Indian Journal of Experimental Biology
Vol. 59, April 2021, pp. 270-274

Immunohistochemical studies on progressive pathology of ischaemia reperfusion acute kidney injury in male Sprague Dawley rats

Amita Dubey[1]*, KS Prajapti,[2] BP Joshi[2] & Madhu Swamy[1]

[1]Department of Veterinary Pathology, College of Veterinary Science and AH, Jabalpur, Madhya Pradesh, India

[2]Department of Veterinary Pathology, College of Veterinary Science and AH, Anand, Gujarat, India

Received 15 April 2018; *19 February 2021*

Acute kidney injury (AKI) is a major problem in ICU patient, majority of the cases even after functional recovery ended with chronic kidney disease. In the present study, we investigated the immunohistochemical change in progressive pathology of ischaemia reperfusion induced acute kidney injury. Male Sprague Dawley (SD) rats were subjected to 35 min of renal ischemia followed by reperfusion periods of day 1, 4, 7, 14 and 28. Renal injury was confirmed by the increase serum creatinine level. The vascular and tubular changes in kidneys were studied by the CD31 and vimentin immunohistochemistry respectively. In this study, no significant changes were observed in CD31 positive cells, though lower densities of capillaries were noticed in the tubulo-interstitial lesion on day 28 of reperfusion. Significant increase in vimentin positive cells, which is a marker of undifferentiated cells, was observed on day 4 and day 7 of reperfusion. The immature cells were also present on day 28 of reperfusion indicating that these cells may would have led further progressive pathological changes in kidney even after the functional and morphological normalization.

Keywords: CD31, Vimentin, image analysis, chronic kidney disease

Acute kidney injury (AKI) conventionally known as acute renal failure (ARF), is a potentially catastrophic complication in hospitalized patients and affects millions of people worldwide. Despite advances in preventative strategies, therapeutic option and support measures, this disease continue to be associated with significant high morbidity, mortality and cost[1-3]. Clinically, AKI is a characterized by abrupt and reversible kidney dysfunction. It associates with rapid decline in glomerular filtration rate (GFR) and retention of nitrogenous waste product such as creatinine and blood urea nitrogen (BUN). Descriptions of AKI had been found date back to the ancient Greek period, when the diagnosis was possible only by observing a reduction in urine volume. It is usually an asymptomatic disease and diagnosed only on routine biochemical screening of hospitalized patients[4-6]. Though AKI is a worsening problem, but its true incidence is unknown worldwide, majorly due to underreporting or different criteria used for definition and diagnosis, especially in developing countries. However, epidemiological data from various recent multicentre observational or administrative database and retrospective studies, showed that AKI occurred in 5-7% of all patient admitted to the hospital and up to 36-37% in critically ill patient admitted to an intensive care unit . In India, a yearly incidence of 6.4 per 1000 admission has been reported for patients with most severe AKI and has an independent effect on the risk of death[7,8]. The incidence of AKI is especially high in patients (5-30%) undergoing cardiac surgery, with five fold higher mortality rate and cost.

Etiologically AKI may be classified into pre renal (55-60%) (functional response of structurally normal kidneys to hypoperfusion), intrinsic renal (35-40%) (involving structural damage to the renal parenchyma), and postrenal (<5%) (urinary tract obstruction). In the present study we have addressed the intrinsic AKI which has emerged as the most common and serious subtype in hospitalized patients and pathologically associated with acute tubular necrosis (ATN) and poor prognosis with a mortality rate of 40 to 80% in the intensive care setting[9,10].

It is calamitous problem in ICU patients world wide. Patients survived from the acute kidney injury consider being recovered completely but recently experimental studies in laboratory and epidemiological data from the field did point the fact that recovery from AKI is often incomplete and may end up as chronic kidney disease or end stage renal failure[1]. Ischemia reperfusion (I/R) AKI results from the complex reaction between tubules, vessels and interstitium which may further generate the after events[8-10]. Scientific community is debating on the culprit and eager to understand the progressive pathological changes of AKI to identify the patient at risk. A group of thought blame to the renal tubular factors, in-contrary to the vascular factors by other scientists. Considering this lacuna, the present study

*Correspondence:
Phone (Mob.) +91 9407426863
E-Mail: amiabhishek@rediffmail.com..

was designed to understand the changes in renal tubules and peritubular capillaries immuno-histochemicallly after I/R AKI.

