## Research Article
# Different Protective Effects of Trimetazidine against Renal Ischemia/Reperfusion Injury in Rats

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**Abstract:** Ischemia/Reperfusion (I/R) injury during kidney transplantation is a major clinical problem which leads to delayed graft function or even graft rejection. Therefore, the aim of the present study was to explore the possible protective effects of trimetazidine, an anti-ischemic agent, against experimentally-induced renal I/R injury in rats. Ischemia was induced by bilateral clamping of renal pedicles for 45 min followed by 24 h of reperfusion. Trimetazidine (10 mg/kg) was orally administered twice daily for 14 successive days before induction of ischemia. At the end of the reperfusion period (24 h), blood and renal tissue samples were collected for biochemical and histopathological examination. Trimetazidine showed nephroprotective effects as evidenced by significant decrease in blood urea nitrogen and serum creatinine levels as well as renal content of tumor necrosis factor-α, malondialdehyde and myeloperoxidase. In addition, trimetazidine replenished renal content of adenosine triphosphate and glutathione that was confirmed by the improvement in histopathological features. The current study demonstrated the nephroprotective effects of trimetazidine against renal I/R injury, which might be mediated through its anti-ischemic, anti-inflammatory or antioxidant activities.

**Keywords:** Anti-inflammatory, anti-ischemic, antioxidant, renal ischemia/reperfusion, trimetazidine

## INTRODUCTION

Acute renal failure induced by ischemia/reperfusion (I/R) injury, especially occurs during renal transplantation, is associated with high morbidity and mortality rate. Subsequent to renal transplantation, I/R injury represents the major cause of early graft rejection and adversely affects the long-term survival of transplanted kidney (Avlan et al., 2006).

Multiple pathophysiological mechanisms are involved in I/R injury, including the modulation of mitochondrial function with subsequent depletion of intracellular adenosine triphosphate (ATP) and changes in intracellular calcium levels, activation of neutrophils, generation of inflammatory cytokines and increased production of reactive oxygen species, which could lead to cell death through necrosis and apoptosis (Grossini et al., 2012).

Trimetazidine (TMZ) is an anti-ischemic agent, where its anti-ischemic effects have been experimentally evaluated in different models including cell cultures, isolated and perfused organs and also in vivo (Sulikowski et al., 2008). TMZ has the ability to restore mitochondrial ATP synthesis, which was stopped by the $Ca^{2+}$ overload during ischemia (Domanski et al., 2006). In addition, TMZ decreases the excessive release of reactive oxygen species, reduces membrane lipid peroxidation and inhibits neutrophil infiltration after ischemia and reperfusion (Onbasili et al., 2007).

The aim of the present study was to explore the different protective effects of TMZ against renal I/R injury induced in rats.

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## MATERIALS AND METHODS

**Animals:** AdultmaleWistar rats, weighting 200-250 g each, were used in the present study. The animals were obtained from the animal house of Faculty of Pharmacy, Beni-Suef University and were kept under appropriate laboratory conditions throughout the experimental work. They were fed a standard chow diet and allowed free access to water. All animal use and handling were done in accordance with protocols approved by the Faculty of Pharmacy, Beni-Suef University Animal Care and Use Committee.

**Experimental design:** Rats were randomly divided into 3 groups, each consisting of 8 rats. The first group received 1% Tween 80 once daily "sham control group", the second group received 1% Tween 80 once daily "I/R control group". The third group received TMZ (10 mg/kg) twice daily "TMZ group" (Domanski *et al*., 2006; Sulikowski *et al*., 2008).

On the 14th day, rats were anesthetized with thiopental (60 mg/kg, i.p.) after fasting overnight with free access to water, then a medline laparotomy was made, using minimal dissection, the right and left renal pedicles were exposed and occluded with a surgical clip for 45 min to induce ischemia followed by 24 h of reperfusion. This was done in all groups except the 1st one which was just subjected to sham operation.

At the end of the reperfusion period, blood samples were collected from the retro-orbital plexus for estimation of Blood Urea Nitrogen (BUN), serum creatinine and calcium ($Ca^{2+}$) levels. Animals were sacrificed by cervical dislocation and the kidneys were removed. One kidney was homogenized in ice-cold saline 20% (w/v) to be used for determination of ATP, myeloperoxidase (MPO), tumor necrosis factor-α (TNF-α), malondialdehyde (MDA) and reduced glutathione (GSH). The other kidney was used for histopathological examination.

