



Experimental Research

Analysis of mRNA and protein kidney injury Molecule-1 (KIM-1) expression in a kidney model during the initiation phase of ischemia reperfusion injury

Jerny Dase^a, Haerani Rasyid^b, Rina Masadah^c, Muhammad Husni Cangara^c, Agussalim Bukhari^{d,*}, Ressay Dwiyantri^e, Mochammad Hatta^f

^a Departement of Forensic & Medicolegal Faculty Medicine Hasanuddin University, Dr. Wahidin Sudirohusodo Hospital, Makassar, Indonesia

^b Nephrology and Hypertension Division, Department of Internal Medicine, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

^c Department of Anatomical Pathology, Faculty Medicine Hasanuddin University, Makassar, Indonesia

^d Department of Nutritional Sciences, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

^e Departement of Microbiology, Faculty Medicine Universitas Tadulako, Palu, Indonesia

^f Department of Microbiology, Faculty Medicine Universitas Hasanuddin Makassar, Indonesia

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ABSTRACT

Background: Kidney injury molecule-1 (KIM-1) is a transmembrane glycoprotein expressed predominantly on the proximal tubular epithelium.

Objective: We wanted to see if there was a critical time for increased tubular damage and its related biomarker, KIM-1 mRNA, and protein expressions during the first 24 h of ischemia-reperfusion injury.

Method: An Experimental research used five male Rattus Norvegicus rats in each group. Bulldog clamp was used to clamp renal arteries and veins to create renal ischemia. Immunohistochemistry was used for the analysis of KIM-1 protein expression. While Tubular Injury Score was examined by Histopathology. RT-PCR was used for KIM-1 mRNA expression.

Results: Tubular Injury Score (TIS) was significantly higher in ischemia than control. TIS remained similar after IR 30 min, peaked at IR 2 h, and decreased to the level of IR 30 min at IR 24 h.

The KIM-1 mRNA expression was also higher in ischemia than in control. Similarly, KIM-1 mRNA expression increased more after IR 30 min, IR 2 h, and IR 24 h.

The KIM-1 protein expression was higher in ischemia than in control. KIM-1 protein increased more after IR 30 min, IR for 2 h, and remained similar at IR for 24 h.

KIM-1 mRNA and protein expressions at IR 2 h were significantly different compared to ischemia but not significantly different compared to that in IR 24 h.

Conclusions: KIM-1 mRNA and protein expressions increased within 24 h IR with the critical time was in the 2 h IR.

1. Introduction

The proximal renal tubular epithelium is the part of the kidney that is most susceptible to injury in ischemic conditions. This section requires the most ATP, but the glycolytic capacity of this epithelium is minimal, so the availability of ATP is highly dependent on aerobic mitochondrial respiration [1]. The proximal tubular epithelium is susceptible to injury due to ischemia [2].

Reperfusion results in the reoxygenation of cells, but the injured cells

become more injured [3,4]. Therefore, renal ischemia-reperfusion injury (IRI) contributes to increased morbidity and mortality [5]. On the other hand, ischemia-reperfusion (IR) injury causes acute tubular necrosis (ATN) through oxidative stress mechanism [6–8], inflammation [8–13], and the “no-reflow” phenomenon [14–18].

KIM-1 is a transmembrane glycoprotein that is localized to the apical membrane of the proximal tubular epithelium and is highly expressed in the injured epithelium [19] [–] [23]. KIM-1 level correlates with the severity of the tubular injury [24,25]. Furthermore, the duration of the

* Corresponding author. Department of Nutritional Sciences, Faculty of Medicine, Hasanuddin University, Jl. Perintis kemerdekaan KM.10, Makassar, 90245, Indonesia.

E-mail addresses: jerny.pdf@gmail.com (J. Dase), haeraniabdurasid@yahoo.com (H. Rasyid), rinasadah@yahoo.com (R. Masadah), drhusni1977@gmail.com (M.H. Cangara), agussalim.bukhari@med.unhas.ac.id (A. Bukhari), ressy_chan@yahoo.co.id (R. Dwiyantri), hattaram@yahoo.com (M. Hatta).

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IR affects the severity of the IR injury in animal and human studies [26]. Therefore, this study wanted to evaluate the crucial time for increased tubular damage and its associated biomarkers, KIM-1 mRNA, and protein expressions during 24 h of IR.

