## Potential activity of GIT-27 against renal ischemia reperfusion injury: an experimental study in male rats

Najah Hadi[1*], Huda jabber

## Abstract

The detail mechanisms in which renal ischemia reperfusion IRI happens are still indistinct. Various elements required in the pathogenesis include oxidative stress, inflammation, cellular necrosis, apoptosis, renal pathophysiological changes etc. To understand the pathway of ischemia reperfusion in renal we tested the hypothesis that GIT-27 attenuated renal ischemia reperfusion injury as specific Toll-like receptor inhibitors. Adult (3 to 5 month) male Sprague Dawely rats, and their weights ranged from 180 to 390 gm, were pre-treated with Git-27 intra peritoneal, plasma NGAL and cytokines mediator's in plasma and renal were analyzed by enzyme-linked immunosorbent assay (ELISA). And the pathological changes and cells injury in the renal were examined using hematoxylin and Eosin staining. Improvement of renal ischemia reperfusion by GIT-27 resulted in improved renal function and greater reductions in inflammatory mediators, and kidney injury. Taken together, GIT-27 significantly improved renal function following I/R through down-regulation of Toll-like receptor and serve as a potential therapeutic in ischemia reperfusion induced acute kidney injury.

**Keywords:** Renal ischemia reperfusion; Git-27; NGAL

## Introduction

Renal ischemia reperfusion (I/R) is an inflammatory process that causes acute kidney injury (AKI). AKI may not just happen with regards to kidney transplantation in which I/R is inescapable, but on the other hand is an outcome of hindered kidney perfusion e.g. amid real surgery or sepsis. For the kidney, IR is either because of heart failure (systemic hypo perfusion), surgical intercessions prompting nearby renal hypo perfusion, for example, and aortic cross-clipping, incomplete nephrectomy and in addition transplantation. AKI is freely connected

with expanded unpleasantness and mortality and additionally expanded length of clinic stays [1]. AKI is freely connected with a 2-to 5-fold expanded danger of death [2]. The detail mechanisms in which renal IRI happens are still indistinct. Various elements required in the pathogenesis include oxidative stress [3] inflammation [4] cellular necrosis [5], apoptosis [6] etc. Taking into account these conceivable components, a great deal of competitor medicines have been accounted for protective affecting renal IRI [7, 8, 9, 10]. GIT-27unusual immune modulator, is under development for the management of rheumatoid arthritis [11]. Suppresses TNF-α emission by means of intervention of macrophage toll-like receptor (TLR) 4 and TLR 2/6 signaling pathway. Toll-like receptors (TLRs) are pattern-recognition receptors that principally work as initiators of the distinguishing innate immune response [12].Additionally decreases the release of pro-inflammatory cytokines IL1-β, and IFN-γ. Anti diabetogenic; anticipates IL-β and IFN-γ-affected pancreatic islet cell demise in vitro. The flexibility of the agent's action, its high efficacy, and low toxicity and suggests that GIT-27 is a candidate for anticancer drug of the future [13].

## Materials and method

### Experimental animals
Adult (3 - 5 months) male Sprague Dawely rats, and their weights ranged from 180 to 390gm gained from Animal Resource Center, establishment of collection life examination and treatment of unprofitability, Al-Nahrain University. Rats were adapted for two weeks in light/dark cycle (12:12-h) with availability of free access to water and regular chow diet in animal house of Medical College, Kufa University and this investigation conforms to according to the Guide for the Care and Use of Laboratory Animals.

### Renal ischemia reperfusion procedure in rats
Renal ischemia reperfusion (R I/R) was established to induce acute kidney injury in murine as described previously [14]. In brief, rat was anesthetized by i.p. injection mixture of ketamine and xylazine (Dreieich, Leverkusen, Germany). A 2-cm abdominal midline incision was performed to exposed kidneys, did already right kidney nephrectomy. Then we clamped left kidney for 30 minute and reperfused for 3 hour using a 4.0 surgical suture (Norderstedt, Germany). Rats monitoring during reperfusion time for various signs of sickness. Sham rat received anesthesia and laparotomy without renal I/R served as the surgical control group. The animals were assigned to the following experimental groups: sham group, vehicle group, R I/R group, RI/R + Pretreated with GIT-27 ($n = 7$ in each group).

