The nephroprotective potential of diosgenin against ischemia-reperfusion acute renal damage via suppression of oxidative stress and downregulating inflammatory mediators

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ABSTRACT
Renal ischemia/reperfusion injury (I/R) is caused by short impairment of the blood supply to the kidney. Acute renal damage (ARD) is still a major problem in today’s clinical practice. Diosgenin (DG), a steroidal sapogenin mainly present in fenugreek, yams, Costus species in high amounts. DG as a leading phytoconstituents is beneficial in several disease conditions due to its multifunction including antioxidant, hypolipidemic, anti-inflammatory, hypoglycemic, anticancer and anti-proliferative effects. The purpose of this study was to investigate the nephroprotective potential of DG on ischemia-reperfusion induced kidney damage in rats. Thirty male Wistar rats were divided into five groups. DG was given orally at doses of 20, 40 and 80 mg/kg for 10 days. On day 11th ARD was induced by unilateral ischemia for 45 min followed by 24-hour reperfusion. The levels of creatinine, blood urea nitrogen, total proteins, pro-inflammatory cytokines were decreased in DG + I/R rats compared to NC rats, along with a significant amelioration of oxidative stress. Histopathological analysis exhibited less histological damage in DG + I/R rats as compared with NC rats. Pretreatment of rats with DG (40 and 80 mg/kg) was able to improve these damages significantly, though DG (20 mg/kg) was insignificantly effective. In conclusion, DG proved nephroprotective potential against kidney damage induced by I/R injury via mitigation of inflammation, oxidative stress, and histopathologic injuries.

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Introduction
Acute renal damage is defined as a rapid and unpredicted decline in kidney physiology, causing accretion of nitrogen waste products like urea, uric acid and creatinine with or without affecting the diuresis process [1]. ARD is linked with a higher risk of morbidity and mortality and is resulted in manifold organ failure [2]. Research studies indicate that the overall incidence rate of ARD in hospital admitted patients was about 20% and 67% in intensive care unit patients [3,4]. Ischemic kidney damage may be resulted due to renal transplantation [4], partial nephrectomy [5], renal artery revascularization [6], trauma [7] and hydronephrosis [8]. Various undesirable situations like elevated blood pressure and chronic kidney failure may result in renal ischemia. Further kidney tissue damage is prevented by the restoration of kidney blood circulation, reperfusion frequently aggravates kidney dysfunction encouraged by reperfusion injury, a situation called as oxygen paradox [9]. Renal ischemia is a multiphase disease, prompting

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deficiency in oxygen, nutrient source and removal of waste products from the kidney. Successive reperfusion encourages dysfunction of endothelial cells and will be the main reason for renal damage [10–12]. Subsequently, I/R injury resulted in inflammation and oxidative stress, causing apoptosis and necrosis of renal tubular cells: provoking deterioration of kidney role. Hence, primary prevention and treatment of patients at high risk of acute kidney injury are paramount important [13]. Succeeding renal ischemia/reperfusion injury, the process of inflammation initiates as a consequence of cell injury. The cell injury results in the generation of molecular products which causes activation of kidney dendritic and parenchymal cells, initiating the secretion of cytokines and chemokines [14–16]. As well as renal ischemia resulted in accumulation of leukocytes at the site of injury leading to up-regulation of intracellular adhesion molecules-1 (ICAM-1) in vascular endothelium and secretion of inflammatory cytokines such as interleukin (IL)-1, IL-6 and Tumor necrosis factor-α (TNF-α) [17]. Furthermore, renal I/R injury augments the expression of inducible nitric oxide synthase and nitric oxide (NO) that leads to the formation of peroxynitrite radicals by reacting with reactive oxygen species. Also, it inhibits nitric oxide synthase enzyme with the declining formation of NO in renal endothelium and results in vascular endothelium constriction [18–20].

