A Study on the Effect of Sildenafil Citrate on Acute Renal Ischemic-Reperfusion Injury in Male Rats


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Abstract

Several studies suggest that acute ischemia reperfusion (IR) of the kidney results in deterioration of its performance resulting in bad outcome of kidney transplantation operations and major operations in which the kidneys are exposed to acute ischemia reperfusion.

The present study was designed to investigate if sildenafil citrate (SC) has a protective role in renal I/R injury and to study its possible mechanisms of action in rats by inducing bilateral renal ischemia followed by one hour reperfusion and measurement of serum creatinine, blood urea nitrogen and nitric oxide (NO) level in renal tissues. 60 rats were used & were subjected to sham operated (control group-1), 50min renal ischemia (group-2), ischemia for 50 minutes then reperfusion for 2 hours (group-3), SC (1mg/kg orally) + sham operated group (group-4). Ischemia of the kidneys for 50 minutes, one hour after SC oral treatment (Group 5), I/R of the kidneys, one hour after SC oral treatment (Group 6).

Results: The study documented that I/R resulted in a significant increase \( (p<0.05) \) in the serum creatinine, blood urea nitrogen levels, rat tail systolic blood pressure and a significant decrease \( (p<0.05) \) in NO level in renal tissues.

SC prophylactic treatment resulted in partial reversal of measured parameters compared to the groups untreated with the same drug. There was a significant decrease \( (p<0.05) \) in serum creatinine, blood urea nitrogen and a significant increase \( (p<0.05) \) in NO level in renal tissues in I/R after SC prophylactic treatment compared to the groups untreated with the same drug.

It can be concluded from this study that renal I/R resulted in deterioration of renal function, elevation of systolic blood pressure and decreased NO level in renal tissues. SC provided a partially protective role against renal I/R injury as after prophylactic treatment with SC kidney functions were ameliorated and the level of NO in kidney tissue increased suggesting that this protective effect of SC may be mediated through NO. However, there was no significant difference in rat tail systolic blood pressure after SC prophylactic treatment suggesting that this drug has no significant secondary systemic effects.

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Key Words: Renal ischemia reperfusion – Sildenafil – Nitric oxide – Rats

Introduction

ISCHEMIC-reperfusion (I/R) injury is a common event associated with high mortality and morbidity. Post-ischemic acute tubular necrosis occurs in many clinical settings including shock, renal transplantation, cardiovascular and renal surgery. Despite considerable efforts to discover the cellular and molecular pathogenesis and treatment of the I/R injury, no effective treatment is currently [1].

Renal ischemia leads to depletion of cellular energy, accumulation of intracellular sodium, calcium and reactive oxygen species, activation of multiple enzyme systems including protease, nitric oxide synthases, phospholipase and endonuclease and this results in cell damage and death [2].

Re-establishing blood flow to ischemic organs is vital to prevent tissue death [3]. Tissue damage is mediated by cytokines, local imbalance in nitric oxide (NO) levels, endothelial-cell adhesion molecules, platelet activating factors and free radicals [1].

NO is a key molecule involved in a variety of physiological and pathological conditions. Since NO has regulatory, protective and deleterious effects on cellular functions, NO donors and antagonists have been tested in several studies against I/R injury. NO has been shown to act via a variety of second-messenger cascades, although most of these effects are mediated by cGMP [4].

NO stimulates generation of cGMP, not only in the vasculature, but also in the renal tubules, including the proximal tubules, thick ascending limb and collecting ducts [8].
Phosphodiesterase type 5 (PDE5) enzyme converts cGMP into the inactive GMP and terminates the action of c-GMP. Sildenafil Citrate (SC) is a potent inhibitor of PDE5 and widely used to treat male erectile dysfunction. Studies have shown that apart from its role in erectile dysfunction, SC is also effective in pulmonary hypertension and esophageal motor disorders [6,7]. PDE5 is widely distributed in the body in the vasculature, platelets, kidneys and other tissues. In the rat kidney, PDE5 enzyme is localized in glomeruli, mesangial cells, cortical tubules and inner medullary duct cells [8].

The present study relates to a method for prophylaxis against ischemia and/or reperfusion injury of the kidney using an effective amount of sildenafil citrate (SC).

**Material and Methods**

**Experimental animals and protocol:**

A Total of 60 male adult albino rats weighting 150-200gm (approximately five months of age) were housed in wire mesh cages at room temperature, veterinary care was provided by Laboratory Animal House Unit of Faculty of Medicine, Cairo University. Rats were housed with normal light and dark cycle and were allowed to acclimatize to their environment for five days before start of the experiments. The experiments were done in January 2011. All animals were kept under the same environmental conditions and had free access to food and water.