### Collection of samples
The samples of blood were drawn directly from the heart using needle, with heparin as anticoagulant and stored at 4°C. The blood centrifuged at 3000rpm for 15 min at 4°C, and collected plasma stored at -20°C. Also at 3000 rpm for 10 minutes and collected serum stored at -20°C until

used for further analyses for the right kidney of the rat was excised. Renal tissues were cut into two parts: upper half of kidney was snap-frozen (-70) until use, while the remaining parts were used for histological analysis.

## ELISA

The upper part of kidney treated in PBS containing 0.5% Triton X100 with a protease inhibitor cocktail, tissue was homogenized and the supernatant used to quantify (interlukin-18 level) in addition to the plasma neutrophil gelatinase associated lipocalin (NGAL) and serum(urea, creatinine, TNF alpha level) according the instruction of commercial ELISA kits (R&D Systems). The spectrophotometry of micro plate reader (Bio-Rad Laboratories, USA) was used to determine the absorbance of standards and samples at 450 nm.

## Histological examination

The renal tissue samples were fixed in 4% paraformaldehyde for 24 h, as described previously [15]. Briefly, 5 μm sections in thickness of renal tissue were paraffin embedded stained with the hematoxylin and eosin (H&E) through the standard procedure. The scoring of renal injury was examined according to the protocol of McWhinnie [16], quantitative measurements of tissue damage by a blinded observer. Tubular damage was defined as tubular epithelial swelling, loss of brush border, vacuolar degeneration, necrotic tubules, cast formation, and desquamation. The degree of kidney damage was estimated at ×200 magnification, using five randomly selected fields for each animal, by the following criteria were used to scoring the renal injury: 0, normal; 1, area of damage <25% of tubules; 2, damage involving 25–50% of tubules; 3, damage involving 50–75% of tubules; and 4, 75–100% of the area being affected.

## Statistical analysis

The data statistically analyzed by using Kolmogorov-Smirnova test and Shapiro-Wilk test to investigate differences between rats. Statistically the present data significance was defined as $P < 0.05$. The results were presented in the form of a table and a graph for each variable among different groups. P1 for control versus sham, P2 for control versus vehicle, P3 for control versus GIT-27.

## Results

### GIT-27 improved renal function after I/R injury

To investigate the treatment effects of GIT-27on renal function following acute kidney injury by using renal I/R protocol. Renal function dropped in I/R and vehicle ($P < 0.05$) (Tab. 1A, B) (Fig. 1A, B) Furthermore, GIT-27 pre-treated groups improved renal function through increased renal output ($P < 0.05$).

**Table 1A:** Mean serum urea in all groups

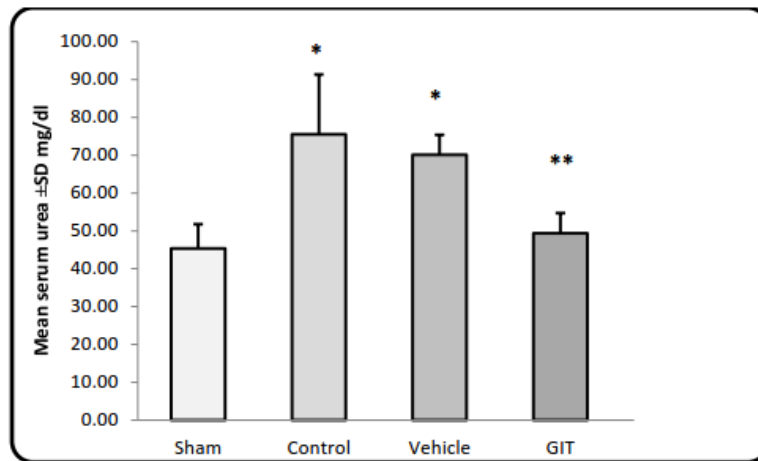| Group | Mean | SD | P1 | P2 | P3 |
|---|---|---|---|---|---|
| Sham | 45.40 | 6.38 | | | |
| Control | 75.51 | 15.84 | | | |
| Vehicle | 70.10 | 5.28 | 0.003 | 0.277 | 0.009 |
| GIT-22 | 49.38 | 5.32 | | | |



