

## The Association of Phosphodiesterase 5 Inhibitor on Ischemia-Reperfusion Induced Kidney Injury in Rats

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**Purpose:** Ischemia-reperfusion (IR) causes various damage in renal tissues. The aim of the present study was to evaluate the renoprotective effect of phosphodiesterase 5 inhibitor (PDE5I) on IR induced renal injury in a rat model.

**Materials and Methods:** Thirty adult male, 12-week-old, Sprague-Dawley rats were divided into three groups. Renal IR injury was induced by occlusion of the bilateral renal pedicle for 45 min followed by reperfusion for 24 h. The rats were sacrificed for collecting blood and tissue specimens. IR rats were administered daily oral Tadalafil (group I) or no pills (group II), while sham-operated animals were treated with no pills (sham group). The pill was diluted with distilled water and administered to rats for 15 days, orally. Renal histopathology, function, pro-inflammatory and inflammatory cytokines and mediators were assessed by serum creatinine, western blot assay and immunohistochemistry.

**Results:** Compared with sham group, rats that underwent renal IR operation exhibited a significant increase in concentration in serum creatinine ( $P < .01$ ) and tissue pro-inflammatory and inflammatory mediators. In group I, however, tadalafil significantly suppressed elevation of the serum creatinine and increased the levels of endothelial nitric oxide synthase and decreased the level of intercellular adhesion molecule-1 (ICAM-1) compared to group II ( $P < .05$ ). Moreover, tadalafil prevented IR-induced expression of pro-inflammatory mediators such as monocyte chemoattractant protein-1 (MCP-1) ( $P < .05$ ).

**Conclusion:** Tadalafil significantly promotes functional recovery after renal IR injury and effectively inhibits the induction of pro-inflammatory and inflammatory mediators. The results substantiate Tadalafil as a protective agent against IR-induced renal injury.

**Keywords:** kidney; ischemia; reperfusion; tadalafil; nephrectomy

### INTRODUCTION

Ischemia is an irreversible tissue injury process in which there is a loss of blood supply in a tissue due to impeded arterial flow or reduced venous drainage.<sup>(1)</sup> Reperfusion after the ischemic period can cause severe tissue injury, which is termed ischemia reperfusion (IR) injury.<sup>(2)</sup>

IR-induced renal injury, which occurs in various conditions, such as renal transplantation, nephron sparing surgery, renal angioplasty, shock and heart failure, is a common cause of acute renal insufficiency.<sup>(3,4)</sup> Although several decades of research have greatly improved the understanding of the mechanisms underlying renal IR injury, effective drugs for treating it are still unavailable. The mechanisms of renal IR injury include hypoxia, vascular endothelial injury, infiltration of inflammatory cell, accumulation of free radicals such as reactive oxygen species (ROS) and generation of inflammatory mediators. IR aggravates renal structural damages.<sup>(5,6)</sup> Sildenafil citrate, vardenafil HCl and tadalafil are wide-

ly used primary treatment of erectile dysfunction and various other disorders including hypertension, prostatic hyperplasia and coronary heart disease.<sup>(7,8)</sup> These drugs are phosphodiesterase 5 inhibitors (PDE5Is); they enhance cyclic guanosine monophosphate and nitric oxide (NO)-mediated vasodilation with resulting improvement of erectile dysfunction.<sup>(7,8)</sup> Experimental studies have demonstrated that PDE5Is improve endothelial function and reduce infarct size in rat models of myocardial infarction.<sup>(8)</sup> Hence, these inhibitors may also be nephroprotective in renal IR injury.

A recent study using a renal IR model demonstrated that PDE5Is improve endothelial function and protect nephrons.<sup>(9)</sup> In this study, we further explored the protective effects of a PDE5I (Tadalafil) in a rat model of renal IR injury.