2. Materials and methods

2.1. Study design

An experimental animal study was conducted from August 2019 to January 2020. All experimental protocols employed in this study were approved by the Medical Research Ethics Committee of Hasanuddin University Makassar, Indonesia (Reference No. 1010/UN4.6.4.5.31/PP36/2019). In addition, the work has been reported according to the ARRIVE guidelines [27].

2.2. Experimental animals

Male *Rattus Norvegicus*, Wistar strain, bodyweight 200–300 mg, aged 10–12 weeks received from Animal Biopharmaceutical Laboratory, Faculty of Pharmacy, Hasanuddin University. A simple random sampling procedure was used to select the sample. According to Federer's formula, 25 samples were determined and were separated into five groups: negative control ($n = 5$), ischemia ($n = 5$), ischemia-reperfusion for 30 min ($n = 5$), ischemia-reperfusion for 2 h ($n = 5$), and ischemia-reperfusion for 24 h ($n = 5$). The animals were kept at a constant temperature of 25 °C, 12 h light-dark cycle, diet and water were provided *ad libitum*. The rats were acclimatized and treated under the Ethical principles of animal use and care.

2.3. Experimental procedures

Using all techniques and hands-on protocols at the Anatomy Laboratory, Gajah Mada University, and Hasanuddin University Medical Research Centre (HUMRC) Laboratory. Anesthesia was achieved by intraperitoneal injection of 0.1 mL per 20 g of body weight mixture of 1.75 mL of ketamine (100 mg/mL) + 0.25 mL of xylazine (100 mg/mL) + 8 mL of sterile water. The renal arteries and veins were clamped on the right and left kidneys using a non-traumatic vascular micro-clamp (bulldog clamp). If a uniform ischemic appearance were shown in both kidneys, the ischemia induction was considered successful and was maintained for 30 min. Renal arteries and veins were not clamped in the control group.

Release of arterial and venous clamps allowed for reperfusion. The induction of reperfusion was successful if the ischemic appearance in the kidney returned to normal appearance.

The rats were sacrificed by intraperitoneal injection 0.1 mL/20 g BW mixture of 1.75 mL ketamine (100 mg/mL) + 0.25 mL xylazine (100 mg/mL) + 8 mL sterile water. An incision was made from the median line to the lateral side of the abdominal wall to the peritoneal cavity after complete anesthesia. The ribs were cut from the lower to the higher lateral side to open the anterior thoracic wall. A needle connected to a perfusion pump machine hose was used to puncture the heart at the apex while still beating. A small incision was made in the right atrial wall (auricula) to relieve intracardiac pressure. The heart was then perfused with normal saline solution through the apex of the heart until the blood in the circulation was clear (marked by the liver, kidneys, and other organs being paler in color).

2.4. Experiment outcomes

Immunohistochemistry (IHC) for KIM-1 protein expression and histopathological studies for tubular injury score were examined using the right kidney's tissue. The left kidney was removed for RT-PCR analysis of KIM-1 mRNA expression.

3. Sample collection and examination

3.1. KIM-1 mRNA expression

The left kidney was removed and stored at -80 °C for RT-PCR to measure the quantity of KIM-1 mRNA. KIM-1 F CTGGAATGGCACTGTTGACATCC, KIM-1R GCAGATGCCAACATAGAAGCCC (Cat No. MCG0050); iSCRIPT cDNA Synthesis, 25R, BioRad Laboratories (Cat No. 1708890); EVA Green SMX 200R, BioRad Laboratories (Paint No. 1708890); EVA Green (Paint No. 1725200). All of these items were ordered from PT Scienwerke Jakarta. RT-PCR was performed using the PCR-Bio-Rad BR004129USA machine. A mixture of 22.5 μ L PCR Mastermix and SYBR green QRT was prepared. DNA extract of 2.5 μ L was added to a 22.5 μ L mixture of PCR mix. First stage amplification was performed at 94 °C for 2s and continued up to 40 cycles 60s at 94 °C, and 45s at 57 °C. The expression of mRNA was calculated according to the previous study [28].