Scientists Fujii and Matsukawa in 1935 have discovered DG from the plant Dioscorea Tokoro Makino belonging to the family Dioscoreaceae DG, a steroidal sapogenin (Figure 1) mainly present in fenugreek, yams and Costus species in high amounts. They are obtained from numerous plant species such as Smilax menispermoidea, Costus speciosus, Trigonella foenum and many other Dioscorea species [21,22]. DG is used as a major precursor bioactive molecule for the synthesis of steroidal drugs in the pharmaceutical industry [23]. In this regard, primarily over the past 20 years, extensive animal and mechanistic experimentation have been done to know the benefits and importance of DG as a leading phytocconstituents in several disease conditions like antioxidant, hypolipidemic, anti-inflammatory, hypoglycemic, anticancer, anti-proliferative, allergic diseases, skin aging, neurodegenerative and menopause [24–27]. Kanchan et al. (2016) have reported the renoprotective role of DG in streptozotocin-induced diabetic rats via restoration of antioxidant biomarkers in kidney tissue after the administration of DG [28]. Therefore, nutraceutical, cosmetic and pharmaceutical industries have attracted significant devotion toward DG.

**Material and methods**

**Drugs and chemicals**

Diosgenin, Pyrogallol, Thiobarbituric acid, Trichloroacetic acid (20%), 5, 5-dithio-bis-2-nitro benzoic acid, O-dianisidine hydrochloride were purchased from Sigma Aldrich, USA. Creatinine, total protein, and blood urea nitrogen assay kits were procured from Accurex Biomedical, Thane. Thiopental was purchased from Merck specialties Pvt. Ltd, Mumbai. Tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β enzyme-linked immunosorbent assay (ELISA) kits were bought from Krishgen Biosystem (Mumbai). All other chemicals used were of analytical grade.

**Animals**

Thirty healthy male Wistar rats (200–220 g) were purchased from the National Institute of Biosciences, Pune. Animals were maintained under 12 h light/dark cycle at a constant...
temperature, with free access to water and food ad libitum. The Institutional Animal Ethics Committee of Poona College of Pharmacy, Pune has approved all the experiment details and animal study (CPCSEA/PCL/13/2014-15).

**Experimental groups and treatment**

The rats were randomly divided into five groups of six rats (n = 6).

1. Sham Control (SC): Animals received saline orally for 10 days and on the 11th day underwent midline incision
2. Nephropathy Control (NC): Animals received saline orally for 10 days and on the 11th day underwent surgical process for induction of I/R
3. DG (20 mg/kg) + I/R group: Animals received DG (20 mg/kg) orally for 10 days and on the 11th day underwent surgical process for induction of I/R
4. DG (40 mg/kg) + I/R group: Animals received DG (40 mg/kg) orally for 10 days and on the 11th day underwent surgical process for induction of I/R
5. DG (80 mg/kg) + I/R group: Animals received DG (80 mg/kg) orally for 10 days and on the 11th day underwent surgical process for induction of I/R [29].

**Ischemia-reperfusion renal injury and sampling**

Animals were anaesthetized with Thiopental (50 mg/kg) via intraperitoneal injection. Then, abdominal cavities of rats from NC and DG + I/R groups were opened with a small midline incision. The ischemia was induced by clamping the left renal artery for 45 min followed by reperfusion for 24 hours after removal of clamps. On the 11th day, animals were sacrificed to obtain blood samples for biochemical estimations and cytokines analysis. The left kidney was removed for histopathological analysis and measurement of antioxidant parameters. The blood samples were centrifuged at 3500 g (15 min) for the separation of serum. The serum samples were used to evaluate the levels of renal markers such as Cr, TP, BUN and pro-inflammatory cytokines like TNF-α and IL-1β. Kidney tissue samples were weighed immediately after the collection of blood samples. One part of the tissue was fixed in 10% formaldehyde for histopathological evaluation using hematoxylin and eosin (H & E) stain and the other part was used for measurement of oxidative stress markers like malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) and myeloperoxidase (MPO) [29]. The schematic representation of ischemia/reperfusion renal injury induction and drug treatment in rats is shown in Figure 2.