### MATERIALS AND METHODS

#### PDE5I

Tadalafil powder (Eli Lilly and Company, Indianapolis,

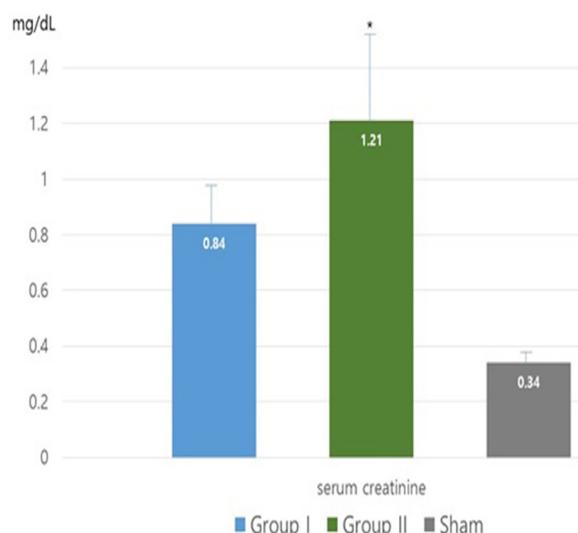
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**Figure 1.** The effects of tadalafil on renal function during renal IR. Serum creatinine was less increased by tadalafil pre-treatment (Group I) compared by Group II. Group II compared with the group I and sham group. \*,  $P < .05$ .

lis, ID) was dissolved in dimethyl sulfoxide (DMSO, 10mg/mL) and diluted 1:100 in ethanol. The dilution was added to 28 mL of drinking water (28mL). Tadalafil was administered to rats every day for 15 days. The animal house technicians ensured that the tadalafil-laden water was completely consumed each day. Tadalafil dosing for 15days was 5 mg/kg/day.<sup>(10)</sup>

#### Rat model

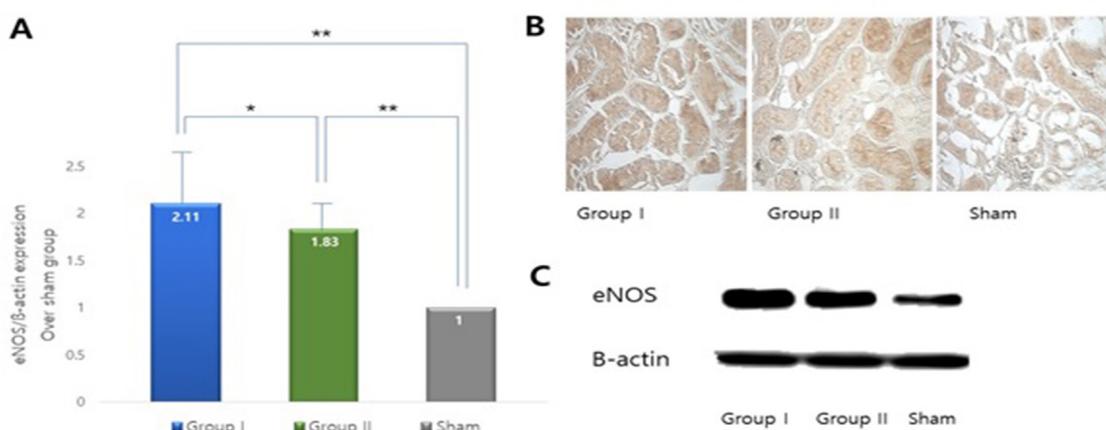
The experimental study was carried out after obtaining the approval of the Ethics Committee [IRB No. 06-2014-006]. Thirty adult, male, 12-week-old Sprague-Dawley rats were allowed free access to food and drink, and were housed under the specific pathogen-free conditions in alternating 12 h periods of light and dark, with 35-75% humidity at 20-26°C. Renal IR injury was induced by clamping of the bilateral renal pedicle and

subsequently with reperfusion.<sup>(11,12)</sup> In brief, rats were anesthetized using an intramuscular injection of ketamine 100mg/kg. With each rat in the supine position, midline incision was conducted and the both kidneys were subjected to 45minutes of ischemia by both renal pedicles clamping with atraumatic method followed by reperfusion for 24 h. The rats were sacrificed and then, immediately blood and tissue specimens were gathered and stored at -75°C. The rats were allocated to three experimental groups at random. The rats in group I and II underwent IR injury surgery, with oral tadalafil supplied (group I) or not supplied (group II). Rats in sham group underwent a sham operation with no medication.