**Determination of serum levels of BUN, creatinine and $Ca^{2+}$:** BUN and serum creatinine levels were determined using Diamond Diagnostics kit regents (Egypt), while serum $Ca^{2+}$levels were determined using Spinreact kit reagents (Spain).

**Estimation of renal ATP:** Renal ATP content was estimated as previously mentioned (Lowry *et al*., 1964). The method depends on the formation of glucose-6-phophate (G-6-P) from the reaction of ATP with glucose in the presence of hexokinase. The resultant G-6-P is then oxidized in the presence of $NADP^+$ by the aid of glucose-6-phosphate dehydrogenase activity. The increase in fluorescence intensity due to formation of $NADPH^+$ was measured at 460 nm after excitation at 365 nm, using a spectrofluorometer (FP-6200,JASSCO[®], Japan).

**Measurement of renal TNF-α:** TNF-α content in tissue homogenate was determined using rat TNF-α enzyme-linked immunosorbent assay kit (Quantikine, R and D Systems, USA) as mentioned before (Salama *et al*., 2014).

**Assessment of renal MPO activity:** MPO is located within the primary granules of neutrophils, hence extraction of MPO depends upon procedures that disrupt the granules and render MPO soluble in aqueous solution. This could be achieved by freezing and re-thawing followed by sonication in phosphate buffer (pH 6) containing 0.5% (w/v) hexadecyltrimethyl ammonium bromide (HTAB), where HTAB is a detergent that releases MPO from the primary granules of the neutrophils (Bradley *et al*., 1982). This method is based on measuring hydrogen peroxide-dependent oxidation of *o*-dianisidine, which is catalyzed by MPO. The reaction results in the formation of a compound exhibiting an increased absorbance at 460 nm (Arab *et al*., 2014).

**Determination of renal MDA:** The method depends on the colorimetric measurement of the intensity of the pink color, resulting from the reaction of MDA with thiobarbituric acid in an acidic medium at high temperature (Uchiyama and Mihara, 1978; Mehany *et al*., 2013) using a spectrophotometer (UV/Visible-1601PC, Shimadzu[®], Japan).

**Measurement of renal GSH:** Renal GSH content was determined by the reaction with Ellman's reagent [5, 5'-dithiobis (2-nitrobenzoic acid)] to form a stable yellow color of 5-mercapto-2-nitrobenzoic acid, which can be measured colorimetrically at 412 nm (Engelhardt and Homma, 1996).

**Histological assessment of nephrotoxicity:** Kidney samples were taken from rats in different groups and placed in vials containing 10% formaldehyde for at least 24 h. Fixed kidneys were dehydrated through graded alcohols. Specimens were cleared in xylene and embedded in paraffin, then paraffin sections (4 μm) were stained by Hematoxylin and Eosin (H&E) for histopathological examination by the light microscope (Bancroft and Gamble, 2008).

**Statistical analysis:** Values are expressed as mean ± standard error of mean (SEM). Results were analyzed using one way Analysis of Variance (ANOVA) followed by Newman-Keuls for comparison between different groups. Differences between values were considered significant if $p < 0.05$. Prism GraphPad software, version 5 was used to carry out all statistical tests.

## RESULTS

**Effect on BUN and serum creatinine, $Ca^{2+}$levels:** Rats subjected to renal I/R injury showed a significant

Table 1:  Effect of TMZ on BUN, serum creatinine and Ca$^{+2}$ levels in I/R

| Groups | BUN (mg/dL) | Creatinine (mg/dL) | Ca$^{+2}$ (mg/dL) |
|---|---|---|---|
| Sham control | 12.79±0.40 | 0.66±0.01 | 7.08±0.38 |
| I/R control | 65.25±2.50[a] | 2.42±0.18[a] | 3.63±0.20[a] |
| TMZ | 38.32±2.90[a,b] | 1.19±0.11[a,b] | 5.41±0.44[a,b] |

Data are presented as mean of 6-8 experiments±SEM and compared by one way ANOVA followed by Newman-Keuls multiple comparisons test; [a]$p<0.05$ vs. sham control; [b]$p<0.05$ vs. I/R control