**Figure 1A.**

The mean serum urea (mg/dl) among four (*n*=7) experimental groups. GIT-27(25 mg/kg) suppressed the level of serum urea after renal I/R injury. Data are expressed as mean ± standard error; $^*P$ <0.05 versus corresponding sham; $^{**}P$ <0.05 versus I/R rats.

*Effective role of GIT-27 on pro-Inflammatory cytokines after renal I/R*

We next investigated the importance effects of GIT-27 on the local and systemic pro-inflammatory responses during renal I/R. At the end of the experiment (3 hour after renal I/R), the levels of pro-inflammatory mediators including (TNF-α, IL-18) in serum and renal tissue are measured by ELISA according to manufacture protocol. The resulted data showed that all pro-inflammatory cytokines are increased in both renal I/R and vehicle treatment rat group compared with sham group (P < 0.05) in both serum and renal tissue (Tab. 2A, B) (Fig. 2A, B).

**Table 2A.**

Mean serum TNF- α in all groups

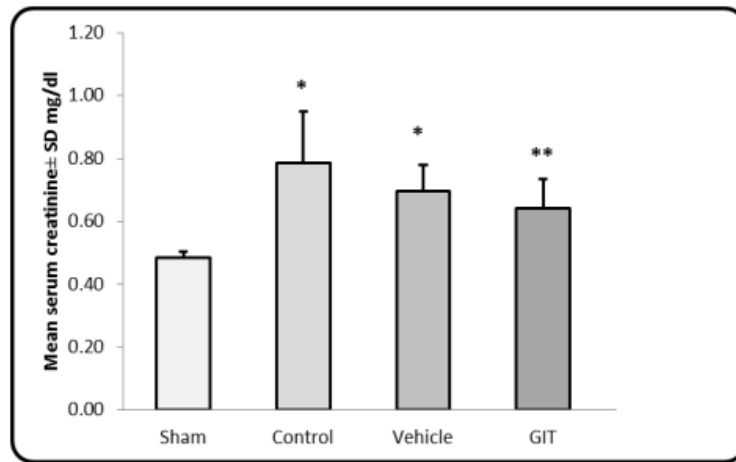| Variable | Mean | SD | P1 | P2 | P3 |
|---|---|---|---|---|---|
| Sham | 25.64 | 4.63 | | | |
| Control | 45.33 | 35.39 | | | |
| Vehicle | 40.41 | 3.45 | 0.013 | 0.047 | 0.565 |
| GIT-27 | 31.24 | 3.66 | | | |

**Figure 2A.**

**The mean serum TNF-α (pg/ml) among four (*n*=7) experimental groups**. GIT-27 (25 mg/kg) suppressed the level of serum TNF-α after renal I/R injury. Data are expressed as mean ± standard error; [*]*P* <0.05 versus corresponding sham; [**]*P* <0.05 versus I/R rats.

