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# Ameliorative, Antioxidant and Immunomodulatory Potential of Vitamin D on Aminoglycoside Induced Acute Kidney Injury in Wistar Rats

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## ABSTRACT

**Background:** Acute kidney injury causes an abrupt decline in renal filtration and affects animals in a similar way to humans. Diagnosis can be made based on urinalysis, serum biochemistry and various biomarkers. The present study was conducted to evaluate the ameliorative, antioxidant and immunomodulatory potential of vitamin D in rats induced with acute kidney injury.

**Methods:** In the present study, group A rats were taken as healthy control, group B rats were given gentamicin @ 100 mg/kg BW intraperitoneally for 8 days and were considered as disease control and group C rats were treated with Vitamin D @ 0.4 µg/kg/day subcutaneously for 8 days along with intraperitoneal gentamicin injection. Reduced glutathione (GSH), lipid peroxide (LPO), catalase and superoxide dismutase (SOD) were estimated in erythrocytes on day 0, 4 and 8. Tumor necrosis factor alpha (TNF α) and interleukin 10 (IL 10) were also estimated along with urine and serum biochemistry on day 0, 4 and 8. Kidney tissue samples were collected on day 8 for histopathological examination.

**Result:** The mean values of GSH, catalase and SOD were significantly (P<0.05) higher whereas the mean value of LPO was significantly (P<0.05) lower in group C compared to group B on day 4 and 8. On day 4 and 8, the mean value of TNF α was significantly (P<0.05) lower, while the mean value of IL-10 was significantly (P<0.05) higher in rats treated with vitamin D as compared to disease control. Histopathological examination along with urine and serum biochemistry revealed protective efficacy of vitamin D in acute kidney injury. Based on the findings of the present study, it is concluded that vitamin D is having ameliorative efficacy along with antioxidant and immunomodulatory potential in case of gentamicin induced acute kidney injury in Wistar rats. However, detailed studies are required to explore the therapeutic potential of vitamin D in clinical cases of kidney diseases.

**Key words:** Acute kidney injury, Gentamicin, IL-10, TNF-α, Vitamin D.

## INTRODUCTION

Worldwide all racial and ethnic groups are affected by kidney injury, which can be divided into acute kidney injury (AKI) and chronic kidney disease (CKD) (Zhang *et al*. 2012; Gyurászová *et al*. 2019). In AKI, loss of renal function is rapid and can be reversed. Depending on severity, clinical findings and other concurrent diseases, there is variation in prognosis of AKI (Lewington *et al*. 2013). Various rat and mice models of AKI have been developed to study the therapeutic potential of certain drugs (Bao *et al*. 2018). Gentamicin is the most widely used aminoglycoside for experimental AKI models (Lopez-Novoa *et al*. 2011; Bae *et al*. 2014). After metabolism, gentamicin is filtered by glomerulus and due to its polycationic structure, small amount can be reabsorbed in renal proximal cells (Bae *et al*. 2014). Overdose of gentamicin stimulates reactive oxygen species (ROS) in renal tissues, leading to oxidative stress (Kushwaha *et al*. 2016; Gyurászová *et al*. 2019).

The renal tubular cells are rich in mitochondria which helps to meet the high energy requirement of reabsorption process. As mitochondria is the chief site for intracellular free radical production via respiratory chain, the renal tubules are highly vulnerable to oxidative damage (Eirin *et al*. 2016). Vitamin D is a pleiotropic hormone having its effects on

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various tissues of body (Andrew and Christopher, 2013). Vitamin D can protect animals against AKI through an anti-inflammatory mechanism by inhibiting toll like receptor-4 (TLR-4) and interferon gamma (IFN-γ) (Hamzawy *et al*. 2019). The present study was undertaken to study the ameliorative, antioxidant and immunomodulatory effects of vitamin D in rats induced with acute kidney injury.

## MATERIALS AND METHODS

### Study area

The present study was conducted at College of Veterinary and Animal Sciences, G.B.P.U.A.T, Pantnagar in October, 2019. Study animals were kept in laboratory animal house of the college.