Table 2: Effect of TMZ on renal content of MDA and GSH in I/R

| Groups | MDA (nmol/g wet tissue) | GSH (µg/g wet tissue) |
|---|---|---|
| Sham control | 69.22±6.55 | 431.70±5.85 |
| I/R control | 111.20±14.59[a] | 371.60±7.08[a] |
| TMZ | 76.40±5.76[b] | 419.00±10.95[b] |

Data are presented as mean of 6-8 experiments±SEM and compared by one way ANOVA followed by Newman-Keuls multiple comparisons test; [a]$p<0.05$ vs. sham control; [b]$p<0.05$ vs. I/R control

increase in BUN and serum creatinine levels compared with sham control animals. Prophylactic treatment with TMZ produced a significant reduction in BUN and serum creatinine levels compared with rats subjected to I/R only (Table1).

I/R control rats showed a significant reduction in serum Ca$^{2+}$ level, compared to sham control. Pretreatment with TMZ significantly increased serum Ca$^{2+}$ level compared to I/R control (Table 1).

**Effect on renal ATP content:** Rats subjected to I/R injury showed a significant reduction in renal ATP content, compared to sham control rats. Pretreatment with TMZ twice daily for two weeks replenished the reduced ATP content (Fig. 1).

**Effect on renal TNF-α content:** Figure 2 demonstrated a significant increase in renal TNF-α content in rats subjected to I/R injury, compared to sham control rats. Prophylactic treatment with TMZ significantly decreased TNF-α content to about 76%, compared to I/R control group.

**Effect on renal MPO activity:** I/R injury resulted in a significant elevation in renal MPO activity, compared to sham control. Prior administration of TMZ significantly reduced MPO activity to about 64%, compared to I/R control, showing similar response to that of sham control group (Fig. 3).

**Effect on renal oxidative stress biomarkers (MDA and GSH):** Rats subjected to renal I/R injury showed a significant elevation in MDA content, compared to sham control rats. Pretreatment with TMZ significantly reduced renal MDA content (Table 2).

Renal GSH content was significantly decreased by induction of I/R injury, compared to sham control. Prophylactic treatment with TMZ protected against the decline in renal GSH content (Table 2).
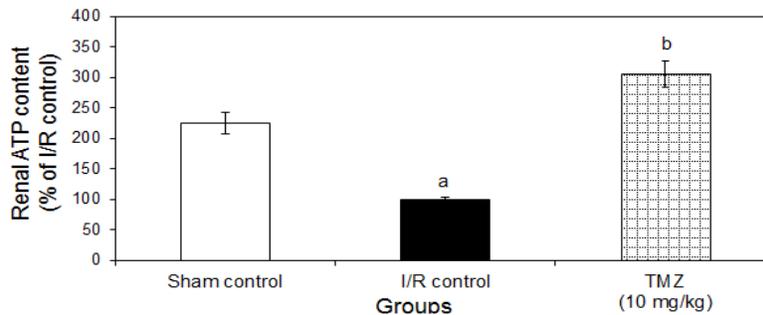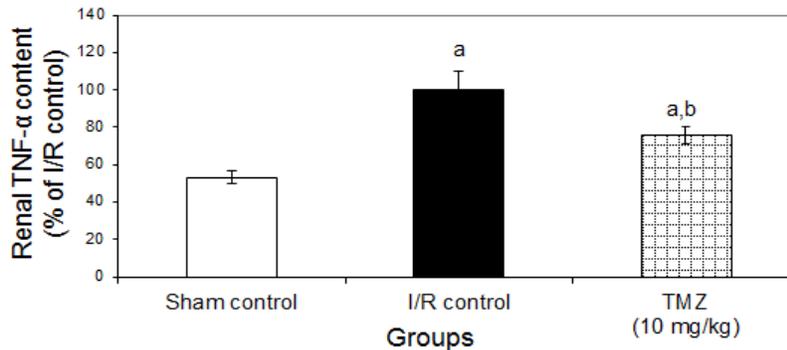


Fig. 1:  Effect of TMZ on renal ATP content in I/R; Data are presented as mean of 6-8 experiments±SEM and compared by one way ANOVA followed by Newman-Keuls multiple comparisons test. [a]$p<0.05$ vs. sham control; [b]$p<0.05$ vs. I/R control