**Acute toxicity study**

DG’s acute toxicity study was performed in compliance with Organization for Economic Cooperation and Development (OECD) guidelines (OECD 425). SG at doses of 55 mg/kg, 175 mg/kg, 550 mg/kg, 1750 mg/kg and 2000 mg/kg body weight were administered orally to overnight fasted healthy female Swiss albino mice and continuously monitored for behavioral changes, restlessness, respiratory, salivation, tremors and diarrhea or any signs of toxicity or mortality from 2 h to 48 h and up to 14 days [30].

**Assessment of biochemical parameters**

On the 11th day, rats were anaesthetized again, and the blood samples were collected from the retro-orbital plexus. The samples were subjected to centrifugation at 3000 g (10 min) at 4°C for collection of serum and kept at −80°C for biochemical study. The estimation of Cr, TP and BUN were carried out by using Accurex assay kits according to the manufacturer instructions.

**Assessment of kidney weight**

After the collection of blood samples, kidney tissues were removed immediately and weighed.
Assessment of oxidative stress markers

**Determination of malondialdehyde**
Determination of MDA in the tissue was determined by the method of Ohkawa et al. (1979) [31].

**Determination of reduced glutathione**
Estimation of GSH was carried out by the method of Ellman (1959) [32].

**Determination of superoxide dismutase**
Determination of SOD activity was done according to the method of Marklund and Marklund (1974) [33].

**Determination of myeloperoxidase**
The MPO activity was determined by the method of Barone et al., (1991) [34].

**Assessment of serum proinflammatory cytokines**
Estimation of TNF-α and IL-1β cytokines were carried out by using enzyme-linked immunosorbent assay (ELISA) kits, as per the manufacturer’s protocol.

Histopathological study

The kidney tissue samples from each group were fixed immediately in 10% formalin and embedded in paraffin blocks. Slices of four to five μm were made and stained with H & E to evaluate kidney inflammation. According to the damage intensity, the scoring of samples was done in between 1 and 4 by the blind observer as described below: 0: no abnormality; 1: low kidney damage (<25%), 2: mild kidney damage (26–50%), 3: moderate kidney damage (51–75%), 4: severe kidney damage (>75%) [35].

Statistical analysis

The statistical analysis was performed by using Graph Pad Prism. Results were expressed as mean ± SEM. One-way analysis of variance (ANOVA) and post hoc Tukey’s multiple comparison tests were used to analyze the data. A P-value of less than 0.05 was considered statistically significant.

Figure 2. Schematic representation of ischemia/reperfusion injury induction and drug treatment in rats.
Results

Effect of DG on Serum creatinine

As shown in Figure 3a, the Serum Cr level was increased significantly when compared with SC group rats ($p < 0.001$). However, the Cr level exhibited a remarkable reduction ($p < 0.01$; $p < 0.001$) in all the three DG treatment groups (20, 40 and 80 mg/kg). The reduction in serum Cr level was found to be more prominent in 80 mg/kg ($p < 0.001$) DG + I/R treatment groups when compared with the NC group.

Effect of DG on blood urea nitrogen

As specified in Figure 3b, the serum BUN level of NC rats ($p < 0.001$) was increased significantly when compared with the SC rats. On the other hand, BUN level was declined significantly ($p < 0.01$; $p < 0.001$) in DG + I/R treatment rats (40 and 80 mg/kg) when compared with SC rats. However, there was a non-significant decrease in the BUN level was found in rats treated with DG (20 mg/kg) when compared to NC rats.

Effect of DG on total protein

In this study, the TP level in the NC rats was found to be increased significantly ($p < 0.001$) when compared with the SC rats. However, TP level was found to be decreased significantly ($p < 0.01$; $p < 0.001$) in rats treated with DG + I/R (40 mg/kg and 80 mg/kg) when compared to NC rats (Figure 4).