#### Assessment of renal function

The diagnosis of clinical acute kidney injury (AKI) due to IR injury is typically based on an elevation in plasma creatinine. There is a lag period between the onset of kidney injury and the rise in creatinine; typically, creatinine levels peak 6-24 h after the ischemic insult. Prior experimental data showed that 45 min of bilateral renal pedicle ischemia resulted in a significant rise in serum creatinine at 6-24 h.<sup>(13)</sup> Therefore we measured serum creatinine level and mediators at 24 h. The samples were measured using a Technicon RA-1000 autoanalyzer (Bayer, Tarrytown, NY).

Immunohistochemical staining and Western blot assay  
Immunohistochemical staining was performed according to the manufacturer's instructions. Paraffin-embedded renal tissues were cut into 3  $\mu$ m thickness, deparaffinized and hydrated. The antibodies against intercellular adhesion molecule-1, (ICAM-1, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), endothelial nitric oxide synthase (eNOS, 1:1000; Santa Cruz Biotechnology) or monocyte chemoattractant protein-1 (MCP-1, 1:1000; Santa Cruz Biotechnology) used for immunohistochemical staining. The immunohistochemical staining protocols by Shi et al. were used.<sup>(14,15)</sup> Total protein in rat kidney was withdrawn with radio-immunoprecipitation assay lysis buffer containing 1% phenylmethanesulfonyl fluoride (Beyotime, Nanjing, China). A kit (Beyotime) was used to extract nuclear and cytoplasmic protein antibody against ICAM-1, eNOS, MCP-1 or  $\beta$ -actin (all 1:1000; Santa Cruz Biotechnology), or to glyceraldehyde-3-phosphate dehy-



**Figure 2.** The effect of tadalafil on eNOS during renal IR detected by Immunohistochemical staining (x400) and Western blot. At 24 h after reperfusion, the expression of eNOS was enhanced by tadalafil (A). Immunohistochemical staining (B) and Western blot results (C) showed the expression of eNOS in Group I was increased compared by Group II and sham group. \*,  $p < .05$ , \*\*  $p < .01$ .

drogenase (GAPDH, 1:500; Santa Cruz Biotechnology) were used for Western blot assay. Membranes were then examined with HRP-conjugated secondary antibody (1:1,000; Santa Cruz Biotechnology). By using an enhanced ECL Detection System (Millipore), target proteins were visualized and by using Quantity One 4.6.2 software (Bio-Rad, Hercules, CA), band densitometries were quantified. The Western blot protocol by Mahmood et al. were used.<sup>(16,17)</sup>

### Statistical analysis

The statistical data were analyzed using Statistical Package for the Social Sciences Version 13.0 for Windows software (SPSS, Chicago, IL). One-sample Kolmogorov Smirnov tests were used to determine that the quantitative data for every group were normally distributed. Statistical significance for multiple comparisons was analyzed by one-way analysis of variance test. Statistical test results were considered significant at a  $p$ -value  $< .05$ .

## RESULTS

### Effect of tadalafil on renal function after IR injury

To investigate the protective effect of the tadalafil on renal function caused by IR injury, plasma creatinine levels were measured 24 h after renal reperfusion. Plasma creatinine concentrations were significantly higher in group I and II than in the sham group. Compared with the group II, group I rats exhibited a significant difference in concentration in creatinine level (**Figure 1**) ( $P = .008$ ).

### Effect of tadalafil on eNOS after IR injury

eNOS concentrations in the renal tissue were markedly increased after reperfusion, and remained at significantly higher level than in sham operated group (**Figure 2A**) ( $P < .01$ ). This was significantly different relative to group I and II ( $p = .032$ ). IR injury increased the expression of eNOS in the kidneys compared to the sham operated group. Pre-treatment with tadalafil enhanced the increase in eNOS concentration (**Figure 2A, B and C**). After reperfusion, the numbers of positive cells stained with eNOS antibodies were increased compared with the sham group (**Figure 2 B and C**).