3.2. Tubular injury and KIM-1 protein expression

The right kidney was removed and stored/soaked in a 4% PFA (paraformaldehyde) solution for histological examination and immunohistochemistry (IHC). For histological examination, tissue sections were stained with HE using a Hematoxylin-Eosin Staining kit. Histological alteration in the renal tubular interstitium was assayed according to the quantitative measurements of tubular damage, characterized by tubular epithelial swelling, degeneration, necrosis, tubular ectasia, inflammatory cell infiltration, and tubular interstitial fibrosis, evaluated at a magnification of 200x. The degree of tubular damage was scored using the following criteria: 0, normal; 1, damage area of 25% of tubules; 2, damage area of 25–50% of tubules; 3, damage area of 50–75% of tubules; 4, damage area of 75–100% of tubules. KIM-1 protein expression was analyzed using IHC with Novusbio TIM-1/KIM-1/HAVCR polyclonal antibodies, size 100 L, Catalog No.: NBP1-76701 were used. PT Vantagebio Scientific Solution in Jakarta was responsible for ordering the antibodies. Semiquantitative analysis was categorized as 0 (no staining or extremely weak expression), 1 (mild staining area, expression 1–25%), 2 (expression 26–50%), 3 (expression 51–75%), 4 (expression >75%) [44].

3.3. Statistical analysis

As data were not normally distributed, the differential expression values of KIM-1 mRNA, KIM-1 protein, and histology were analyzed using the Kruskal Wallis and Mann-Whitney test. The data was presented as median \pm interquartile range. The Spearman correlation test was used to examine the correlation between KIM-1 mRNA, KIM-1 protein, and Tubular Injury Score. p -value < 0.05 was considered significant. IBM SPSS 23 (IBM, New York, United States) was used for statistical analysis.

4. Results

4.1. Kidney tubular injury after ischemia and reperfusion

The median score of tubular injury was significantly higher ($p = 0.011$) in the ischemia group (2.00 ± 1.00) than in the control group (0.00 ± 0.50). In addition, tubular injury scores remained similar after 30 min of IR (2.00 ± 1.00) and increased after 2 h IR (4.00 ± 2.00) but subsequently decreased after 24 h IR (2.00 ± 0.50). Tubular injury scores increased after reperfusion IR for 30 min, IR for 2 h, and IR for 24 h, and they were significant when compared to ischemia and control ($p < 0.05$). The details are shown in Table 1, Table 2, and Fig. 1.

4.2. KIM-1 mRNA and protein expression after ischemia and reperfusion

The average expression of KIM-1 mRNA was significantly higher (p

Table 1
Comparison of KIM-1 mRNA and KIM-1 protein expressions and Tubular Injury Score.

Groups	KIM-1 mRNA (fold change) Median Mean ± IQR n = 5	KIM-1 mRNA (fold change) Mean ± SD	KIM-1 protein (score) Median Mean ± IQR n = 5	KIM-1 protein (score) Mean ± SD n = 5	Tubular Injury Score (score) Median Mean ± IQR n = 5	Tubular Injury Score (score) Mean ± SD n = 5
Control	0.35 ± 0.51	0.42 ± 0.26	1.00 ± 1.00	0.60 ± 0.44	0.00 ± 0.50	0.20 ± 0.40
Ischemia	1.14 ± 0.41	1.28 ± 0.29	1.00 ± 1.50	0.80 ± 0.83	2.00 ± 1.00	1.60 ± 0.54
IR 30 min	3.14 ± 2.69	2.60 ± 1.41	2.00 ± 1.50	2.20 ± 0.83	2.00 ± 2.00	2.00 ± 1.00
IR 2 Hours	6.15 ± 2.72	5.12 ± 2.06	3.00 ± 1.00	2.60 ± 0.89	4.00 ± 2.00	3.20 ± 1.09
IR 24 Hours	9.01 ± 8.58	7.45 ± 4.74	3.00 ± 0.50	2.80 ± 0.44	2.00 ± 0.50	2.20 ± 0.44
p-value*	0.006		0.003		0.004	

IR: Ischemia-Reperfusion, IQR: Interquartile range, *Kruskal Wallis test.

Table 2
Comparison of Tubular Injury Score, KIM-1 mRNA, and KIM-1 Protein Expressions amongst the groups.