**Table 2B.**

Mean tissue IL-18 in all groups

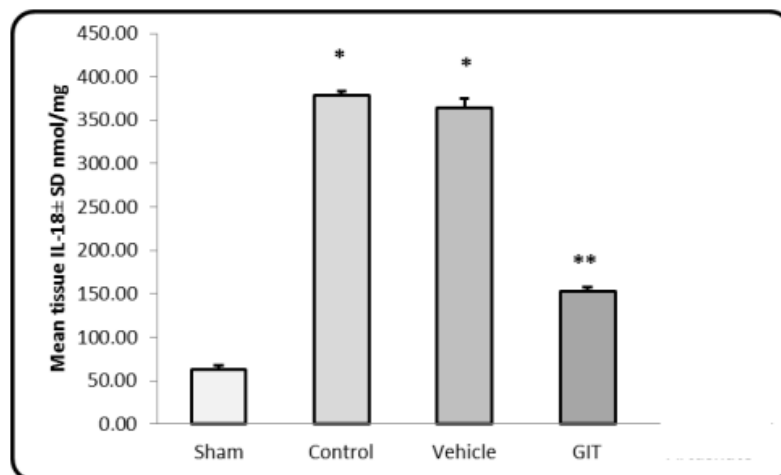| Variable | Mean | SD | P1 | P2 | P3 |
|----------|--------|-------|-------|-------|-------|
| Sham | 62.71 | 4.35 | | | |
| Control | 378.29 | 5.25 | | | |
| Vehicle | 364.43 | 10.71 | 0.002 | 0.018 | 0.002 |
| GIT-27 | 152.71 | 4.42 | | | |



**Figure 2B.**

**The mean tissue IL-18 (n mol/mg) among four (*n*=7) experimental groups**. GIT-27(25 mg/kg) suppressed the level of tissueIL-18after renal I/R injury. Data are expressed as mean ± standard error; [*]*P* <0.05 versus corresponding sham; [**]*P* <0.05 versus I/R rats.

*GIT-27 suppresses the expression level of NGAL*

Renal I/R lead to upstream release of NGAL expression in plasma. We assayed the expression of NGAL in plasma by ELISA. (Tab. 3) (Fig. 3) showed that the levels of NGAL are markedly increased by renal I/R, whereas GIT-27 treatment attenuates plasma NGAL levels ($P < 0.05$).

**Table 3.**

Mean plasma Tissue NGAL in all groups

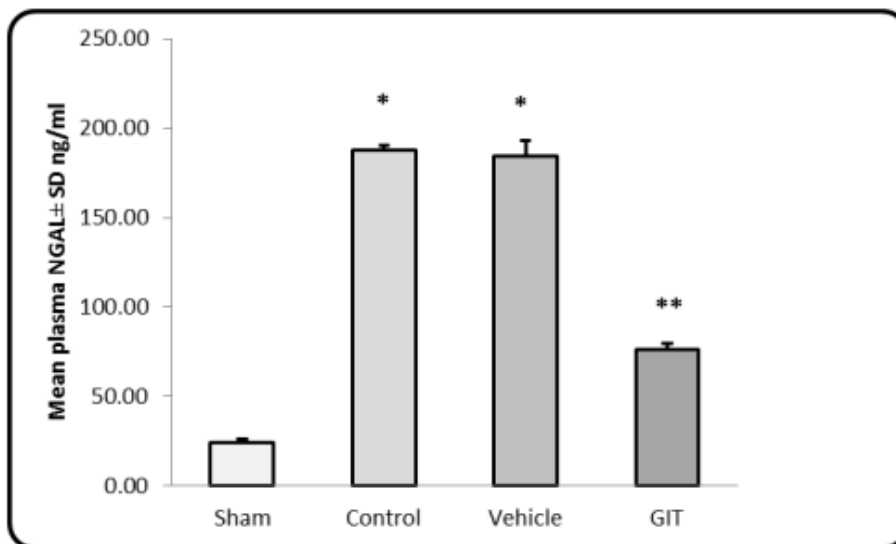| Variable | Mean | SD | P1 | P2 | P3 |
|----------|-------|------|-------|-------|-------|
| Sham | 24.54 | 1.91 | | | |
| Control | 188.00 | 2.58 | | | |
| Vehicle | 184.43 | 8.56 | 0.002 | 0.847 | 0.002 |
| GIT-27 | 76.00 | 3.51 | | | |



**Figure 3.**
**The mean plasma NGAL (ng/ml) among four (*n*=7) experimental groups**.
GIT-27(25 mg/kg) suppressed the level of tissue NGAL after renal I/R injury. Data are expressed as mean ± standard error; [*]$P$ <0.05 versus corresponding sham; [**]$P$ <0.05 versus I/R rats