Material and Methods

Male Sprague Dawley rats, weighing 250 to 350 gram after a brief acclimatization period, under went 35 minutes of bilateral renal ischemia reperfusion surgery as described by Chattergee and Thiermann[10] in pentobarbitone anesthesia with all aseptic precautions. Rats were maintained under standard care and management practices of "Committee for the purpose of control and supervision of experimental animals (CPCSEA)", provided with adlib feed and water. Kidney injury was confirmed by analysing serum creatinine level 24 hour after surgery and rats showing serum creatinine level 3-4 mg/dL were grouped (6 rats/group) according to their reperfusion period i.e. day 1, 4, 7, 14 and 28[11-13]. Experimental procedures were reviewed and approved by the institutional animal ethical committee (IAEC).

Histotechnique

At the end of reperfusion period rats were humanely euthanized and subjected to detailed necropsy examination. Kidneys from all the rats were collected in 10% neutral buffered formalin, middle portion approximately 2 mm thickness was processed for paraffin embedding and sectioning[14]. Four micron thin serial sections were taken for CD31 and vimentin immunohistochemistry.

Immunohistochemistry

Paraffin sections of rat kidney were deparaffinized in xylene and hydrated serially in graded alcohol to water for immunohistochemical staining of endothelial marker (CD31) and immature tubular marker (vimentin). In brief, endogenous peroxidase activity was blocked by immersing the slides in 3.5% H_2O_2 in methanol for 30 min. Antigen retrieval was performed in 1X EDTA-Tris buffer under pressure. Non specific antigens were blocked by the incubation of section with 1 X Rodent block R (Biocare, RBR962) for 60 min at room temperature of 25ºC. Then after wiping excess rodent block R, section was incubated with 1: 6000 dilution of primary polyclonal antibody –PECAM-1(CD-31), Clone M-20 (Santa Cruz Biotechnology, Inc, sc-1506-R) or 1:200 dilution of primary antibody – Anti-vimentin monoclonal antibody, clone V9. (Biogenex, MU074-UC) for over night at 2-8ºC. Next day after thorough washing with tris buffer saline (TBS) rabbit on rodent HRP- polymer (Biocare, RMR 622) or mouse on rat HRP-polymer (Biocare, MRT621) with XR factor (Biocare, XRF964) was applied for 30 min. Then TBS washed sections were incubated with peroxidase substrate diaminobenzidine (DAB) (Biocare, BDB2004) for 5 min in dark chamber. Lastly, slides were rinsed in deionized water and counterstained with hematoxylin. Negative control was run by omitting the primary antibody to check the specificity and sensititivey of the antibody[14,15].

These immunhistochemically stained sections were subjected for microscopic evaluation using Leica DM2500 microscope DFC295 camera and further subjected to image analysis by Image proplus software version 6.0, to calculate the cappilary density and area of immature cells.

Measurement of capillary density

Inner medulla of CD31 immunostained kidney sections were photographed under high power field (HPF) i.e. 40X. With the help of image analysis software, manual tagging method, number of peritubular capillaries per HPF were counted[15,16]. Entire renal section was evaluated qualitatively under light microscope for capillary distribution.

Measurement of vimentin positive area

The outer stripe of renal medulla in vimentin immunostained kidney section was evaluated for this parameter. Three random fields from each renal section were photographed under LPF i.e. 10X. By colour segmentation technique of calibrated image analysis software vimentin positive brown color % area per LPF were measured[17-19].

Statistical analysis

Data was subjected to statistical analysis by "One way ANOVA" followed by Dunnett test to study the between and within the group changes.

Results

Microscopical Examination

Severe acute tubular necrosis was evident in kidney of day 1 reperfusion. By day 4 of reperfusion, hyperplasia of basophilic tubules as solid masses were clearly evident and by day 7 of reperfusion, reorganization of the tubular structure with mineralization of necrosed materials were observed. Whereas, by day 14 of I/R, these tubules regenerated near to normal structure. At the end of 28 days of reperfusion period, majority of renal tubules became functionally normal but foci of tubulointerstitial lesion with increased interstitial space, dilated tubules were still clearly evident. (Table 1)

Table 1 — Image analysis data of CD 31 and Vimentin IHC

Groups	Reperfusion period	No of capillaries/ HPF of medulla	% Area of vimentin positive cells/LPF
I	DAY 1	39.33±4.25**	3.01± 0.56
II	DAY 4	54.50 ±3.21	85.95±1.68**
III	DAY 7	62.00±3.25	52.09±1.96**
IV	DAY 14	64.00±5.21	43.55 ±3.36**
V	DAY 28	50.42±3.48	36.18±4.033**
VI	SHAM control	62.75±4.18	4.29±0.67

[** *P* <0.01 level; HPF, High power field; LPF, Low power field]

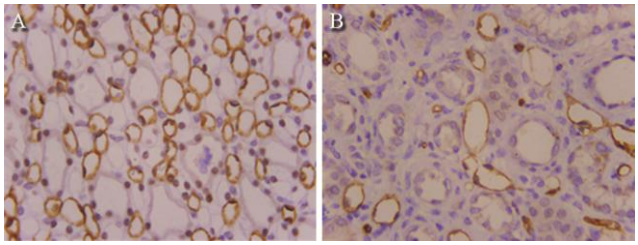

Fig. .1 — Section of rat kidney showing brown colored CD31 positive capillaries, a) normal density, b) decrease density in the area of tubulointerstital lesion on day 28 of reperfusion.CD31 IHCX400.