### Effect of tadalafil on ICAM-1 after IR injury

Pro-inflammatory mediators, such as ICAM-1, amplify the inflammatory response and oxidative stress injury, and deteriorate tissue damage. Presently, the ICAM-1 concentrations in the renal tissue were markedly increased after reperfusion, and remained significantly higher than in the sham operated group (**Figure 3A, B and C**) ( $p < .05$ ). Group II displayed significantly higher levels than group I ( $p = .021$ ). ICAM-1 was significantly decreased by tadalafil pre-treatment. Typical photographs of renal tissue are shown in **Figure 3B**. Compared with group II, group I displayed obvious down-regulation of the expression of ICAM-1.

### Effect of PDE5I on MCP-1 after IR injury

Pro-inflammatory factors, such as MCP-1, reportedly play an important role in renal injury induced by IR. Presently, MCP-1 concentrations in the renal tissue were increased after reperfusion, with group I being significantly lower than group II (**Figure 4A**). Group I and II displayed significantly higher MCP-1 expression than the sham operated group (**Figure 4A, B and C**). The tadalafil pre-treatment group displayed signif-

icantly lower decrease than group II. These findings indicated that tadalafil lowered the local inflammatory response.

## DISCUSSION

The present study demonstrated that eNOS and ICAM-1 antibodies were markedly increased compared with the sham group. Compared with ischemia reperfusion group (group II), tadalafil pretreated group (group I) displayed obvious down-regulation of the expression of ICAM-1 and up-regulated expression of eNOS. Moreover, MCP-1 in the tadalafil pre-treatment group displayed significantly lower decrease than ischemia reperfusion only group. These findings indicated that tadalafil lowered and attenuated the local inflammatory response.

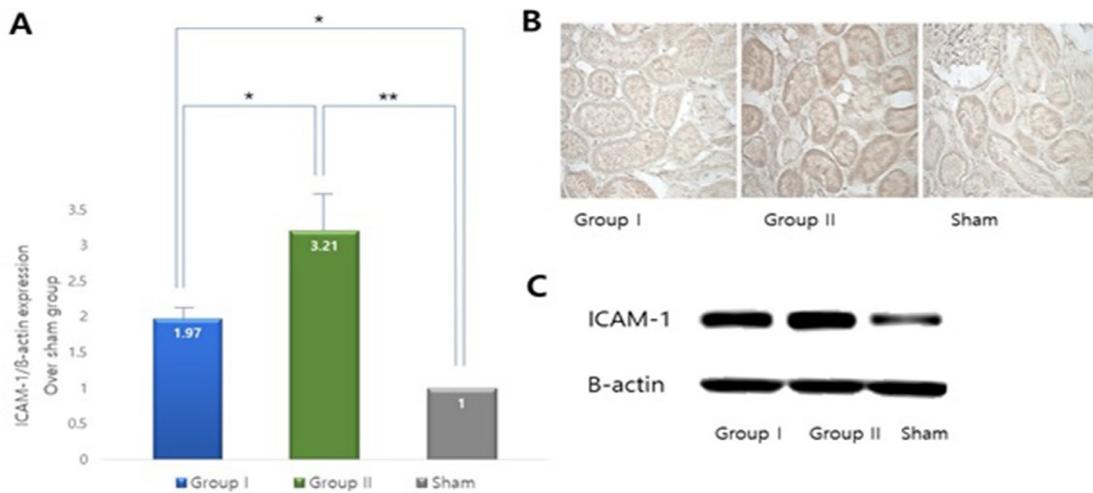
Recovery of renal function following nephron sparing surgery is associated with reduced ischemic damage during operation, pre-operative preparation and post-operative management.

Minimally invasive techniques have been developed to reduce warm ischemia time and to preserve functional renal volume in nephron sparing surgery. However, nephron sparing surgery of difficult cases are usually conducted in long ischemia time to allow for tumor resection and renal reconstruction. Long-standing ischemia compromises renal function due to ischemia reperfusion injury. Some patients with solitary kidney and renal insufficiency may need acute or even permanent dialysis.<sup>(18)</sup> The definition of the ideal ischemia time threshold during nephron sparing surgery is still debatable. Based on data from animal models and small, retrospective clinical studies,<sup>(19)</sup> a warm ischemia time of less than 30 minutes has been historically thought to allow full recovery of kidney function. Current evidence suggests that warm ischemia time and residual functional parenchyma after nephron sparing surgery correlates with postoperative renal function.