*Git-27 treatment improved histopathological changes after renal I/R*

Histologically, renal tissue from I/R or vehicle rat after 3 hour of reperfusion period (Fig. 5A, B, C) revealed renal injury by quantitative measurements of tissue damage by a blinded observer. Tubular damage was defined as tubular epithelial swelling, loss of brush border, vacuolar degeneration, necrotic tubules, cast formation, and desquamation. The degree of kidney damage was estimated at ×200 magnification,. While the histopathological changes in GIT-27

treated group showed mild cellular alterations (Fig. 6A, B). According to McWhinnie scoring system; 0, normal; 1, area of damage <25% of tubules; 2, damage involving 25–50% of tubules; 3, damage involving 50–75% of tubules; and 4, 75–100% of the area being affected. Mean histological score of GIT-27 group were significantly lower than that of control group ($P<0.05$) (Fig. 4).
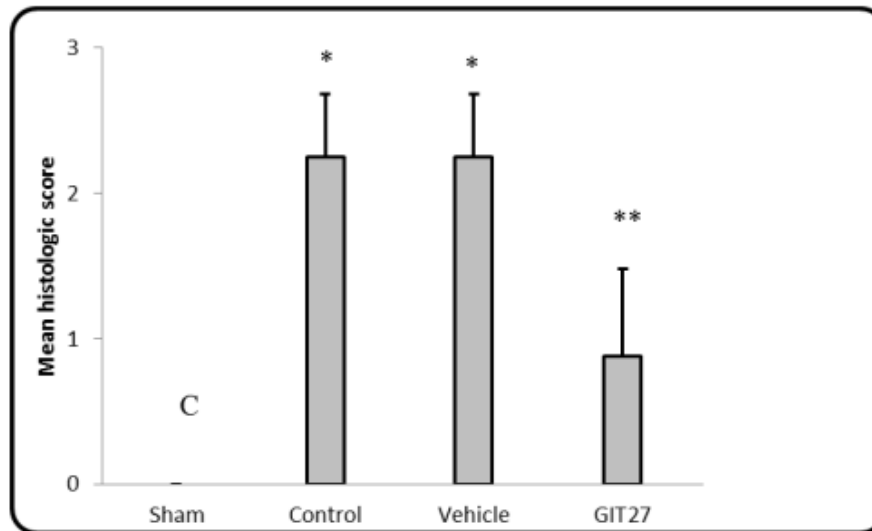


**Figure 4.**

**The mean histological score among four ($n$=7) experimental groups**. GIT-27 (25 mg/kg) treatment improved histopatho-logical changes after renal I/R. Data are expressed as mean ± standard error; [*]$P$ <0.05 versus corresponding sham; [**]$P$ <0.05 versus I/R rats.
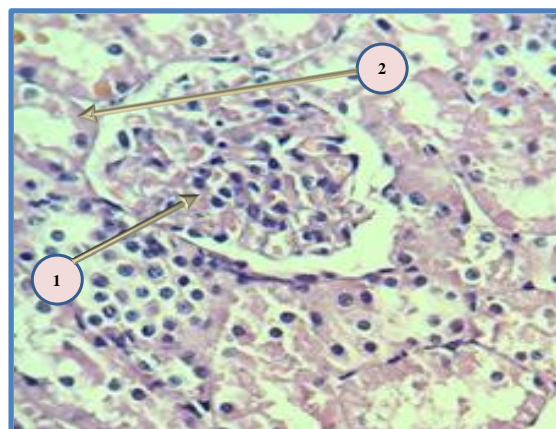