Capillary density by CD31 immunohistostaining

CD31 is a marker of endothelial cells, gives the brown coloration to the endothelial lining of the blood vessels. On day 1 after I/R AKI, number of peritubular capillaries per high power field (HPF) of medulla and area covered by them was found to be decreased (*P* <0.05) as compared to the sham control group. However, at further points of reperfusion period in our experiment, no significant decrease in capillary density of I/R AKI rats was noticed. . Though on day 28, in area of tubulointerstitial lesion in cortex, reduced number of peritubular capillaries were evident (Fig. 1).

Percentage area of immature tubules by vimentin staining

Vimentin is a marker of undifferentiated cells, gives the brown coloration to the undifferentiated mesenchymal tubular cells, which is usually not expressed by the mature renal cells. In renal section of sham control rats and day 1 of reperfusion, few cells showed the vimentin positive staining (<10%). Statistical significant (*P* <0.01) percentage of vimentin positive area was recorded in day 4 and day 7 of reperfusion period. Hyperplastic cells foci and organizing cells on day 4 showed strong vimentin positivity. Dilated tubules were also lined with the vimentin stained cells. Significantly increased (*P* <0.01) vimentin positive area had been noticed even after the complete recovery of functional parameters and as well as microscopically normalization of the most of the renal tubules i.e. on day 14. On day 28 of reperfusion, significantly high (*P* <0.01) vimentin positive cells were still evident, mainly confined to the area of tubulointerstitial lesion (Fig. 2).

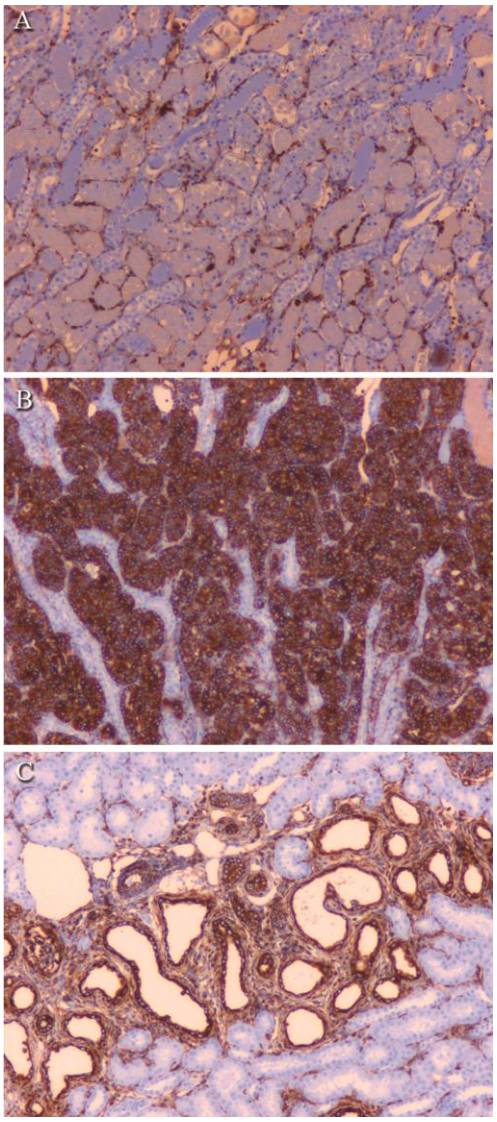

Fig. 2:— Section of rat kidney showing brown coloured vimentin positive renal tubules. (A) Occasional positive cells on day 1 of I/R AKI; (B) Hyperplastic and % increase in area of vimentin positivity on day 4 of I/R AKI; and (C) Vimentin positive tubules in the area of tubulointerstital lesion of day 28 of I/R AKI. Vimentin IHCX100.