Fig. 2:  Effect of TMZ on renal TNF-α contentin I/R; Data are presented as mean of 6-8 experiments±SEM and compared by one way ANOVA followed by Newman-Keuls multiple comparisons test. [a]$p<0.05$ vs. sham control; [b]$p<0.05$ vs. I/R control
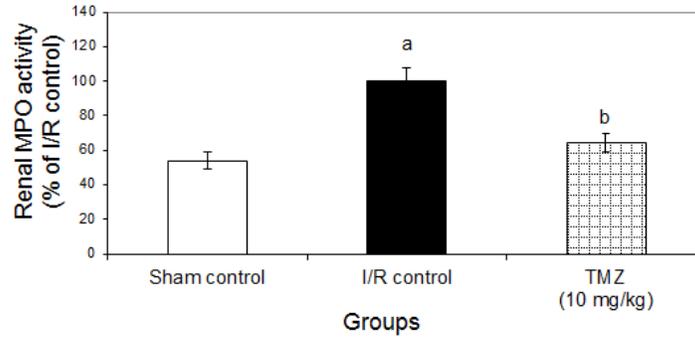
Fig. 3: Effect of TMZ on renal MPO activity in I/R; Data are presented as mean of 6-8 experiments±SEM and compared by one way ANOVA followed by Newman-Keuls multiple comparisons test. [a]$p<0.05$ vs. sham control; [b]$p<0.05$ vs. I/R control
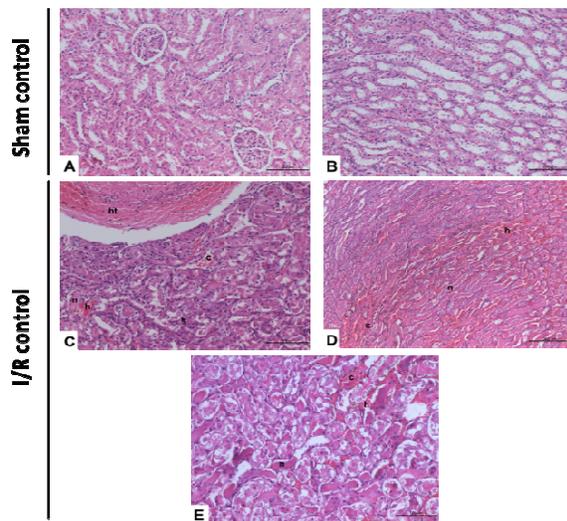


Fig. 4: Photomicrographs showing of H&E-stained kidney sections from sham and I/R control rats; (A, B) Sham control showing normal histological structure of the glomeruli and tubules at the cortex and medulla, respectively (× 200). (C, D, E) I/R control showing severe hemorrhage (h) and hematoma (ht), vacuolar degeneration (arrow) and coagulative necrosis (n) at the cortex (×200), as well as congestion (c), hemorrhage and coagulative necrosis at the medulla (×400) and corticomedullary junction , respectively (×200)
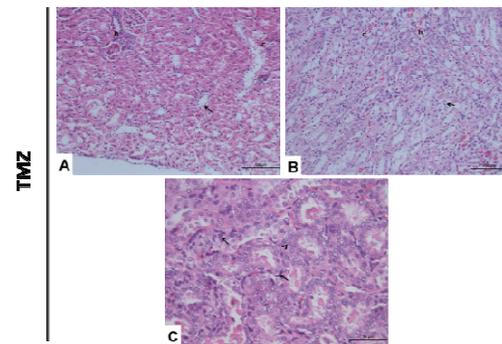


Fig. 5: Photomicrographs of H&E-stained kidney sections from rats treated with TMZ before I/R; (A, B, C) TMZ showing very mild congestion (c) and hemorrhage (h) together with vacuolar degeneration (arrow) at the cortex and medulla (×200), active mitosis and proliferation of basal cells (arrow) and regenerated epithelial cells having faint basophilic cytoplasm and active round vesicular nuclei (arrow head) at the corticomedullary junction, respectively (×400)

