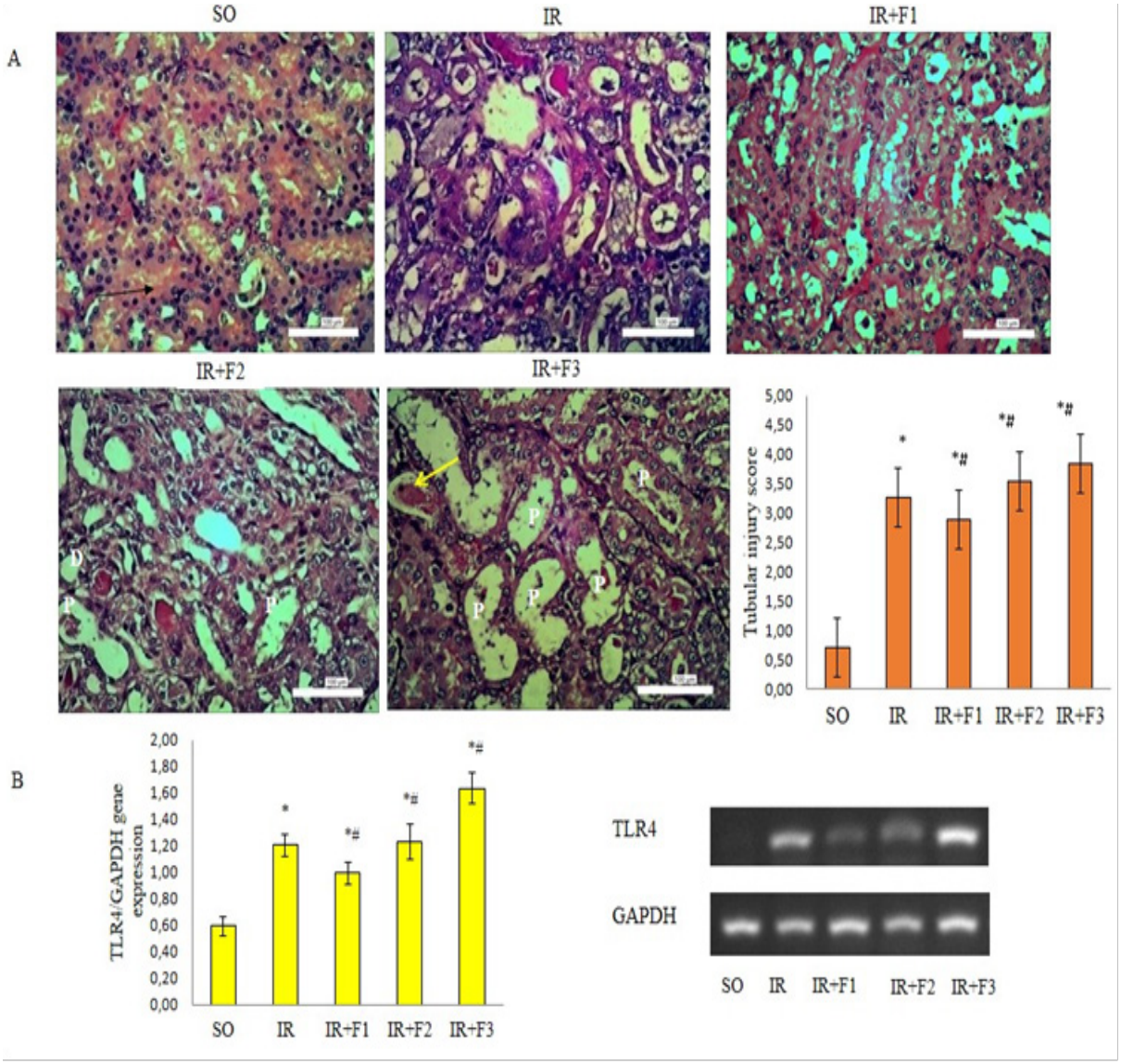


FIGURE 2. Renal histological picture on day 3 with PAS staining and tubular injury score (A). TLR4/GAPDH gene expression (B). * = p<0,05 vs SO, # =p<0,05 vs IR. SO (Sham Operation), IR (Ischemia-reperfusion), IR+F1 (Ischemia-reperfusion+furosemide 3.6 mg/kgBW, IR+F2 (Ischemia-reperfusion+furosemide 7.2 mg/kgBW), IR+F3 (Ischemia-reperfusion+furosemide 14.4 mg/kgBW). Black arrows showed brush border. Yellow arrows showed intraluminal cast. Yellow circle showed tubular dilatation.