### Study animals

Male Wistar rats of 12-13 weeks age, weighing around 150-200 g were included in the study. Animals were housed in polypropylene cages at room temperature with 12-hour light-dark cycle and were provided standard ration with *ad libitum* water. The study was conducted as per the guidelines of Institutional Animal Ethics Committee (IAEC) and the experimental protocol (IAEC/CVSc/VMD/341) was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

### Experimental protocol

Animals were provided an acclimatization period of 10 days before experiment and were divided into three groups, each having 6 animals. Group A rats were taken as healthy control, group B rats were given gentamicin @ 100 mg/kg BW intraperitoneally for 8 days and were considered as disease control, and group C rats were treated with Vitamin D @ 0.4 µg/kg/day subcutaneously for 8 days along with intraperitoneal gentamicin injection.

### Collection of samples

### Blood samples

Blood samples (1.5 mL each) were collected from rats by venipuncture of tail vein into vials with heparin and clot activator, respectively on day 0, 4 and 8. Blood samples collected in clot activator containing vials were allowed to clot and centrifuged at 3000 rpm for 20 minutes at 4°C to retrieve serum and the separated serum samples were stored at -20°C till analysis.

### Urine samples

Rats were housed in individual metabolic cages and 24-hour urine samples were collected on day 0, 4 and 8.

### Tissue samples

Kidney tissue samples were collected at the end of the experiment (day 8) and fixed in 10% buffered formalin for histopathological examination.

### Laboratory analysis

### Preparation of erythrocyte suspension and hemolysate

Blood samples collected in heparinized vials were centrifuged and plasma and buffy coat were removed. Then, erythrocytes were washed thrice in ice cold isotonic normal saline solution (NSS). Erythrocyte pellet was diluted with 1:10 ice cold distilled water for 10% hemolysate preparation and 0.5 mL of leftover erythrocyte pellet was diluted with ice cold NSS (1:1 ratio) to get erythrocyte suspension for the estimation of reduced glutathione (GSH). 10% hemolysate was used for the estimation of superoxide dismutase (SOD), lipid peroxide (LPO) and catalase.

### Hemoglobin estimation

Hemoglobin content in packed erythrocytes was estimated as per the method described by Richterich (1969).

### Estimation of oxidant and anti-oxidant indices

GSH level in erythrocyte suspension was estimated by DTNB method as described by Prins and Loos (1969). LPO level in erythrocyte hemolysate was estimated by the method described by Placer *et al*. (1966). Catalase activity in hemolysate was estimated by using hydrogen peroxide as a substrate by using method given by Bergmayer (1983). SOD was estimated as per method described by Madesh and Balasubramanian (1998).

### Immunomodulatory activity

Immunomodulatory activity of Vitamin D was evaluated by estimating tumor necrosis factor alpha (TNF α) and interleukin 10 (IL10) by using commercially available kits (Krishgen Biosystems, Mumbai) as per manufacturer's instructions.

### Urine biochemistry

Urine creatinine, urine urea nitrogen (UUN), urine total protein and urine albumin were estimated by using commercially available kits (Coral Clinical Systems, Tulip Diagnostics, India) following manufacturer's instructions.

### Serum biochemistry

Creatinine, blood urea nitrogen (BUN), total protein and albumin levels in the serum were estimated by using commercially available kits (Coral Clinical Systems, Tulip Diagnostics, India) as per manufacturer's instructions.

### Histopathology

Kidney tissue samples fixed in 10% buffered formalin were embedded in paraffin and 5 µm sections were cut with microtome and were stained with hematoxylin and eosin as per the standard technique (Culling, 1974). Slides were then examined under light microscope.

### Statistical analysis

The data were analyzed using one way analysis of variance (ANOVA) and paired t test. The data analysis was done by using statistical package for the social sciences (SPSS) version 20. Graphs were drawn in GraphPad Prism 6.0.

For all comparisons, values of P<0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

### Oxidative stress indices

Gentamicin has been known to increase ROS production in renal cortex leading to functional deterioration (Maldonado *et al*. 2003). In the disease control group (group B), there was a significant (P<0.05) reduction in the mean value of GSH compared to healthy control (group A) on day 4 and 8. While, the group C receiving vitamin D was having significantly (P<0.05) higher GSH level in erythrocytes as compared to group B but the value was significantly (P<0.05) lower than group A (Fig 1), which explains the protective effects of vitamin D on renal tubules. The mean value of LPO was significantly (P<0.05) higher in group B when compared to group A and group C on day 4 and 8 (Fig 1). Gentamicin induced kidney injury causes overproduction of free radicals leading to lipid peroxidation (Parlakpinar *et al*. 2005). In group C, LPO level was higher than group A but lower than group B, which may be due to antioxidant effect of vitamin D (Wiseman, 1993).