**Histopathological changes:** Histopathological examination of kidney sections taken from sham control rats revealed that there were no histopathological alterations in the glomeruli and tubules at the cortex (Fig. 4A) as well as at the tubules at the medulla. (Fig.4B). Kidney sections of rats subjected to I/R showed severe hemorrhage and hematoma together with vacuolar degeneration and coagulative necrosis in renal tubular cells at the cortex (Fig. 4C), as well as congestion, hemorrhage and coagulative necrosis at the medulla (Fig. 4D) and the corticomedullary junction (Fig. 4E). In the group of rats treated with TMZ before I/R, The cortical tubules showed mild congestion and hemorrhage together with vacuolar degeneration (Fig. 5A), as well as at the medullary tubules (Fig. 5B). The corticomedullary junction showed active regenerative changes that were indicated by active mitosis and proliferation of basal cells and round vesicular nuclei of regenerated epithelial cells with faint basophilic cytoplasm (Fig. 5C).

## DISCUSSION

In the present study, we demonstrated that prophylactic treatment with TMZ twice daily for 14 successive days significantly reduced renal damage induced by I/R. The improvement in kidney function was demonstrated by reduction in BUN and serum creatinine levels, decreased MPO activity, TNF-α and MDA contents in kidney tissues. In addition, TMZ restored GSH and ATP contents in kidney tissues. In parallel with these biochemical changes, histopathological evaluation of the kidney tissues revealed a significant nephroprotective effect of TMZ against I/R induced-degenerative changes.

Ischemic injury prevents oxidative phosphorylation with depletion of intracellular ATP, increase in anaerobic glycolysis and inhibition of the $Na^+/K^+$ ATPase pump, leading to a progressive increase in the concentration of cytosolic $Na^+$, which in turn activates $Na^+/Ca^{2+}$ exchange, inducing an intracellular accumulation of calcium ions (Morin *et al*., 2001). This is consistent with our results, which revealed that renal ATP content and serum $Ca^{2+}$ levels were reduced after I/R. Pretreatment with TMZ, effectively restored renal ATP content and increased serum $Ca^{2+}$ levels, giving an evidence to its anti-ischemic activity against renal I/R injury.

It has been demonstrated that renal I/R injury promotes an acute inflammatory response characterized by elevation of inflammatory cytokines, particularly TNF-α (Hagar *et al*., 2012). Moreover, it has been reported that TNF-α promotes renal dysfunction by stimulation of other inflammatory mediators, such as interleukin-1, platelet activating factor, oxygen radicals, nitric oxide and prostaglandins, beside its role in the activation and recruitment of neutrophils (Meldrum *et al*., 2002). In our study, there was a significant elevation in renal TNF-α content, which was ameliorated by pretreatment with TMZ. These results demonstrates that the protective role of TMZ in renal I/R could be attributed to the suppression of inflammatory mediators.

Renal MPO activity was used as an indicator of neutrophil infiltration into renal tissues. This enzyme catalyzes the formation of the pro-inflammatory oxidant hypochlorous acid (HOCl) from hydrogen peroxide ($H_2O_2$) and chloride ions (Matthijsen *et al*., 2007). In our study, it was shown that MPO activity significantly increased after I/R and this increase was inhibited by prophylactic treatment with TMZ, giving another evidence to the anti-inflammatory role of TMZ in renal I/R injury.

Oxidative stress, induced by the overproduction of reactive oxygen species and/or the decrease in the antioxidant mechanisms, plays a major role in the formation of I/R injury (Chatterjee, 2007). Furthermore, lipid peroxidation is implicated in the pathogenesis of renal I/R injury (Yun *et al*., 2009).We determined the oxidative injury and lipid peroxidation in the kidney after I/R by measuring MDA content, which is the decomposition product of lipid peroxidation and GSH content, which represents an important antioxidant, protects tissue from oxidative damage. Our results demonstrated that renal MDA content was significantly increased, while GSH was reduced subsequent to I/R injury. Prior administration of TMZ, inhibited the increase in MDA and replenished GSH content, demonstrating that the antioxidant properties of TMZ play a role in its nephroprotective mechanism.

## CONCLUSION

The present study demonstrated that prophylactic treatment with TMZ ameliorates I/R induced renal damage and its protective effects are attributed to its anti-ischemic, antioxidant properties or due to the ability to regulate the inflammatory mediators.

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