Rats were divided into 6 groups (n=10/group):

- **Group I** : Control (sham-operated).
- **Group II** : Ischemic group, rats were subjected to ischemia for 50 minutes.
- **Group III** : (Ischemic reperfusion group), ischemia for 50 minutes then reperfusion for 2 hours [9].
- **Group IV** : This group was sham-operated with SC (SC+Sham).
- **Group V** : Ischemic with SC (SC+Ischemia).
- **Group VI** : Ischemia-reperfusion with SC (SC+I/R).

Rats were treated with SC (Viagra), dissolved in saline solution and given as a single dose (1mg/kg) 60min before the operation [1].

**Sham operation:**

Sham-operated animals received equivalent anesthesia and underwent laparotomy but without clamping. Immediately before sacrifice, rat tail blood pressure was measured and blood samples were withdrawn through retro-orbital route using heparinized capillary tubes in 10ml eppendorf tubes. The blood samples were centrifuged for further determination of serum creatinine and urea. Then animals were sacrificed by cervical dislocation and tissue samples from kidney were dissected and kept frozen at -80°C in liquid nitrogen.

**Blood pressure measurement:**

Systolic blood pressure in rats was measured by the Harvard rat tail blood pressure monitor system. A Hand inflation bulb is used to inflate the cuff that fits over the rat tail to occlude the blood flow, while the optical pick up detects blood flow. A Pressure transducer in the control unit converts the pressure to an analogue signal for recording, i.e. changes in the blood flow are detected by the optical pick up unit and these changes are transmitted to a pressure transducer located in the control unit of the apparatus. The control unit then converts the pressure signals to electronic
analogue signals which are displayed on a PC computer system. At least three consecutive recordings were taken if these three recordings were similar they were taken as systolic blood pressure measurement [11].

**Biochemical analysis:**

- **Serum creatinine** was estimated by QuantiChrom™ creatinine Assay Kit [12].
- **BUN** was estimated by QuantiChrom™ Urea Assay kit (DIUR-500) [13].

**Measurement of nitric oxide NO:**

Kidney tissue was homogenized in 5-10ml cold buffer (50mM potassium phosphate, pH 7.5, 1mM EDTA) per gram tissue. Homogenate was centrifuged and supernatant was removed for assay. NO level was determined indirectly as its metabolic products (nitrate + nitrite ions) spectrophotometrically using BioAssay Systems’ QuantiChromTM Nitric Oxide Assay Kit to measure NO production following reduction of nitrate to nitrite using improved Griess method [14]. Absorbance measurement was done at 540nm against the reagent blank. The levels of nitric oxide were determined by extrapolation from absorbance-concentration curve.

**Statistical analysis:**

Data were coded and entered using the statistical package SPSS version 15. Data was summarized using mean, standard deviation and range for the quantitative variable. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test in normally distributed quantitative variables while non parametrical kruscal-wallis test and mann-whitney test were used for non normally distributed quantitative variables [15]. p-values less than 0.05 were considered as statistically significant.

**Results**

Regarding the effect of renal ischemia and reperfusion, Table (1) showed that serum creatinine, BUN & RSBP were significantly elevated (p<0.05) and NO levels in kidney tissues were significantly decreased (p<0.05) in ischemic group (group II) & IR group (group III) compared to control group. However serum creatinine & BUN & RSBP decreased significantly (p<0.05) & NO increased significantly (p<0.05) in IR group compared to ischemic group (Fig. 1).

Assessment of kidney function, RSBP & NO after SC prophylaxis showed that the levels of serum creatinine, BUN, RSBP and NO level in kidney tissue were not significantly changed in SC group (group IV) compared to control group (group I). However, Serum levels of creatinine and BUN were significantly elevated & NO levels were significantly decreased in Ischemia + SC group (group V) & IR+SC group (group VI) compared to SC group (group IV) (p<0.05). After SC prophylaxis the levels of creatinine & BUN decreased significantly (p<0.05) & the levels of NO increased significantly (p<0.05) in ischemic + SC group & IR+SC group compared to ischemic group & IR group respectively (Fig. 1).

RSBP was significantly elevated in Ischemia + SC group & IR+ SC group compared to SC group (group IV) (p<0.05). The value of RSBP in ischemia + SC group and IR+SC group (group VI) showed no significant change compared to ischemic & I/R group respectively (Fig. 1D).