Preoperative preparation for protection against renal IR injury introduces many substances.<sup>(20-24)</sup> However, currently they are not used clinically because of a lack of data, particularly clinical studies. Methylene blue,<sup>(20)</sup> melatonin,<sup>(21)</sup> dexamethasone,<sup>(22)</sup> sufosalazine<sup>(23)</sup> and beta-carotene<sup>(24)</sup> have been studied for their efficacy in preventing destruction in experimental renal IR injury models.

Phosphodiesterase 5 (PDE5) is the predominant phosphodiesterase in the corpus cavernosum. PDE5I blocks degradative action of cGMP by PDE5, increasing blood flow to the penis during sexual stimulation. In addition, novel therapeutic indications have emerged with the discovery that PDE5 is expressed in various other tissues, such as arterial and venous vasculatures, skeletal and visceral muscles, and platelets.<sup>(25)</sup>

IR induced renal injury occurring with ischemia and restoration of blood flow to post-ischemic tissue may be associated with microvascular injury, particularly due to increased permeability of arterioles and capillaries, leading to an increase of diffusion and an induction of fluid exudation across the tissues.<sup>(1)</sup> The sterile inflammatory response induced by IR is characterized by marked recruitment of neutrophils and the production of cytokines, chemokines, and other proinflammatory stimuli. Activated endothelial cells produce more ROS following reperfusion, which promote leukocyte adhesion to capillaries and venules and subsequent emigra-



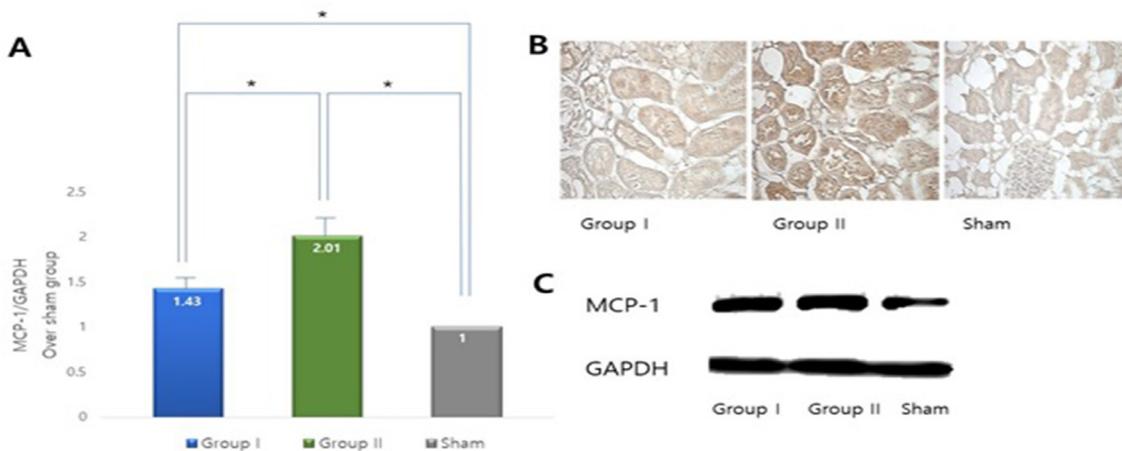
**Figure 3.** The effect of tadalafil on ICAM-1 during renal IR detected by Immunohistochemical staining (x400) and Western blot. At 24 h after reperfusion, the expression of ICAM-1 was suppressed by tadalafil (A). Immunohistochemical staining (B) and Western blot results (C) showed the expression of ICAM-1 in Group I was decreased compared by Group II. \*:  $p < .05$ , \*\*:  $p < .01$ .

tion into the tissues.<sup>(2)</sup> Damage to the cell membrane can release more free radicals. Such reactive species may also act indirectly in redox signaling to stimulate apoptosis. Leukocytes and endothelial cell adhesive interactions may also bind to the endothelium of small capillaries, which precipitate the development of microvascular dysfunction.<sup>(1,2,26)</sup>