Discussion

In this experiment, special immunohistochemical markers were used to study the vascular and tubular changes involved in I/R acute kidney injury. Tubular marker of undifferentiated cells, vimentin were more pronounced in day 4 of reperfusion as the consequence of regeneration. By the end of experiment, when creatinine value was normal as sham control, vimentin positive undifferentiated tubules were still

evident up to 28 days of reperfusion period. In experiments by the earlier workers[20-24] demonstrated that these cells release the autocrine and paracrine or other signaling factor responsible for progressive chronic change. This is further confirmed, as we noticed in our experiment, increased interstitial index, atrophic tubules, infiltration of mononuclear cells and fibroblasts on day 28 of reperfusion period. These pathological findings are mediated by the nexus of events like ATP depletion, increased intracellular calcium, production of ROS, activation of proteases, phospholipase, cytokines and disruption of apical actin cytoskeleton, mislocalization of Na K ATPase etc causing desquamation of brush border, formation of extracellular vesicles (blebs), intraluminal cast, necrosis/apoptosis of tubular epithelium and tubulointerstial inflammation, infiltrate of MNCs, fibroblast etc[25-28]. Where as in contrary to our findings, other workers[29-32], pointed towards the role of vascular changes like decrease capillary density and hemodynamic derangement for the long term consequences of AKI. However, in the present study with use of CD31 marker we could not confirm this theory. This could be due to lack of sensitivity of applied method in our experiment.

Conclusion

Results of the present study reveals that even after the complete functional recovery and microscopically normal renal tubules as evident on day 28 of reperfusion period, presence of vimentin positive cells signifying towards the factors which may further cause the progressive pathology leading to chronic kidney disease. However, we cannot completely deny the role of peritubular capillaries as well.

References

1 Bellomo R, Ronco C, Mehta RL, Asfar P, Boisrame HJ, Darmon M, Diehl JL, Duranteau J, Hoste EJ, Olivier JB, Legrand M, Lerolle N , Malbrain MLNG , Martensson J, Oudemans-van Straaten HM , Parienti JJ, Payen D, Perinel S, Peters E, Pickkers , Rondeau E , Schetz M, Vinsonneau C, Wendon J , Zhang L & Laterre PF, Acute kidney injury in the ICU: from injury to recovery: reports from the 5th Paris International Conference. *Ann Intensive Care*, 7 (2017) 49.

2 Dasta JF, Kane-Gill SL, Durtschi AJ, Pathak DS & Kellum, JA, Costs and outcomes of acute kidney injury (AKI) following cardiac surgery. *Nephrol Dial Transplant*, 23 (2008) 1970.

3 Liu H, Wang H, Tu C, Mao D, Hu Y, Zou R, Min Y, Xiong F & Su W, Kidney damage in COVID-19 patients with or without chronic kidney disease: Analysis of clinical characteristics and related risk factors. *Indian J Exp Biol*, 58 (2020) 811.

4 Kiran G, Nandini CD, Ramesh HP&Salimath PV, Progression of early phase diabetic nephropathy in streptozotocin-induced diabetic rats: Evaluation of various kidney-related parameters. *Indian J Exp Biol*, 50 (2012) 133.

5 Waikar SS, Liu KD, & Chertow GM, Diagnosis, epidemiology and outcomes of acute kidney injury. *Clin J Am Soc Nephrol*, 3 (2008) 844.

6 Thakar CV, Christianson A, Freyberg R, Almenoff P & Render ML, Incidence and outcomes of acute kidney injury in intensive care units: A Veterans Administration study. *Crit Care Med*, 37 (2009) 2552.

7 Kumaş M, Eşrefoğlu M & Güler EM, Protective effects of silymarin against isotretinoin induced liver and kidney injury in mice. *Indian J Exp Biol*, 56 (2018) 158

8 Guzzi F, Cirillo L, Roperto RM, Romagnani P & Lazzeri E, Molecular Mechanisms of the Acute Kidney Injury to Chronic Kidney Disease Transition: An Updated View. *Int J Mol Sci*, 20 (2019) 4941.

9 Polichnowski AJ, Griffin HL, Lan R, Picken MM, Long J, Williamson GA, Rosenberger C, Mathia S, Venkatachalam MA & Bidani AK. Pathophysiology of unilateral ischemia-reperfusion injury: importance of renal counterbalance and implications for the AKI-CKD transition. *Am J Physiol-Renal Physiol*, 318 (2020) F1086.

10 Chatterjee PK & Thiemermann C, An *in vivo* model of ischemia/reperfusion and inflammation of the kidneys of the rat. In: *Inflammation protocols. Methods in Molecular Biology*, (Eds. Winyarad PG & Willoughby DA; Humana press INC,.), 2003, 223.

11 Oxburgh L & de Caestecker MP, Ischemia-reperfusion injury of the mouse kidney. *Methods Mol Biol*, 886 (2012) 363.

12 Fu Y, Tang C, Cai J, Chen G, Zhang D & Dong Z, Rodent models of AKI-CKD transition. *Am J Physiol Renal Physiol*, 315 (2018) 1098.