**Table (1): Comparison between mean±SD of serum creatinine (mg/dl), blood urea nitrogen BUN (mg/dl), rat tail systolic blood pressure RSBP (mmHg) and nitric oxide NO in kidney tissue (µM/mg ptn) in control group (I), ischemic group (II) and ischemic reperfusion I/R Group (III), Sildenafil Citrate SC+sham-operated group (IV), SC+ischemic group (V), SC/I/R group (VI).**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group (I)</th>
<th>Group (II)</th>
<th>Group (III)</th>
<th>Group (IV)</th>
<th>Group (V)</th>
<th>Group (VI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.5±0.07</td>
<td>1.6±0.28*</td>
<td>1.11±0.2*#</td>
<td>0.55±0.11</td>
<td>0.9±0.11$#</td>
<td>0.81±0.11$@</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>36.8±5.78</td>
<td>72.23±6.95*</td>
<td>57.17±7.54*#</td>
<td>34.52±5.79</td>
<td>47.63±5.72$#</td>
<td>42.17±3.95$@</td>
</tr>
<tr>
<td>RSBP (mmHg)</td>
<td>110±2.44</td>
<td>124.7±7.63*</td>
<td>116.9±5.09*#</td>
<td>107.3±6.07</td>
<td>121.11±6.34$</td>
<td>116±3.05$</td>
</tr>
<tr>
<td>NO level in kidney tissue (µM/mg ptn)</td>
<td>29.3±4.3</td>
<td>15.3±2.43*</td>
<td>17.46±1.78*#</td>
<td>27.13±4.23</td>
<td>20.03±2.09$#</td>
<td>20.96±2.35$@</td>
</tr>
</tbody>
</table>

Values are represented as mean±SD

* : Statistically significant compared to corresponding value in group (I) (p<0.05).
# : Statistically significant compared to corresponding value in group (II) (p<0.05).
@ : Statistically significant compared to corresponding value in group (III) (p<0.05).
$ : Statistically significant compared to corresponding value in group (IV) (p<0.05).
So, administration of SC to rats before inducing ischemia and I/R improved renal functions (BUN, serum creatinine) as compared to ischemic group and I/R group respectively. But the level of kidney functions (BUN, serum creatinine) were still higher as compared to the SC+sham operated group. On the other hand, the level of NO in renal tissue was higher in ischemic group and I/R group receiving prophylactic SC as compared to ischemic group and I/R group respectively. But the level of NO in renal tissue were still lower as compared to the SC+sham operated group.

SC has no significant effect on RSBP in ischemic & IR groups.

**Discussion**

Acute renal failure (ARF) caused by renal I/R is an important clinical problem. Even though great progress has been made in patient care, there is still high morbidity and mortality associated with ARF. Renal I/R injury is also an important determinant of allograft survival after transplantation [16].

In the present study, rats in the ischemic group (50min of renal ischemia) and in the I/R group...
(50min of renal ischemia and 2 hours of reperfusion) showed a significant increase in the serum creatinine and BUN levels compared to the control group.

Kadkhodaee et al. [17] reported that both 45 and 60min ischemia followed by 1 hour reperfusion resulted in significant increase in plasma creatinine and BUN compared to the sham-operated group. However, they found that BUN did not change after 30min renal artery occlusion followed by 1 hour reperfusion. So they concluded that renal I/R caused a reduction in renal function and structural alteration in an ischemia-time-dependent manner.

Pechman et al. [18] added support and mentioned that acute I/R injury resulted in a transient decrease in renal function 24 hours after surgery as indicated by a significant increase in plasma creatinine compared to sham control rats and that plasma creatinine in I/R rats returned toward control values and was not significantly different from that of sham controls after 28 days of recovery. These results were also consistent with the finding of Jung et al. [19] who found that serum creatinine and BUN peaked 2 days after the renal I/R injury (maximum increases of 14.3-fold in BUN and 7.4-fold in serum creatinine compared with levels in sham-operated mice).

Sutton et al. [20] reported that renal I/R injury in rats resulted in microvascular injury manifested by disruption of the actin cytoskeleton and adherens junction of endothelial cells after ischemic injury and that this injury increased renal microvascular permeability which in turn induced interstitial edema that contributed to decreased renal blood flow during reperfusion leading to deterioration of renal function.