Effect of DG on left kidney weight

The left kidney weight of NC rats was increased significantly ($p < 0.001$) when compared to SC rats. However, the left kidney weight was significantly reduced ($p < 0.01$; $p < 0.001$) after the treatment with DG + I/R (40 and 80 mg/kg) as compared to NC rats. The rats treated with DG + I/R (20 mg/kg) exhibited a non-significant decrease in the weight of left kidneys (Figure 5).

Effect of DG on Malondialdehyde

Based on Figure 6a, the tissue MDA level of NC rats was increased significantly ($p < 0.001$) after I/R injury compared to the SC rats, but this MDA level was found to be declined significantly ($p < 0.01$; $p < 0.001$) after the treatment of rats with DG + I/R (40 and 80 mg/kg) when compared with NC rats. The rats treated with DG + I/R (20 mg/kg) exhibited a non-significant decrease in the tissue MDA level.

Effect of DG on reduced glutathione

Renal I/R injury resulted in a significant decrease ($p < 0.001$) in tissue GSH concentrations of NC rats as compared with SC rats.

Figure 3. Effect of DG on Serum creatinine and blood urea nitrogen in I/R renal damage in rats. Results were expressed as Mean ± SEM (n = 6). ###$P < 0.001$ as compared to SC rats and **Non-significant** **$P < 0.01$,** ***$P < 0.001$ as compared to NC rats.
Whereas DG + I/R (40 and 80 mg/kg) treatment significantly increased \( p < 0.01; p < 0.001 \) GSH levels as compared to NC rats. Moreover, the treatment of rats with DG + I/R (20 mg/kg) non-significantly increased the tissue GSH concentration (Figure 6b).

**Effect of DG on superoxide dismutase**

As shown in Figure 7 and 9, the tissue SOD level of NC rats was decreased significantly \( p < 0.001 \) after I/R injury compared to SC rats. The SOD level was found to be increased significantly \( p < 0.01; p < 0.001 \) after the treatment of rats with DG + I/R (40 and 80 mg/kg) when compared with NC rats. The rats treated with DG + I/R (20 mg/kg) exhibited a non-significant increase in the tissue SOD level (Figure 6c).

**Effect of DG on myeloperoxidase**

Renal I/R injury resulted in a significant increase \( p < 0.001 \) in tissue MPO concentrations of NC rats compared with SC rats. Whereas DG + I/R (40 and 80 mg/kg) treatment significantly reduced \( p < 0.001 \) MDA levels as compared to NC rats.
Moreover, the treatment of rats with DG + IR (20 mg/kg) non-significantly decreased the concentration of MPO (Figure 7).

**Effect of DG on tumor necrosis factor-α**

As seen in Figure 8a, I/R injury significantly increased ($P < 0.001$) the serum TNF-α level (a marker of inflammatory pathways) compared to the SC group rats. Following DG + I/R treatment (40 and 80 mg/kg), the TNF-α levels were reduced significantly when compared with NC rats. However, the treatment of rats with DG (20 mg/kg) exhibited a non-significant decrease in TNF-α levels.

**Effect of DG on interleukin-1β**

As illustrated in Figure 8b, NC rats that underwent renal I/R exhibited a significant increase ($P < 0.001$) in the serum IL-1β level as compared to SC rats. Treatment of rats with DG + IR (40 and 80 mg/kg) exhibited a significant reduction ($P < 0.01$; $P < 0.001$) in IL-1β level when compared to NC rats. However, the rats treated with DG (20 mg/kg) has a non-significant decrease in IL-1β level.
Effect of DG on histopathological evaluation