Although IR injury triggers the toxic and inflammatory cascades leading to alteration of renal function. Interestingly, in our study, after administration of tadalafil, serum creatinine levels decreased significantly. In addition, our data demonstrate that pre-treatment with tadalafil is renoprotective against IR injury, with affects evident at the serum creatinine level as well as for inflammatory mediators. The up-regulated expression of eNOS and the down-regulated expression of ICAM-1 and MCP-1 were largely modified after pre-treatment. This may reflect tadalafil-mediated inhibition of inflammatory factors at the inflammatory sites. NO synthases (NOSs) are a group of enzymes that catalyze the production of NO from L-arginine. NO pro-

duced by eNOS is a vasodilator identical to the EDRF (endothelium-derived relaxing factor) produced in response to increased blood flow in arteries. This expands blood vessels by relaxing of vascular smooth muscle in linings.<sup>(27)</sup> eNOS is the main regulator of vascular smooth muscle tone.<sup>(27)</sup> eNOS-mediated NO production plays a pivotal protective role in IR injury.<sup>(27,28)</sup> Presently, tadalafil significantly enhanced eNOS in the treated rats compared with non-treated rats. In contrast, others showed that sildenafil has a protective effect that is independent of the NO/cGMP pathway in an IR model.<sup>(29)</sup> This indicates that other protective pathways involving sildenafil could exist. Their identity requires further studies.

ICAM-1 is an endothelial- and leukocyte- associated transmembrane protein that stabilizes intercellular interaction and promotes leukocyte endothelial transmigration. When activated, leukocytes bind to endothelial cells and then transmigrate into tissues. Particularly, ICAM-1 signaling seems to recruit inflammatory immune cells including macrophages and granulocytes.



**Figure 4.** The effect of tadalafil on MCP-1 during renal IR detected by Immunohistochemical staining (x400) and Western blot. At 24 h after reperfusion, the expression of MCP-1 was enhanced by tadalafil (A). Immunohistochemical staining (B) and Western blot results (C) showed the expression of ICAM-1 in Group I was decreased compared by Group II. \*:  $p < .05$ .

<sup>(30,31)</sup> This finding, as well as the increased expression of ICAM-1, has been corroborated by experiments on cultured endothelial cells exposed to IR injury. In our study, ICAM-1 level was significantly decreased by pre-treatment with tadalafil. The findings indicate the effectiveness of tadalafil in reducing leukocyte endothelial transmigration and decreasing the inflammatory response.

MCP-1, also termed chemokine ligand 2, has been implicated in pathogenesis of several diseases characterized by monocytic infiltrates.<sup>(32)</sup> Administration of anti-MCP-1 antibodies in a model of glomerulonephritis reduced the infiltration of macrophages and T cells, and reduced scarring, renal impairment and crescent formation.<sup>(33)</sup> Presently, tadalafil pre-treatment significantly reduced the expression of MCP-1. This finding indicates that tadalafil can alleviate the inflammatory response of the kidney during renal IR by reducing the local inflammatory response.

Previous several studies confirmed the efficacy of some premedication in preventing renal damage in animal models of IR injury.<sup>(20-24)</sup> However, none of the agents are currently used in adjuvant therapy in humans because of lack of clinical data on safety and their restricted availability. In contrast, PDE5I is commonly used and readily available; the current data support its use for reducing IR injury. Clinical studies are still necessary to evaluate the therapeutic properties of PDE5 inhibitors in prevent IR injury.

Limitation to our study, such as the relatively short period of ischemia (45 min) and reperfusion (24 h), leave the prolonged renal function unclear. Also, the mechanisms to explain the renoprotective effects are ambiguous because of missing evaluation of free radicals such as ROS. Furthermore, we did not examine the histologic damage such as, inflammatory cell count and the number of apoptotic cells in renal tissue were increased concomitantly in IR injury. Further studies are needed to resolve the controversy in the present and previous studies.

## CONCLUSIONS

In our study, tadalafil improved the recovery of renal injury during IR by enhancing eNOS expression, and decreasing ICAM-1 and MCP-1 expression. These findings showed that tadalafil lowered the local inflammatory response and enhanced ischemic tolerance. Tadalafil pre-treatment has the potential to attenuate the IR induced renal injury in nephron sparing surgery.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

The authors report no conflict of interest.

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