Body has a defense mechanism against oxidative damage in the form of enzymatic and non-enzymatic systems. Antioxidant enzymes can remove reactive species catalytically (Nandi *et al*. 2019). Catalase and SOD play an important role in these catalytic processes. In the present study, the mean values of catalase and SOD were significantly (P < 0.05) lower in group B on day 4 and 8 compared to group A (Fig 1). In group C, the mean levels of catalase and SOD were significantly (P<0.05) lower than group A but were significantly (P<0.05) higher than group B. Rats with gentamicin induced kidney injury are more prone to ROS damage due to reduction of antioxidant enzymes in body as reported by Pedraza-Chaverrìì *et al*. (2000).

### Immunomodulatory activity

TNF α is a pro-inflammatory cytokine which increase in inflammatory conditions. On day 4 and 8, TNF α levels were significantly (P<0.05) higher in serum as well as urine samples of group B compared to group A (Fig 2). The mean value of TNF α was significantly (P<0.05) lower in group C compared to group B on day 4 and 8. Vitamin D is known to reduce the production of TNF α by acting through macrophages and $CD8^+$ cells (Overbergh *et al*. 2000). IL10 is an anti-inflammatory cytokine produced in body to combat inflammatory conditions. The mean value of serum IL 10 was significantly (P<0.05) higher in group C compared to group B on day 4 and 8 (Fig 2). The anti-inflammatory activity of vitamin D can be attributed to increased production of IL 10 through macrophages and B cells (Heine *et al*. 2008; Korf *et al*. 2012).

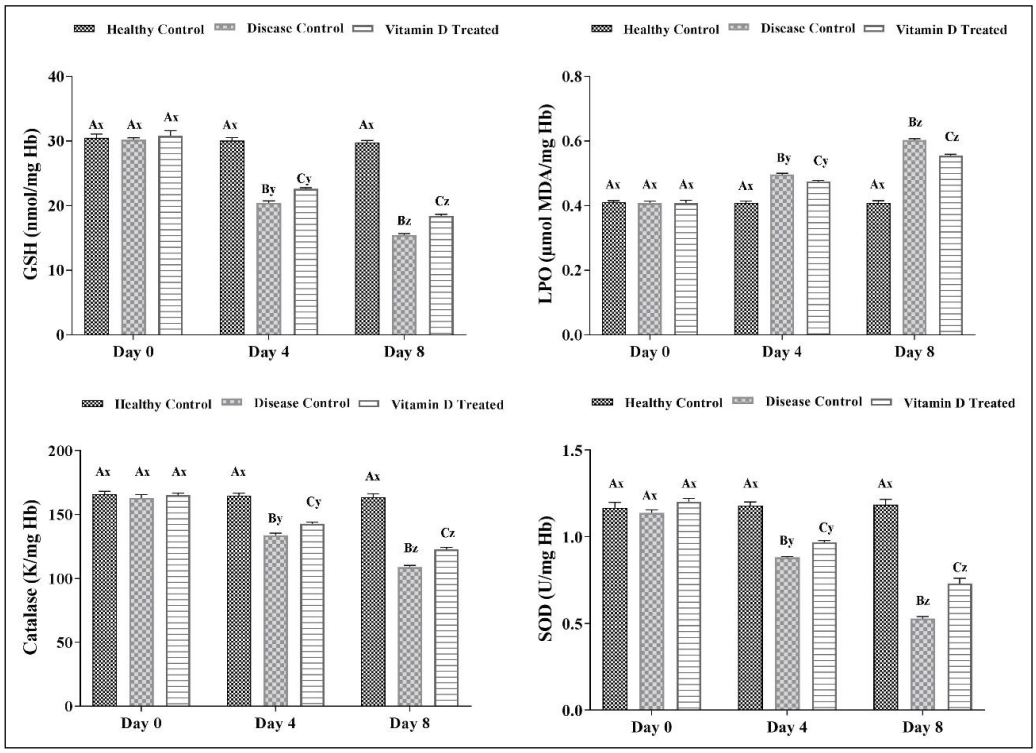


**Fig 1:** Alterations in oxidative stress indices and antioxidant enzymes on different days in disease control (group B) and vitamin D treated (group C) rats in comparison with healthy control (group A).