## DISCUSSION

The volume of urine output in the IR group is higher than the SO group. This result similar with Younan *et al*.[18] study, that in mice IR group had higher urine volume at 24 and 48 h after reperfusion compared with SO. Ischemia-reperfusion causes damage primarily in the proximal tubule S3 segment and the thick ascending loop of Henle. Ischemia causes the cord region becomes increasingly diminished oxygenation, causing severe damage. Due to damage involving the proximal tubules, the renal function related to the formation of urine is impaired, especially reabsorption. If this reabsorption function is impaired by tubular cells damage, then only a little water can be reabsorbed, and most will be excreted. In the IR group that were given furosemide had urine volume higher than the

SO group but lower than the IR group. The volume of urine was higher with the higher dose of furosemide. The timing of giving furosemide may affect the process of kidney damage both the functional and structural. Furosemide guard against partial damage shown by the improvement of the medullary hypoxia during the AKI, the impact is in the early phase after the occurrence of AKI than ongoing AKI. However the greater the dose of furosemide given, the more the urine volume.[19]

Creatinine serum level in IR group was 1.23 (1.10-1.66) higher compared to that of SO group 0.74 (0.48-0.91). As explained by Wu *et al*.[20] and Younan *et al*.[18], there were creatinine level increase in IRI group. In normal condition, creatinine is filtered by glomerulus but not absorbed. About 10-20% creatinine is excreted to proximal tubules. Thus, any damage in tubules will affect the process. Consequently, the creatinine serum level becomes higher. Creatinine serum level in IR group receiving furosemide increased as furosemide dose increase. This is possibly because at the time of ischemia occurs ATP depletion, and when there is furosemide as a ligand which binds to the transporter as a site of action, the ATP should be used for repairs but used to work, and the greater number of ligand binding, ATP getting a much needed so that the kidney getting damaged because the heavier work. In contrast, Lassnigg *et al*.[9] study demonstrated creatinine serum level increase in cardiac surgery. The negative effect of furosemide probably due to neurohormonal activation dan temporary blood pressure increase as the result of sympathetic neural activation dan renin angiotensin system. Those mechanisms might increase peripheral vascular resistance, left ventricle afterload, heart workload, and cardiac output reduction. Thus, they might worsen myocardial ischemia. Moreover, renal blood flow maldistribution induction through medular perfusion diversion due to cortex vascular resistance decline might promote tubular disfunction.[9]

Urine creatinine level was elevated in SO group (5.25±0.69) and depleted in IR group (0.98±0.15). In physiological condition, creatinine was filtrated by the glomerulus and excreted through urine hence, the creatinine level was elevated in urine dan depleted in serum and if there is any damage causing low creatinine level in urine. Urine creatinine level in IR+F1 was higher compared to that of IR group (IR+F1 vs IR (1.76±1.18 vs 0.98±0.15). The greater furosemide dose given the higher urine creatinine level (IR+F1(1.76±1.18), IR+F2 (2.16±1.31), dan IR+F3 (3.18±0.61)). This condition contradicted with serum creatinine level in this study that demonstrated serum creatinine raise as furosemide dose increase.

Creatinine is catabolism yield of muscle creatinine and distributed to entire body fluid. Mostly, creatinine is excreted by the kidney. Creatinine has low molecular weight (113D) that facilitate its simple movement through glomerular filtration barrier into tubular filtrate. Creatinine is not reabsorbed nor affected by urine flow.[21] About 10-20% creatinine is secreted into proximal tubules. Active secretion done by tubular cells is facilitated by that are OAT1 (organic anion transporter), OAT2, OAT3, OAT4, OCT1 (organic cation transporter), OCT2, OCT3, OCTN1 (organic cation transporter novel), OCTN2, MATE1 and MATE2-K.[22–24] Correspondingly, furosemide requires the transporter to reach its target that are OAT1, OAT2, OAT3, and OAT4.[25–27] Kim *et al*.[26] demonstrated OAT1 elevation in rat renal after 7 days furosemide administration. and the rise of OAT1 and OAT3 expression in IR model.[28] There is some similar transporter that involved in creatinine and furosemide secretion. The possible explanation of higher urine creatinine level in higher furosemide dose is due to higher serum creatinine level in IR group receiving furosemide hence, the excreted creatinine in urine is greater.

Creatinine clearance in the SO group was higher than the IR group and differently significant ($p<0.05$), it shows that the function of renal excretion in the SO group is better

than the IR group and vice versa. Creatinine clearance in the IR+F1 and IR+F2 groups were higher than IR group, and IR+F3 equal to IR group ($p>0.05$). In accordance with Heyman *et al*.[29] that creatinine clearance in the group of acute renal failure (ARF) that were given furosemide were higher than the ARF group on the first day ($p<0.05$), and had a tendency to rise on the 3rd day but was not significant ($p>0.05$). Creatinine clearance describes the renal excretory function and can be used to predict GFR. The greater the creatinine clearance value showed the better kidney functions, and vice versa. In this study of kidney function in the group which were given the common dose of furosemide was better than that the group which did not receive furosemide, and the group which was given higher doses of furosemide had worse kidney function than the group that did not receive furosemide. In contrast to Lassnigg *et al*.[9] that administration of furosemide in cardiac surgery showed creatinine clearance lower than for the placebo group. It is associated with the activation of neurohumoral, increased blood pressure as a result of the activation of the sympathetic and the renin-angiotensin system.