Leemans et al., [21] found that renal tubular epithelial cells played an important role in renal I/R induced renal dysfunction. They reported that injury of these cells resulted in a cascade of responses that led to the local production of pro-inflammatory mediators, which in turn increased renal injury and dysfunction. They also reported that renal tubular epithelial cells expressed and up regulated Toll-like receptor 2 after ischemic injury and showed that during I/R injury, Toll-like receptor 2 played an important role in the early development of acute renal failure after renal I/R.

Renal I/R led to vascular and tubular defects that took place after ischemia and during the reperfusion process. The vascular component included intrarenal vasoconstriction that led to a glomerular filtration rate reduction, together with vascular congestion in the outer medulla and activation of tubuloglomerular feedback. The mechanisms implicated in this response were as follows: Increased release of vasoconstrictor factors (mainly endothelin, adenosine, and angiotensin II); decreased production of vasodilators (such as NO, prostaglandin, acetylcholine, and bradykinin); and increased structural damage in endothelial and vascular smooth muscle cells [22].

Occurring at the same time, the tubular component included loss of tubular polarity, tubular obstruction, interstitial inflammation, cytoskeletal breakdown, sublethal cell injury, apoptosis, and tubular necrosis. The factors responsible for these tubular alterations included hypoxia, ATP depletion, increased concentrations of reactive oxygen species, intracellular acidosis, elevated cytosolic calcium concentrations, increased activity of phospholipases, and proteases released from the tubular cell brush border. All these events led to renal dysfunction [23].

The results of the current work demonstrated that, RSBP. was significantly increased in ischemic group (group II) and in I/R group (group III) as compared to the control group (group I).

Similarly the result of the present study was in agreement with the work of Müller et al. [9] who reported that mean arterial pressure was significantly higher in female rats following renal ischemia for 50 minutes and reperfusion for 2 hours than pre-ischemic pressure. The investigators also reported that further investigations were necessary to determine the mediators involved in the significant increase in mean arterial pressure observed in female rats. They reported that several vasoactive agents such as endothelin were released as a result of I/R injury in males. However, these agents were not detected in females.

Pechman et al. [18] demonstrated that male rats recovered from acute renal I/R injury for 5 weeks developed sodium-sensitive hypertension and had a blunted pressure-natriuretic diuretic relationship as they found that post-I/R rats had normal blood pressure when fed 0.4% NaCl but developed hypertension when the salt content of the food was increased to 4.0% NaCl. Further studies are needed to investigate if blunted natriuresis response has a role in systolic blood pressure elevation in this study.

However, our results contradict the findings of Kadkhodaee et al. [17] who reported that mean arterial pressure after 1 hour of renal ischemia...
followed by 1 hour reperfusion was not significantly different from the basal value in Sprague-Dawley rats. This controversy can be explained by different species and experimental protocols used.

In the present study, when renal NO level was assessed in renal tissues in ischemic group (group II) and in I/R group (group III), it was significantly decreased compared to control group (group I).

These results are in accordance with that obtained by Kwon et al. [24] who found that renal NO generation was significantly lower in recipients destined to have sustained acute kidney injury, at least until postoperative day 3. They found also diminished e-NOS expression in cadaveric allografts after I/R compared with control tissues and suggested that vascular endothelial damage, occurring after I/R, could cause an impaired vasodilator ability of the renal vasculature due to a decreased endothelial NO generation and may contribute to the reduction in glomerular filtration in recipients of cadaveric renal allografts.

Goligorsky et al. [25] proposed the key role of endothelial dysfunction in acute renal ischemia, suggesting that the defective production of endothelial NO may eventually lead to the destruction of tubular epithelial cells through vascular congestion or the “no reflow” phenomenon.

The mechanism of the protective effect of NO on renal I/R injury might be from prevention of the neutrophil component of ischemic renal injury by blocking increased expression and function of CD11/CD18 on neutrophils, which are involved in adhesion, activation and migration of neutrophils and in the subsequent respiratory burst [26]. Also NO decreases the expression of the endothelial adhesion molecules ICAM-1, VCAM-1 and E-selectin, and of pro-inflammatory cytokines such as interleukin 6 and interleukin 8 [27].

NO plays a beneficial role in I/R injury by inhibiting platelet adhesion and aggregation, by diffusion into platelets elevating cGMP levels, which in turn, causes an extrusion of intracellular Ca$^{++}$ from platelets. This reduction in cytosolic free Ca$^{++}$, results in inhibition of fibrinogen binding to platelet glycoprotein receptors, which is essential for platelet adhesion and aggregation [28].