The values have been expressed as Mean±SEM. Superscripts A, B and C between the groups within a day and superscripts x, y and z between the days within a group differ significantly (P<0.05).

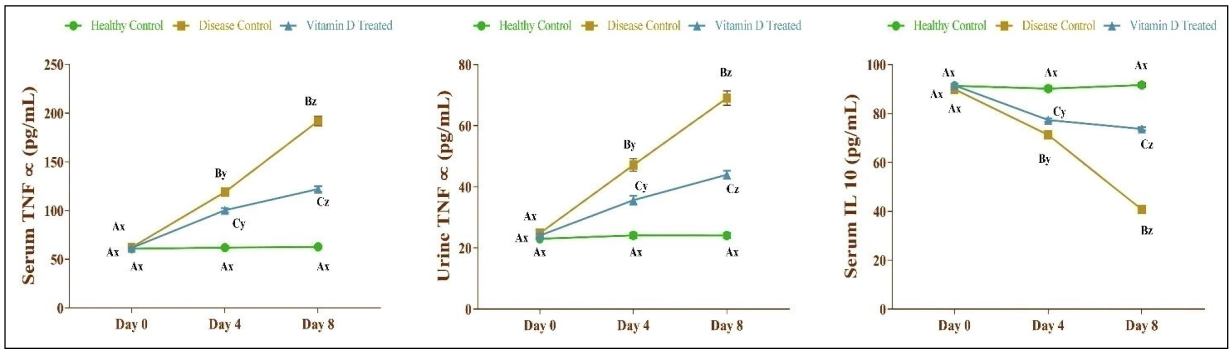


**Fig 2:** Alterations in serum TNF α, urine TNF α and serum IL 10 on different days in disease control (group B) and vitamin D treated (group C) rats in comparison with healthy control (group C).
The values have been expressed as Mean±SEM. Superscripts A, B and C between the groups within a day and superscripts x, y and z between the days within a group differ significantly (P<0.05).

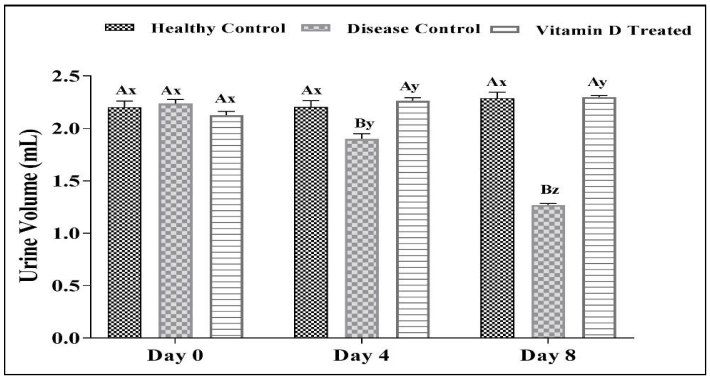


**Fig 3:** Alterations in urine volume on different days in disease control (group B) and vitamin D treated (group C) rats in comparison with healthy control (group A).
The values have been expressed as Mean±SEM. Superscripts A, B and C between the groups within a day and superscripts x, y and z between the days within a group differ significantly (P<0.05).

**Table 1:** Alterations in urine biochemical parameters on different days in disease control (group B) and vitamin D treated (group C) rats in comparison with healthy control (group A).