Variables	Groups	p-value*
Tubular Injury Score	Control vs. Ischemia	0.011
	Control vs. 30 min IR	0.012
	Control vs. 2 h IR	0.006
	Control vs. 24 h IR	0.005
	Ischemia vs. 30 min IR	0.502
	Ischemia vs. 2 h IR	0.031
	Ischemia vs. 24 h IR	0.093
	30 min IR vs. 2 h IR	0.106
	30 min IR vs. 24 h IR	0.734
	2 h IR vs. 24 h IR	0.120
KIM-1 mRNA	Control vs. Ischemia	0.009
	Control vs. 30 min IR	0.009
	Control vs. 2 h IR	0.009
	Control vs. 24 h IR	0.076
	Ischemia vs. 30 min IR	0.251
	Ischemia vs. 2 h IR	0.016
	Ischemia vs. 24 h IR	0.117
	30 min IR vs. 2 h IR	0.047
	30 min IR vs. 24 h IR	0.117
	2 h IR vs. 24 h IR	0.465
KIM-1 Protein	Control vs. Ischemia	0.729
	Control vs. 30 min IR	0.017
	Control vs. 2 h IR	0.014
	Control vs. 24 h IR	0.006
	Ischemia vs. 30 min IR	0.041
	Ischemia vs. 2 h IR	0.021
	Ischemia vs. 24 h IR	0.009
	30 min IR vs. 2 h IR	0.343
30 min IR vs. 24 h IR	0.189	
2 h IR vs. 24 h IR	0.881	

* Mann-Whitney test.

= 0.009) in the ischemia group (1.14 ± 0.41) than in the control group (0.35 ± 0.51). KIM-1 mRNA further increased after IR for 30 min (3.14 ± 2.69), IR for 2 h (6.15 ± 2.72), and IR for 24 h (9.01 ± 8.58) (Table 1, Table 2 and Fig. 2). KIM-1 mRNA expression for IR 30 min was significantly different (p = 0.047) compared to IR 2 h but was not significantly different (p = 0.117) compared to IR 24 h (Fig. 2). The increase of KIM-1 mRNA expressions was accompanied by an increase of KIM-1 protein expressions. However, the KIM-1 protein expression was not significantly different (p = 0.729) in the ischemia group (1.00 ± 1.50) compared with the control group (1.00 ± 1.00). KIM-1 protein expression increased significantly (p = 0.017) after IR for 30 min (2.00 ± 1.50), IR for 2 h (3.00 ± 1.00, p = 0.014), and IR for 24 h (3.00 ± 0.50, p =

0.006) compared to the control group (Table 1, Table 2, Fig. 3, and Fig. 4). KIM-1 protein expression for IR 30 min was not significantly different compared to IR 2 h (p = 0.343) and IR 24 h (p = 0.189). Similarly, KIM-1 protein expression for IR 2 h was not significantly different compared to IR 24 h (p = 0.881).

4.3. Correlation between KIM-1 gene expression, KIM-1 protein expression, and Tubular Injury Score on ischemia, IR 30 min, IR 2 h, and IR 24 h

A Spearman correlation test was used to perform a correlation analysis. Ischemia and reperfusion had a substantial influence on tubular damage, KIM-1 mRNA, and KIM-1 protein expression. Tubular damage was found to be substantially linked with KIM-1 mRNA (r = 0.751; p = 0.000) and protein levels (r = 0.645; p = 0.001). The expression of KIM-1 mRNA was shown to be substantially linked with KIM-1 protein (r = 0.805; p = 0.000). The correlation in detail is shown in Table 3.