**Figure 5A.**

Section through kidney (sham group) showing normal glomerulus (1) and normal renal tubule (2). H and E stain (40X).
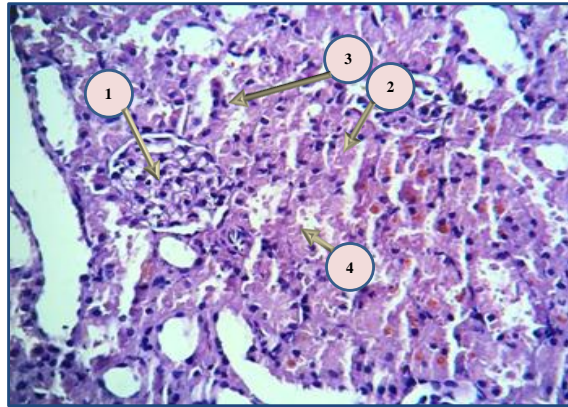
**Figure 5B.**
Section through kidney (control untreated I/R group) showing swelling of epithelial cells of glumerulus and narrowing of urinary space (1), increased cytoplasmic eosinophilia and fragmentation (2) and nuclear kayorhexis (4) of renal tubular cells together with nutrophilic inflammatory infiltrate (3). H and E stain (40X).
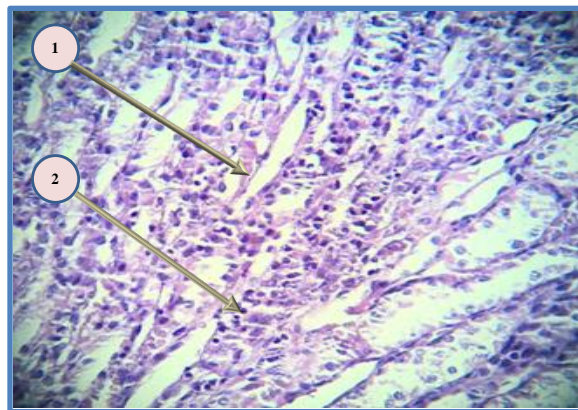


**Figure 5C.**
Section through kidney (DMSO treated I/R group) nuclear fragmentation (karyorhexis) (2) andrenal tubule cell cytoplasmic eosinophilia and degeneration (1). H and E stain (40X).
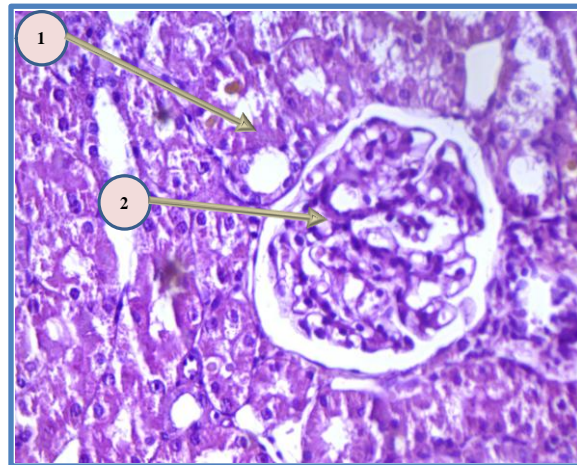
**Figure 6A.**
Section through kidney (GIT 27 treated I/R group) showing nuclear fragmentation (karyorhexis) and increased renal tubule cell cytoplasmic eosinophilia (1) and another near normal renal tubule (2). H and E stain (40X).



**Figure 6B.**
Section through kidney (GIT 27 treated I/R group) showing nuclear fragmentation (karyorhexis) and increased renal tubule cell cytoplasmic eosinophilia (1) and another near normal renal tubule (2). H and E stain (40X).
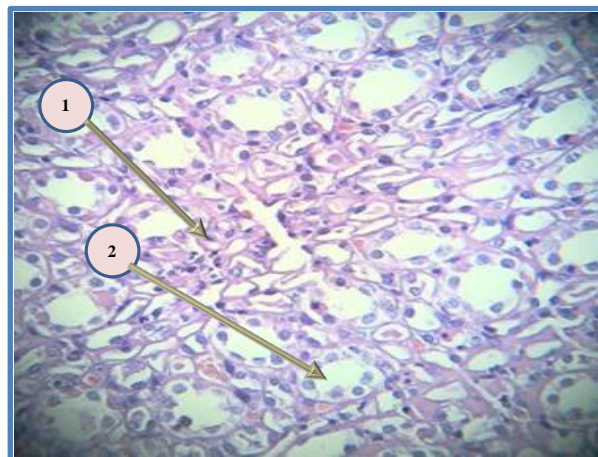
## Discussion