However, Wang et al. [29] contradict our results by reporting that the kidney eNOS and iNOS expression after renal ischemia for 45min and reperfusion for 24 hour were both significantly higher than those of control group in Sprague-Dawley rats.

Also, Salom et al. [30] observed that renal ischemia was followed by a rapid increase in peroxynitrite generation that peaked at 30min of ischemia, reaching a plateau until the end of ischemia, and rapidly dropping to preischemic values on reperfusion. Because peroxynitrite is formed by the reaction of NO with superoxide, the increase in peroxynitrite observed means that NO and superoxide must be generated together in hypoxic conditions when renal blood flow is interrupted. The reduced renal function after I/R was preceded by a significant and correlative increase in outer medullary levels of NO and peroxynitrite during ischemia, indicative of oxidative and nitrosative stress in the outer medulla.

NO has been shown to have several functions in the kidney, depending on its concentration, site of release and duration of action. For example, NO generated by iNOS has been shown to have cytotoxic effects on renal tubular epithelial cells. In contrast, an increased expression of eNOS, which leads to the enhanced production of endothelium-derived NO, may ameliorate ischemic and toxic renal injury by mediating vasodilatation, inhibiting leukocyte adhesion, and reducing platelet aggregation [24].

PDE5 enzyme converts cGMP into the inactive GMP and terminates the action of cGMP. SC (‘Viagra’) is a potent inhibitor of PDE5 and widely used to treat male erectile dysfunction.

The present study revealed that I/R after SC group (group VI), showed a significant decrease in the serum creatinine and the BUN compared to the I/R group (group III) but value of RSBP in I/R after SC (group VI) showed no significant change compared to I/R group (group III). These results were consistent with the finding of Salom et al. [31] and Lledó-García et al. [32] who stated that there is a positive effect of sildenafil in the immediate post-transplantation outcome of warm ischemic kidneys without secondary systemic effects and also stated that NO levels were significantly higher for all periods in the I/R and SC group. (31,32) These results are also consistent with our present study in which we found the level of NO in kidney tissue significantly higher in I/R with SC(group VI),compared to I/R group (group III).

Few studies have so far examined the effects of PDE5 inhibitors on renal I/R injury; however, one such study found that a selective cGMP PDE5 inhibitor, Zaprinast, had anti-platelet effects following ischemia I/R in rats [33]. In another study, the same drug accelerated renal recovery by stim-
ulating regional renal blood flow and increasing intracellular cGMP in ischemic ARF in rats [34]. Although SC does not increase renal blood flow in healthy individuals and in patients with liver cirrhosis and ascites, its effects on renal blood flow after ischemia remain unknown [35,36].

In a more recent study, SC treatment prevented deterioration of renal function, reduced histological damage, inflammation and apoptosis, and preserved renal capillary integrity in a renal ablation model [37].

Also Ozgur et al. [38] reported that SC pretreatment attenuated the renal I/R injury, tubular morphology was significantly better than that in untreated I/R group and it was not different from that in sham group in outer cortex, inner cortex and medullary regions.

Evidence indicates that the major part of tissue injury occurs upon reperfusion and is mediated by activated neutrophils [3]. During ischemia reperfusion, neutrophils are recruited, activated and adhere to the vascular endothelium. Upon neutrophil activation and accumulation, release of reactive oxygen free radicals as well as cytotoxic constituents released from the invading neutrophils cause further damage. Previous studies have shown that inhibitors of neutrophil activation and accumulation attenuate damage to ischemic tissue after reperfusion [39,40] SC pretreatment significantly decreased leukocyte infiltration in the I/R group. As expected, myeloperoxidase (MPO) activity, which is accepted as an indicator of neutrophil infiltration, was significantly higher in the kidney tissue of the I/R group than that of the sham group and furthermore this increase was inhibited by SC treatment [38].

It can be concluded that I/R injury produced renal dysfunction as evidenced by increase in blood urea, serum creatinine and rat tail systolic blood pressure. The I/R injury in the present study was associated with a decrease in NO synthesis. Also, it was found that SC ameliorated I/R injury. Furthermore, our results showed that there is no significant change in blood pressure in groups treated by SC in comparison to the groups not treated with SC suggesting the partial protective role of SC against I/R injury without significant secondary systemic changes. Also the increased level of NO in SC treated groups suggested that the action of SC may be mediated through increase in NO synthesis. However, further studies are required to confirm these findings and reveal the exact mechanism of action of SC in I/R injury before clinical applications.

References

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