5. Discussion

The availability of ATP in the renal proximal tubular epithelial cells is highly dependent on the availability of oxygen. Therefore, the tubular epithelium is highly susceptible to ischemic injury. Brief ischemia (10 min) will result in a 20% decrease in renal ATP levels. In reperfusion, injury is increased as a result of oxidative stress [6–8], inflammation [9–11] and “no-reflow” phenomenon [14–16,18,26]. The causes of “no-reflow” are mainly endothelial oxidative stress, fibrinogen thromboembolic formation, neutrophil adhesion to capillary endothelium, endothelial dysfunction, increased vasoconstrictor, and decreased vasodilator. Thus, the “no-reflow” phenomenon results in prolonged ischemia during the reperfusion period [15,17,18,30]. In the present study, ischemia for 30 min caused significant tubular injury compared to no ischemia (control). After reperfusion for 30 min, the injury was not significantly different compared to that in ischemia. These findings were not in line with a previous study that found severe tubulointerstitial injury occurred after 30 min of reperfusion compared with controls [4]. A significant increase in reperfusion injury over ischemic injury occurred after 2 h of reperfusion, then decreased at 24 h of reperfusion. This shows that 2 h of reperfusion is a critical time for IR injury. This is due to a significant “no-reflow” phenomenon at 2 h of reperfusion, where the severity of this no-reflow correlates strongly with the severity of the microvascular injury. After 24 h, the level of injury was still higher than in control, although not significantly different than those in ischemia and 2 h after IR. This indicated that “no-reflow” might have been improved after 24 h of reperfusion or not as severe as at 2 h of reperfusion [26]. Cell death on reperfusion can be through the mechanisms of necrosis, apoptosis, necroptosis, and autophagic [12,31,32].

KIM-1 is a transmembrane glycoprotein that is found on the apical membrane of the proximal tubular epithelium and is highly expressed on injured epithelial cells [20,23,33–35]. The KIM-1 level was related to the severity of tubular injury [25,36]. Results of this study found that KIM-1 mRNA and protein expressions were increased after 30 min of reperfusion compared to ischemia (Table 1, Table 2, Figs. 2 and 3). This study supports previous studies on KIM-1 expression that found reperfusion at 30 min of ischemia results in more progressive ischemia within 30–180 min of reperfusion, leading to persistent cell death and progressive atrophy [3]. A significant increase in KIM-1 expression occurred after 2 h and not 24 h of reperfusion. We support a previous study that found a progressively increased reperfusion injury with an increase in the extent of the injury after the first 2 h of reperfusion [26]. Elevated KIM-1 also indicates increased tubular inflammation [37]. Thus, increased KIM-1 mRNA and protein expressions at 2 h of reperfusion can increase the migration and proliferation of tubular epithelial cells for the recovery of injured cells [38,39]. This finding is essential for managing acute tubular injury in renal ischemia-reperfusion with the best therapeutic intervention prior to the first 2 h of ischemia-reperfusion.

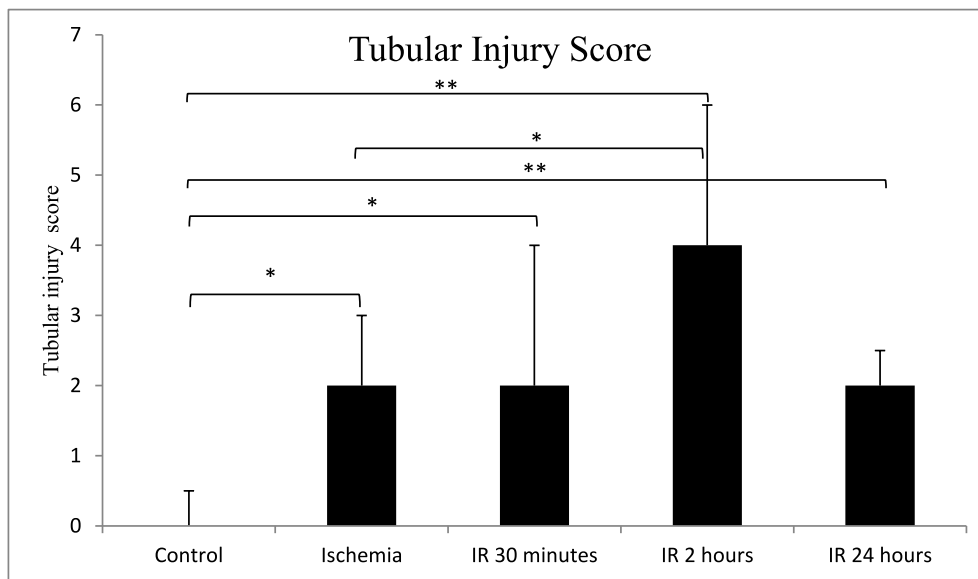


Fig. 1. Comparison of kidney tubular injury score between control, ischemia, ischemia reperfusion (IR) 30 min, 2 h and 24 h groups. * $p < 0.05$, ** $p < 0.01$ using Mann-Whitney test. Groups without asterisk mark (*) or (**) are not statistically significant. Data are expressed as Median \pm IQR. $n = 5$ for each group.