The tubular injury is characterized by tubular dilatation, brush border loss, depletion of epithelial cell and intraluminal cast,[30] so that it causing kidney morphology changes.[31] Epithelial cell injury due to ischemic-reperfusion especially in the S3 segment of proximal tubules.[20] Our study showed that increasing of tubular injury score was in-line with increasing of furosemide dose. Heyman *et al*.[32] show that furosemide decreased structural and functional of tubular injury in S3, especially in the middle and the outer zone of the inner stripe of outer medulla kidney that has isolated and perfused. The decline in structural damage assessed from the decrease fragmentation in S3 tubules. The protective effect of furosemide in the kidney was correlated with of active reabsorption and reduced of oxygen required by the mTAL cells with limited oxygen supply. Loop diuretics increase the oxidation potential of cytochrome oxidase in whole perfused kidney and increases oxygen pressure in the outer medulla of kidney. It is showed protective effects toward the proximal tubule in-line with research Heyman *et al*.[32] Several clinical studies in-line with the results of this study, Cantarovich *et al*.[19] showed that high doses of furosemide can maintain urine output but has no effect on median survival and kidney repair in patients with AKI. It was showed by the improvement of medulla hypoxia during AKI. The impact may be more significant in the initial phase of the AKI than after AKI.[19] Furosemide has a weak inhibitor carbonic anhydrase enzyme impact.[33] Carbonic anhydrase enzymes play a role in the reaction between $CO_2$ and $H_2O$ into $H^+$ and $HCO_3^-$. Hydrogen ions that are secreted into the lumen of the kidney tubules to replace reabsorbed $Na^+$. The hydrogen ions in the luminal kidney tubules react with $HCO_3^-$ to form $H_2CO_3$. Hydrogen ions also react with the $NH_3^-$ to form $NH_4$. If the carbonic anhydrase enzyme is inhibited, $H^+$ will not be secreted into the tubular lumen. Therefore $NH_3^-$ will not be neutralized to $NH_4$, so $NH_3^-$ will damage kidney.[34]

TLR4 is a pattern recognition molecule doe to ischemic injury. Ischemic caused tubular and microvascular injuries, thus the integrity of cytoskeleton and cell polarity will be lost, brush border of proximal tubules loss, loss of polarity followed by the change of the adhesion molecules location and other membrane proteins such as $Na^+K^+$-ATPase and β-integrin.[35] The microvascular injury causes the disruption of blood flow, increased of leukocyte adhesion and increased of blood vessels permeability that causes a response inflammation.[36] Activation and epithelial damage led to the formation of inflammatory and vasoactive mediators, which provide feedback on the vasoconstriction and inflammation blood vessel. Furthermore, activation of innate immune system plays an important role in the initiation of acute injuries and acute on chronic.[14] Ischemic-reperfusion injury causes the activation of the innate immune system.

Activation of the innate immune system begins to bond TLR by endogenous ligands. The TLR4 has expressed by the kidney is a potential mediator of innate immune system activation and inflammation. Appropriate research Wu *et al*.[15] TLR4 gene expression in the kidney increased after ischemic, mainly expressed by cells tubulus. In this study, TLR4 gene expression was higher in IR group than SO group. TLR4 mediates the expression of pro-inflammatory cytokines and chemokines in the kidney during an IRI. There are two mechanisms that signaling pathway in TLR4: MyD88-dependent and MyD88-independent, until the process of transcription. In this study, IR+F3 has the highest TLR4/GAPDH among others. Group IR+F3 has the highest score of ischemic injury score in-line with the most severe inflammation. To determine the role of renal tubular epithelial cells in the inflammatory process may need to do further research on markers of epithelial cell damage in the renal tubules.

The results of this study were AKI rat models which were given 3.6 mg/kgBW and 7.2 mg/kgBW of furosemide (0.014±0.001 and 0.012±0.007 mL/min) showed higher creatinine clearance compared to IR (0.009±0.003) ($p>0.05$) while administration of 14.4 mg/kgBW furosemide (0.009±0.004) indicated equal creatinine clearance to IR ($p>0.05$). Kidney tubular injury score of 3.6 mg/kgBW furosemide (2.89±0.13) was lower than IR (3.26±0.19) ($p<0.05$) while 7.2 mg/kgBW and 14.4 mg/kgBW furosemide (3.55±0.26; 3.83±0.19) were higher than IR ($p<0.05$). Giving of 3.6 mg/kgBW furosemide (0.99±0.08) showed lower TLR4 gene expression than IR (1.20±0.08) ($p<0.05$) whereas 7.2 mg/kgBW furosemide (1.23±0.13) was non-significantly higher ($p>0.05$) and 14.4 mg/kgBW furosemide (1.63±0.12) was significantly higher than IR ($p<0.05$).

## CONCLUSION

Administration of 3.6 mg/kgBW furosemide reduces kidney damage in AKI rat models while higher dosages (7.2 mg/kgBW and 14.4 mg/kgBW) increase kidney damage in the used models. It shows that administration of 40 mg furosemide in the early phase of human AKI reduces kidney damage, but not to be increased of the dosage.

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