| Groups | Day 0 | Day 4 | Day 8 |
|---|---|---|---|
| **Urine creatinine (mg/dL)** | | | |
| Group A | 33.513±0.440$^{Ax}$ | 33.275±0.398$^{Ax}$ | 32.968±0.756$^{Ax}$ |
| Group B | 32.508±0.929$^{Ax}$ | 24.531±1.182$^{By}$ | 16.769±0.638$^{Bz}$ |
| Group C | 34.568±0.507$^{Ax}$ | 27.360±0.575$^{BCy}$ | 22.760±0.296$^{Cz}$ |
| **Urine urea nitrogen (mg/dL)** | | | |
| Group A | 708.365±6.628$^{Ax}$ | 714.051±5.296$^{Ax}$ | 711.846±3.978$^{Ax}$ |
| Group B | 689.807±14.117$^{Ax}$ | 602.555±3.910$^{By}$ | 388.416±8.539$^{Bz}$ |
| Group C | 719.222±10.505$^{Ax}$ | 648.169±5.957$^{Cy}$ | 543.657±2.194$^{Cz}$ |
| **Urine total protein (g/L)** | | | |
| Group A | 0.164±0.002$^{Ax}$ | 0.162±0.002$^{Ax}$ | 0.165±0.002$^{Ax}$ |
| Group B | 0.160±0.003$^{Ax}$ | 0.212±0.004$^{By}$ | 0.317±0.005$^{Bz}$ |
| Group C | 0.167±0.002$^{Ax}$ | 0.195±0.001$^{Cy}$ | 0.253±0.007$^{Cz}$ |
| **Urine albumin (g/L)** | | | |
| Group A | 0.085±0.002$^{Ax}$ | 0.081±0.002$^{Ax}$ | 0.081±0.002$^{Ax}$ |
| Group B | 0.084±0.002$^{Ax}$ | 0.123±0.005$^{By}$ | 0.170±0.003$^{Bz}$ |
| Group C | 0.084±0.001$^{Ax}$ | 0.112±0.003$^{Cy}$ | 0.136±0.002$^{Cz}$ |

The values have been expressed as Mean±SEM. Superscripts A, B and C between the groups within a day and superscripts x, y and z between the days within a group differ significantly (P<0.05).

**Table 2:** Alterations in serum biochemical parameters on different days in disease control (group B) and vitamin D treated (group C) rats in comparison with healthy control (group A).

| Groups | Day 0 | Day 4 | Day 8 |
|---|---|---|---|
| **Creatinine (mg/dL)** | | | |
| Group A | $0.805\pm0.008^{Ax}$ | $0.822\pm0.005^{Ax}$ | $0.830\pm0.019^{Ax}$ |
| Group B | $0.874\pm0.033^{Ax}$ | $1.312\pm0.003^{By}$ | $2.055\pm0.052^{Bz}$ |
| Group C | $0.907\pm0.028^{Ax}$ | $1.155\pm0.021^{Cy}$ | $1.410\pm0.005^{Cz}$ |
| **Blood urea nitrogen (mg/dL)** | | | |
| Group A | $18.629\pm0.256^{Ax}$ | $18.899\pm0.147^{Ax}$ | $18.784\pm0.210^{Ax}$ |
| Group B | $17.345\pm0.470^{Ax}$ | $31.906\pm0.394^{By}$ | $69.341\pm0.507^{Bz}$ |
| Group C | $19.476\pm0.828^{Ax}$ | $26.148\pm0.375^{Cy}$ | $47.145\pm0.467^{Cz}$ |
| **Total protein (g/L)** | | | |
| Group A | $6.855\pm0.099^{Ax}$ | $6.812\pm0.158^{Ax}$ | $7.071\pm0.061^{Ax}$ |
| Group B | $6.891\pm0.140^{Ax}$ | $5.128\pm0.105^{By}$ | $4.165\pm0.057^{Bz}$ |
| Group C | $6.850\pm0.078^{Ax}$ | $5.862\pm0.061^{Cy}$ | $5.136\pm0.052^{Cz}$ |
| **Albumin (g/L)** | | | |
| Group A | $4.166\pm0.040^{Ax}$ | $4.224\pm0.051^{Ax}$ | $4.099\pm0.067^{Ax}$ |
| Group B | $4.215\pm0.054^{Ax}$ | $3.335\pm0.070^{By}$ | $2.378\pm0.092^{Bz}$ |
| Group C | $4.259\pm0.052^{Ax}$ | $3.585\pm0.149^{BCy}$ | $3.114\pm0.124^{Cy}$ |

The values have been expressed as Mean±SEM. Superscripts A, B and C between the groups within a day and superscripts x, y and z between the days within a group differ significantly (P<0.05).