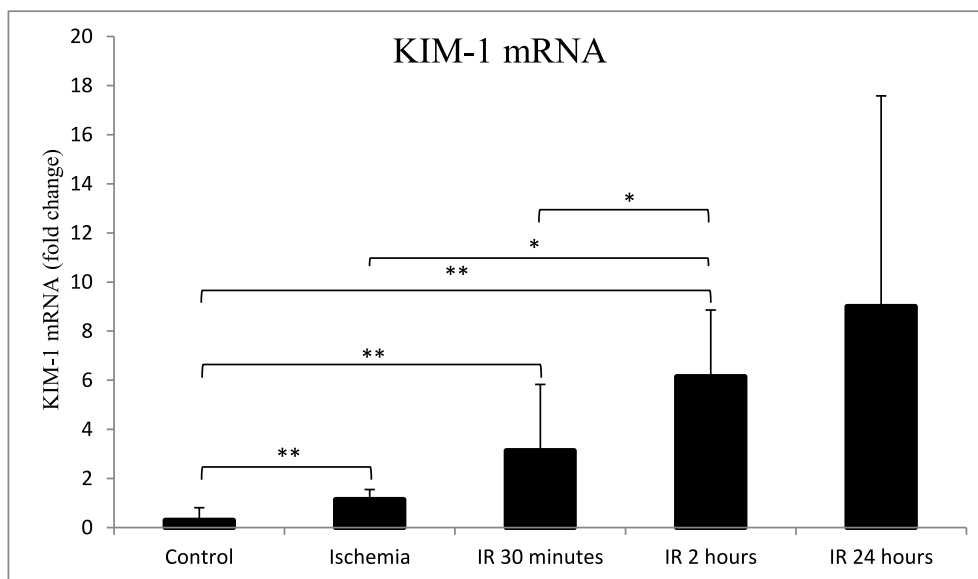


Fig. 2. Comparison of kidney mRNA KIM-1 expression between control, ischemia, ischemia reperfusion (IR) 30 min, 2 h and 24 h groups. * $p < 0.05$, ** $p < 0.01$ using Mann-Whitney test. Groups without asterisk mark (*) or (**) are not statistically significant. Data are expressed as Median \pm IQR. $n = 5$ for each group.

The limitations of this study were that the sample size was small, and the expressions of KIM-1 and the level of tubular injury at the time points between 2 h and 24 h of reperfusion were not measured.

6. Conclusion

Tubular injury, KIM-1 mRNA, and protein expressions increased linearly up to 2 h and decreased after 24 h, indicating that the critical time may be the first 2 h of reperfusion.

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Ethical approval

Ethical approval has been approved by the ethics commission of Faculty of Medicine, Hasanuddin University reference no. 1010/UN4.6.4.5.31/PP36/2019.

Consent

Not applicable to the study.

Author contribution

Jerny Dase (JD), Haerani Rasyid (HR), Rina Masadah (RM), Muhammad Husni Cangara (MHC), initiated and designed the study. JD, HR, RM, Agussalim Bukhari (AB), Mochammad Hatta (MH), Ressay Dwiyantri (RD), drafted and wrote the manuscript. MHC, Haerani Rasyid