During renal I/R, the inflammatory responses mediate renal injury, including dysfunction and renal pathophysiological changes. It is important to create novel medications to protect kidney from IRI [17]. Previous studies reported that expression of inflammatory mediators (TNF-α, IL-18) was higher following renal injury [18, 19]. The present study investigated the GIT-27 to improve the renal function following ischemia reperfusion injury. According to our knowledge there was no data published

discussed the relationship renal ischemia reperfusion injury and effective role of GIT-27 on improved renal function following I/R model in rat. A number of published paper have investigated and confirmed that renal dysfunction during ischemia reperfusions related with inflammatory mediator's expression, including TNF-α and IL-1β [19]. Interestingly, we observed in this study that pre-treatment with GIT-27 results in significantly reduction in cytokines with improvement in renal function. The results revealed plasma NGAL was significantly higher in control than that in sham group (P<0.05) and reduced plasma level of NGAL in GIT-27 pretreated group. Many clinical studies have shown that increases in urinary and plasma NGAL are powerful and independent predictors of AKI when compared with serum creatinine [20, 21, 22]. Clearly, NGAL represents to a novel predictive biomarker for AKI and its results. To best of our knowledge there is no research has measured this parameter regarding the use of GIT-27 against renal ischemia reperfusion injury. However, [23] up regulation of NGAL in renal tubule cells may be induced by local release of cytokines from monocytes in the microcirculation after ischemic injury [24] Nils and others [25] demonstrated that in vascular smooth muscle cells NGAL expression is induced in response to vascular injury and depends on nuclear factor kappa B (NF-kB) expression [26], and upon activation of Toll-like receptors (TLRs) on immune cells establishing an acute phase response [27]. In I/R group, generally all intersections of the most of rat sections

of this group indicated tubular cell swelling, brush border loss, nuclear condensation, with more than 66% of the tubular profile demonstrating nuclear loss. Also intersections of rat sections of GIT-27 pretreated group showed significant improvement in kidney Parenchyma I/R causes significant elevation (P<0.05) in serum TNF-α, tissue IL-18 and which are pro-inflammatory cytokines that cause adhesion, activation, and transmigration of polymorph nuclear leukocytes (PMNs) into renal tissues and their oxidative burst, which results in excessive ROS production and kidney damage (neutrophil-mediated tissue injury ). Data available in present study showed significant reduction in serum TNF-α, tissue IL-18. Cong and others [28] demonstrated that I/R in rat model revealed morphologic variations from the norm including cytoplasmic vacuolization, cell necrosis of the proximal convoluted tubule, and tubular lumen obstruction and impairments were discovered generally in I/R group. Recently, researchers showed less albuminuria, mesangial expansion, infiltration of macrophages and pro-inflammatory and extracellular matrix-associated gene expression in glomeruli as histological changes by GIT-27 [29].

**In conclusion,** this work found that Toll-like receptor, have critical role in renal I/R and lead to attenuated renal function. Further, it was found that Toll-like receptor antagonist (GIT-27) has valuable Reno-protective role related to the changes of pro inflammatory mediators (TNF-α, and IL-18), further reduced renal function, and all of these suggest that Toll-like receptor could be a novel target for

therapy in patients with acute kidney injury induced ischemia reperfusion lead to improved renal function. These experimental results let us believe that Toll-like receptor deactivation led to decreased level NGAL with sequential signal caused renal cell injury.

## Competing interests

The authors declare that there is no conflict of interest.

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Editor-In-Chief
Amit Kumar Tyagi MSc, PhD
Department of Experimental Therapeutics
The University of Texas MD Anderson Cancer Center,
1515 Holcombe Blvd
Houston TX 77054, USA
Tele: 713-792-6543
         281-716-0484

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