13 Scarfe L, Menshikh A, Newton E, Zhu Y, Delgado R, Finney C & de Caestecker, MP Long-term outcomes in mouse models of ischemia-reperfusion-induced acute kidney injury. *Am J Physiol Renal Physiol*, 317 (2019) 1068.

14 Anderson G & Bancroft JD, Tissue processing and microtomy, In: *Theory and practice of histological techniques*. (Eds. Bancroft JD & Gamble M; Harcourt Churchill Livingstone, London), 2002, 85.

15 Basile D & Yoder M, Renal endothelial dysfunction in acute kidney ischemia reperfusion, *Cardiovasc Hematol Disord Drug Targets*, 14 (2014) 3.

16 Lovisa S, Fletcher SE, Sugimoto H, Hensel J, Lahiri S, Hertig A & Kalluri R, Endothelial-to-mesenchymal transition compromises vascular integrity to induce Myc-mediated metabolic reprogramming in kidney fibrosis. *Sci signaling*, 13 (2020) 635.

17 Zhou D, Fu H, Liu S, Zhang L, Xiao L, Bastacky SI & Liu Y, Early activation of fibroblasts is required for kidney repair and regeneration after injury. *FASEB J*, 33 (2019) 12576.

18 Venkatachalam MA, Weinberg JM, Kriz W & Bidani AK, Failed tubule recovery, AKI-CKD transition, and kidney disease progression. *J Am Soc Nephrol*, 26 (2015) 1765.

19 Segev G, Nivy R & Kass PH, A retrospective study of acute kidney injury in cats and development of a novel clinical scoring system for predicting outcome for cats managed by hemodialysis. *J Vet Intern Med*, 27 (2013) 830.

20 Schödel J & Ratcliffe PJ, Mechanisms of hypoxia signalling: new implications for nephrology, *Nat Rev Nephrol*, 15 (2019) 641.
21 Gaut JP & Liapis H, Acute kidney injury pathology and pathophysiology: a retrospective review. *Clin Kidney J*, 14 (2021) 526.
22 Bonventre JV, Maladaptive Proximal Tubule Repair: Cell Cycle Arrest. *Nephron Clin Pract*, 127 (2014) 61.
23 Leslie G, Roy Z & Ambra P, Progression of chronic kidney disease: too much cellular talk causes damage. *Kidney Int*, 91 (2017) 552.
24 Dubey A, Prajapti KS, Joshi BP, Swamy M & Dubey A, Pathology of ischaemia reperfusion induced acute kidney injury in rats. *Indian J Vet Pathol*, 39 (2015) 136.
25 Chen y, Lin L, Tao X, Song, Y, Cui J, & Wan J, The role of podocyte damage in the etiology of ischemia-reperfusion acute kidney injury and post-injury fibrosis. *BMC Nephrol*, 20 (2019) 106.
26 Black LM, Lever JM, Traylor AM, Chen B, Yang Z, Esman SK & Agarwal A, Divergent effects of AKI to CKD models on inflammation and fibrosis. *Am J Physiol Renal Physiol*, 315 (2018) 1107.
27 He L, Wei Q, Liu J, Yi M, Liu Y, Liu H & Dong Z, AKI on CKD: heightened injury, suppressed repair, and the underlying mechanisms. *Kidney Int*, 92 (2017) 107.
28 Sun X, Nkennor B, Mastikhina O, Soon K & Nunes SS, Endothelium-mediated contributions to fibrosis. *Semin in cell dev biol*, 101 (2020) 78.
29 Afsar B, Afsar RE, Dagel T, Kaya E, Erus S, Ortiz A & Kanbay M, Capillary rarefaction from the kidney point of view. *Clin kidney J*, 11 (2018) 295.
30 Menshikh A, Scarfe L, Delgado R, Finney C, Zhu Y, Yang H & Caestecker MP, Capillary rarefaction is more closely associated with CKD progression after cisplatin, rhabdomyolysis, and ischemia-reperfusion-induced AKI than renal fibrosis. *Am J Physiol Renal Physiol*, 317 (2019) F1383.
31 Kumar S, Cellular and molecular pathways of renal repair after acute kidney injury. *Kidney Int*, 93 (2018) 27.
32 Jemai H, Feryeni A, Mahmoudi A, Fki I, Bouallagui Z, & Sayadi S, Oleuropein protects kidney against oxidative and histopathological damages in subchronic cadmium intoxicated mice. *Indian J Exp Biol*, 57(2019) 602.