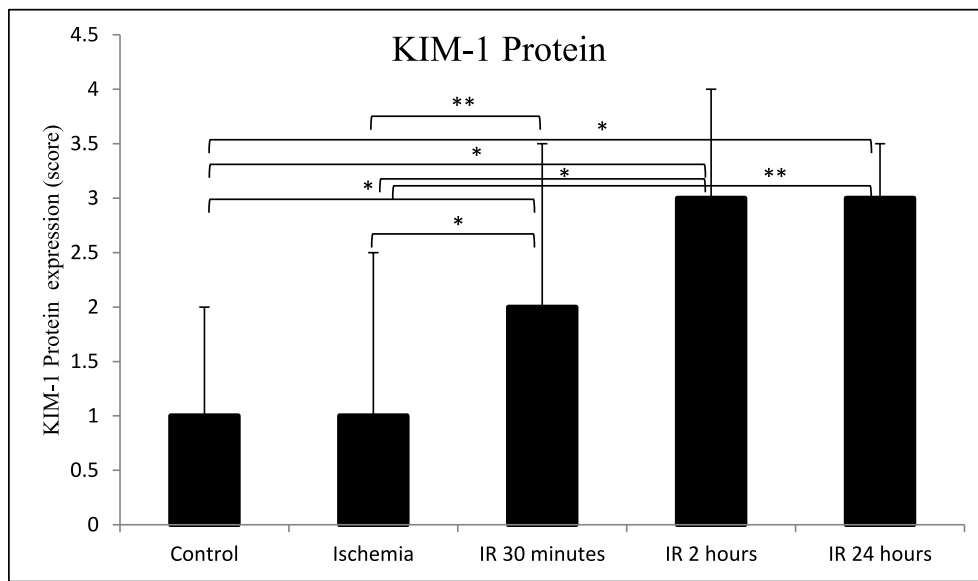


Fig. 3. Comparison of kidney KIM-1 protein expression between control, ischemia, ischemia reperfusion (IR) 30 min, 2 h and 24 h groups. * $p < 0.05$, ** $p < 0.01$ using Mann-Whitney test. Groups without asterisk mark (*) or (**) are not statistically significant. Data are expressed as Median \pm IQR. $n = 5$ for each group.

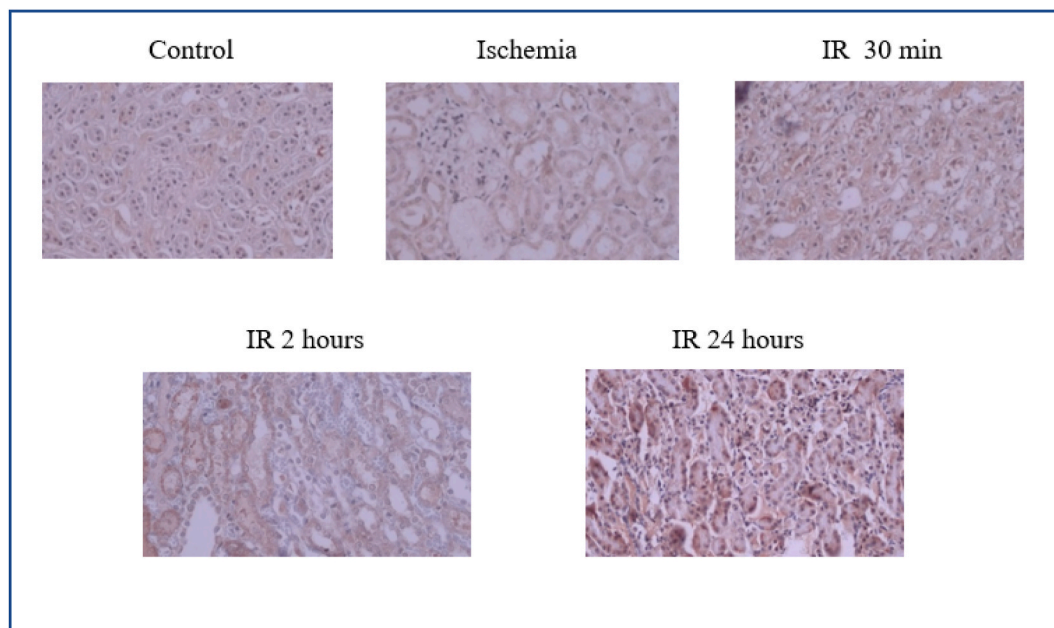


Fig. 4. Level expression of kidney KIM-1 protein in control, ischemia, ischemia reperfusion for 30 min, 2 h, and 24 h groups. $n = 5$ for each group.

Table 3
Correlation amongst KIM-1 mRNA, KIM-1 protein expressions and Tubular Injury Score.

Groups	r	P^a
Tubular injury vs. KIM-1 mRNA	0.751	0.000
Tubular injury vs. KIM-1 Protein	0.645	0.001
KIM-1 mRNA vs. KIM-1 Protein	0.805	0.000

^a Spearman Correlation test.

(HR), Rina Masadah (RM), contributed in the data processing. All authors have read and approved the final manuscript.

Registration of research studies

This study was not involving human participants. Registration was not applicable.

Guarantor

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Provenance and peer review

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Declaration of competing interest

The authors declare there is no conflict of interest.

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