In the histopathological analysis, the renal section of sham control rats exhibited normal glomerular structure (Figure 9a). Whereas NC rats that underwent I/R injury (NC rats) showed severe tubular injury with swollen kidney tubules, tissue edema and neutrophils accumulation in the renal tissue (Figure 9b). The rats treated with DG + I/R (20 mg/kg), displayed moderate renal tubular swelling and tissue edema (Figure 9c). The treatment of rats with DG + I/R (40 to 80 mg/kg), diminished tubular injury, tissue edema and neutrophils accumulation in the renal tissue with preservation of glomerular architecture as compared to NC rats (Figure 9d,e). The above results revealed the protective effect of DG on kidney histopathological changes during I/R injury. Histological scoring in I/R renal injury is shown in Table 1.

Discussion

I/R renal injury is a multifaceted pathological procedure that arises in numerous situations, like transplantation of kidney, cardiac arrest, heminephrectomy and vascular surgery and is a major reason for renal failure, death of the renal cell and renal graft rejection [36]. The mechanisms behind I/R renal damage comprises oxidative stress, hypoxic injury to renal cells, inflammation response, apoptosis and necrosis [37,38]. Hence, there is an urgent need for the discovery of novel drugs proficient in protecting the renal cells from I/R injury. In this study, we have investigated the nephroprotective activity of DG in the I/R model of renal injury mainly through the anti-oxidant system and inhibition of pro-inflammatory cytokines.

In clinical practice, kidney function is assessed by measuring the amount of urea and Cr in blood. It is proposed that Cr will remain at its normal levels even though about 50% of the kidney role is already lessened [39]. The levels of plasma Cr and BUN were reduced dose-dependently after DG treatment in I/R rats in the present investigation. Measurement of TP levels is an indicator of assessment of kidney function. The levels of TP got enhanced due to I/R damage that was significantly reduced in DG rats when compared with NC rats. Kidney weight was identified as the best marker of vascular permeability and tissue edema [40]. In this study, the kidney weight was remarkably elevated in the NC group due to I/R injury as compared to the SC group reflecting kidney edema, inflammation, the apparent increase in collagen accretion and kidney hypertrophy [41]. The administration of DG exhibited a significant reduction in kidney weight as compared to NC rats.

Here, we have focused on the investigation of the DG effect on oxidative stress in rat kidneys that underwent ischemic injury. It is

Figure 8. Effect of DG on Tumor necrosis factor-α and Interleukin-1β in I/R renal damage in rats. Results were expressed as Mean ± SEM (n = 6). ###*P < 0.001 as compared to SC rats and *ns Non-significant, **P < 0.01, ***P < 0.001 as compared to NC rats.
suggested that overproduction of reactive oxygen species (ROS) after I/R injury causes the generation of numerous oxidative damages at cellular levels like peroxidation of lipids [42] and carbonylation of proteins [43]. By this information, our study results showed that the rats undergoing I/R injury exhibited enhanced MDA and SOD levels along with the reduction in SOD and GSH enzyme levels in renal tissue. Malondialdehyde is an end product of lipid peroxidation and is used as a common marker of oxidative stress [44]. MPO is an enzyme stored in neutrophilic granular cells, primarily released from activated neutrophils, and therefore used as a marker of the inflammation process [45]. In the present study, the rats who underwent I/R injury had a significant elevation in the MDA and MPO concentration when compared with

**Figure 9.** Histopathological results of kidney sections stained with H & E after 24 h reperfusion (400 X). (a) SC; Group (b) NC Group; (c) DG + I/R (20 mg/kg); (d) DG + I/R (40 mg/kg); DG + I/R (80 mg/kg).
SC rats. At the time of kidney ischemic situation, neutrophilic infiltration occurs at the site of damaged kidney tissue (owing to raised oxidative stress). This method would result in blockage of blood circulation in the kidney tissue, resulting in additional renal tissue damage and altering the glomerular filtration rate [46,47]. Hence, elevated MDA and MPO concentrations reveal the induction of oxidative stress in kidney tissue after I/R injury. Another reason behind the generation of oxidative stress is the reduction of adenosine triphosphate as a result of ischemia causes activation of damaging enzymes like phospholipases and proteases during reperfusion injury [48]. Furthermore, after I/R injury, the hypoxic area that exists along with regular blood circulation can interact and results in the formation of increased ROS [49,50]. Adding to that, ROS enhance vascular responsiveness to vasoconstrictors, thus showing increased vascular resistance [51]. Therefore, DG treatment to I/R rats resulted in significantly decreased MDA and MPO levels in comparison with NC rats.