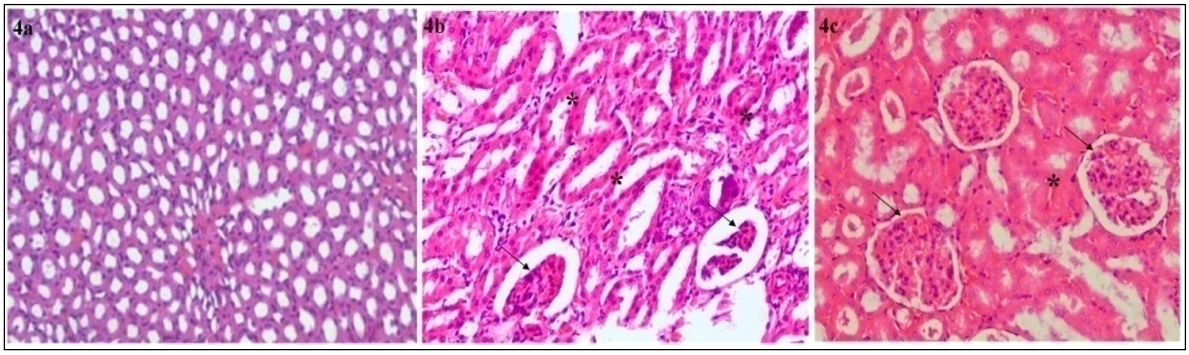


**Fig 4:** a) Normal kidney tubules seen in healthy rats of group A (× 200). b) Coagulative necrosis of tubular epithelium (*) and degeneration of glomerular tuft (arrow) in disease control (group B) rats (× 200). c) Normal glomerulus (arrow) with mild tubular generation (*) in some areas in vitamin D treated (group C) rats (× 200).

### Urine volume and biochemistry

The urine volume was significantly (P<0.05) lower in group B compared to group A and C on day 4 and 8. However, there was no significant difference noticed between group A and C (Fig 3). A mandatory mediator for renal injury is renal angiotensin system (RAS) and vitamin D suppresses renin expression, thus having a negative regulatory effect on RAS (Li *et al*. 2002). Augmented urine output in vitamin D treated group may be due to RAS inhibitory effect of vitamin D and similar finding has also been reported by Hur *et al*. (2013). Urine creatinine and UUN levels were significantly (P<0.05) lower in group B compared to group C on day 8 (Table 1). Creatinine is excreted by tubular secretion and the decrease in urine creatinine level indicates reduced renal efficiency. In renal damage, urea is not properly excreted in the urine and is reflected in the form of decreased UUN level (Udupa and Prakash, 2019). In the present study, decreased urine creatinine and UUN levels noticed could be correlated to renal tubular damage. On day 8, urinary total protein and albumin levels of group B were significantly (P<0.05) higher than group C indicating severe proteinuria due to renal tubular damage. Increased excretion of total protein and albumin in the urine could be associated with the injury to primary site of drug accumulation *i.e*., proximal tubular cells (Silverblatt and Kuehn, 1979). Filtered albumin is normally reabsorbed by proximal tubule but increased albumin excretion points towards injury to proximal tubules. In group C, urinary total protein and albumin levels were significantly (P<0.05) lower than group B which may be attributed to nephroprotective effects of vitamin D on proximal tubular cells.

### Serum biochemistry

Serum creatinine and BUN levels were significantly (P<0.05) higher in group B compared to group A and C on day

4 and 8 (Table 2). The increased concentration of creatinine and BUN in serum could be due to decreased excretion of these products from the renal tubules (Rahman *et al*. 2012). Serum total protein and albumin levels were significantly ($P<0.05$) lower in group B compared to group A and C on day 8 (Table 2). Hypoproteinemia and hypoalbuminemia noticed in the disease control group could be due to renal tubular damage leading to excess loss of protein in the urine. Group C showed significantly ($P<0.05$) lower levels of BUN and creatinine and higher levels of total protein and albumin compared to group B on day 8, which may be attributed to reno-protective effects of vitamin D.

### Histopathology

Histopathological study of kidney tissue sections from group A revealed normal structure of tubular epithelial cells (Fig 4a). In group B, coagulative necrosis of tubular epithelium and degeneration of glomerular tuft was noticed (Fig 4b). In group C, normal glomerulus with mild tubular generation in some areas was noticed (Fig 4c). Similar histopathological findings of acute kidney injury have also been reported by earlier workers (Safa *et al.* 2010; Udupa and Prakash, 2019). Histopathological findings of the present study were correlated with the results of urinary and serum biochemistry.

## CONCLUSION

It is concluded that vitamin D is having ameliorative effect on gentamicin induced acute kidney injury in rats. Vitamin D inhibits the overproduction of ROS and TNF $\alpha$ and enhances IL 10 production leading to reduced renal tubular damage. Further, detailed studies are required to explore the therapeutic potential of vitamin D in clinical cases of kidney diseases.

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### Conflict of interest

The authors declare that they have no conflict of interest.

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