Glutathione is a main intracellular reducing agent that causes detoxification of lipid peroxides and is associated with other antioxidants like NADPH, vitamin C and vitamin E, protecting against oxidative stress [52]. In association with SOD that enhances the generation of oxygen and hydrogen peroxide from superoxide anion [53]. The treatment of rats with DG significantly increased SOD and GSH, indicating its antioxidative characteristics. It has been shown that DG significantly enhances the antioxidative capability of renal tissues, reduction in lipid peroxidation [54], decreases endoplasmic reticulum oxidative stress [55] and enhances the concentration of SOD and GSH in kidney tissue after I/R induced renal toxicity [56] through its antioxidant capacity.

TNF-α is a pro-inflammatory cytokine secreted by mononuclear macrophages and natural killer cells, which is an important mediator in the kidney inflammation process. TNF-α can stimulate kidney smooth muscle and encourage the adhesion of eosinophils and neutrophils inflammatory cells at the site of inflammation [57]. IL-1β is an added important contributor in kidney I/R injury. It causes activation of the NF-κB signaling pathway for TNF-α which initiates the process of inflammation and renal tissue injury along with late retrieval of renal function [58,59]. TNF-α and IL-1β cytokines played a significant role in kidney dysfunction of the I/R injury model [60]. The serum levels of TNF-α and IL-1β were increased noticeably in the NC rats as compared to SC rats, whereas the rats treated with DG were found to be decreased the levels of TNF-α and IL-1β.

Generalized damage along with severe tubular injury, tissue edema and neutrophilic infiltration in the renal tissue was observed in rat kidneys subjected to I/R injury. Kidneys from the SC group did not show any structural damage. In this study, pretreatment of rats with different doses of DG diminished the tubular injury. The treatment of rats with DG (40 to 80 mg/kg) exhibited conserved cellular structure and normal glomeruli that were found to be reliable on the results of the biochemical and antioxidant studies. Hence, the results of the histopathological analysis proved that DG exhibits nephroprotective potential in rats who underwent I/R injury.

Table 1. Histopathology scoring in I/R renal damage in rats.

| Treatment | Microscopic Changes | Score |
|-----------|---------------------|-------|
| SC        | Absence of tubular injury | 0     |
| NC        | Severe tubular injury, tissue edema and neutrophilic infiltration in the renal tissue | 3.15 ± 0.25 |
| DG (20 mg/kg) | 50% reduction in the tubular injury, tissue edema and neutrophilic infiltration in the renal tissue as compared with NC rats | 2.60 ± 0.25 |
| DG (40 mg/kg) | 85% reduction in the tubular injury, tissue edema and neutrophilic infiltration in the renal tissue as compared with NC rats | 1.45 ± 0.25 |
| DG (80 mg/kg) | 100% reduction in the tubular injury, tissue edema and neutrophilic infiltration in the renal tissue as compared with NC rats | 0.41 ± 0.25 |
Conclusion

It can be concluded that DG proved antioxidant and anti-inflammatory potential against kidney damage induced by I/R injury via mitigation of inflammation, oxidative stress and histopathologic injuries. In this study, a dose of 40 and 80 mg/kg of DG was found to be more efficient than 20 mg/kg. In divergence, 20 mg/kg of DG did not show any significant protection, highlighting a dose-dependent role of DG on kidney I/R injury.

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