Effects of various durations of post-treatment with trimetazidine against ischemia-reperfusion: early and long-term effects in a rat kidney model

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Directed by Professor Young Lan Kwak

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ABSTRACT

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Acute kidney injury (AKI) is a well-known risk factor for poor prognosis in hospitalized patients and believed to be often the consequence of ischemia-reperfusion (I/R) injury. Trimetazidine (TMZ, 1 - [2, 3, 4 - trimethoxybenzyl] piperazine, dihydrochloride), an anti-ischemic agent used for decades, has a theoretical potential to reduce I/R injury through modulations of various cell survival pathways. Yet, the majority of studies including clinical studies have concentrated on the early effects of TMZ pre-treated before I/R injury, limiting its clinical applicability. The aim of this study was to elucidate the effects of various durations of TMZ administered after renal I/R injury on renal recovery in long-term as well as short-term aspects with the evaluation of relevant signaling pathways in rats.

In short-term study, Sprague-Dawley (SD) rats were randomly assigned to four groups: Sham, untreated control I/R (IRC), TMZ treatment before I/R (TMZ-pre), TMZ treatment after I/R (TMZ-post). TMZ (3 mg/kg, intraperitoneally) was administered 1 hr before ischemia (pre) or upon reperfusion (post). All rats except in the Sham group underwent left side nephrectomy and the right renal pedicle was clamped for 45 min and then reperfused for 24 hr when the rats were euthanized for analysis. In intermediate and long-term studies, rats were randomly assigned to four groups: Sham, IRC, TMZ post-treatment after I/R (TMZ-single), daily TMZ post-treatment after I/R (TMZ-daily). TMZ (3 mg/kg, intraperitoneally) was administered once immediately upon reperfusion (TMZ-single), or administered once a day for a scheduled period (TMZ-daily).

The rats were euthanized 5 days or 8 weeks after I/R injury. The effect of post I/R treatment with TMZ was determined using different variables: renal function (serum blood urea nitrogen [BUN] and creatinine), renal histologic change including apoptotic cell death and fibrosis, and related signaling proteins including hypoxia inducible factor (HIF)-1α, vascular endothelial growth factor (VEGF), phosphorylated-Akt (P-Akt), endothelial nitric oxide synthase (eNOS), Bcl-2, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs).

Post I/R treatment with TMZ significantly reduced serum levels of BUN and creatinine, apoptotic cell death through up-regulation of HIF-1α-VEGF and P-Akt/eNOS, and Bcl-2 related apoptotic signaling pathways 24 hr after renal I/R injury. Although serum levels of BUN and creatinine recovered to baseline values, post I/R treatment with TMZ reduced renal tubular cell necrosis and apoptosis via up-regulation of HIF-1α-VEGF, P-Akt/eNOS, and TIMPs, attenuation of MMPs activities, and alteration of the ratio of Bax for Bcl-2 at 5 days after renal I/R injury regardless of the treatment protocol. Post I/R treatment with TMZ, however, did not suppress the progression of renal fibrosis and expression of related signal pathways, 8 weeks after renal I/R injury.

These results indicate that post I/R treatment with TMZ has beneficial effect on renal protection against I/R injury at early and intermediate period, while it failed to inhibit the long-term renal fibrotic change.

Key words: cell signaling pathway, early and long-term, ischemia-reperfusion injury, renal, trimetazidine

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I. INTRODUCTION

Acute kidney injury (AKI) is a well-known risk factor for poor prognosis in hospitalized patients and believed to be often the consequence of ischemia-reperfusion (I/R) injury.[1,2] Although not clearly elucidated, multiple mechanisms including renal vascular dysfunction and reduced renal blood flow, and further aggravation of the initial renal injury through augmented vasoconstriction and renal tubular epithelial damage following I/R injury are believed to be critical in the development of AKI.[3,4] Of importance, renal dysfunction would be gradually aggravated even after complete recovery from AKI during early periods,[5,6] which worsens long-term survival of patients with AKI than that of patients without AKI.[7] Therefore, it is crucial to establish treatment strategies to induce proper renal recovery from I/R injury. Most of the studies employing ischemic-, and pharmacological preconditioning for AKI, however, have focused on the renal function at immediate period after I/R injury,[8-10] and only a scanty of information regarding the long-term influence of the applied therapies in renal function after I/R injury exists.

The cell survival pathways against renal I/R injury involve various anti-apoptotic, anti-inflammatory, and anti-oxidative pathways. Most notably, hypoxia inducible factor (HIF)-1α stimulates various cell survival signaling pathways under renal hypoxic condition like I/R injury,

including a main pathway attenuating oxidative stress through the expression of vascular endothelial growth factor (VEGF) and activation of Akt/endothelial nitric oxide synthase (eNOS) pathways.[11-15] In addition, in case of tubular epithelial apoptosis after I/R injury, anti-apoptotic proteins such as Bcl-2 have been shown to exert affirmative influence as well.[16]

Trimetazidine (TMZ, 1 - [2, 3, 4 - trimethoxybenzyl] piperazine, dihydrochloride), an anti-ischemic agent used for decades, has a theoretical potential to reduce I/R injury at both cellular and mitochondrial levels.[17,18] Indeed, TMZ has provided cardio- and renoprotective effects in various subsets of patients exposed to circumstances associated with I/R injury. In view of its cytoprotective action mechanisms, TMZ reduced intracellular acidosis, preserved ATP stores and inhibited inflammatory reaction in association with promoted release of HIF-1α and enhanced expression of eNOS through activation of Akt.[19,20] In experimental models, TMZ pre-treatment reduced the progression of renal I/R injury via mitigating apoptosis and interstitial fibrosis as well as oxidative stress.[21-23] Still, the majority of studies including clinical studies were focused on the early effects of TMZ pre-treated before the I/R injury,[17,20,24] while pre-treatment with TMZ from 1 day to 1 month before the I/R injury might not be clinically feasible.

This study aimed to elucidate the effects of TMZ administration for various durations after the onset of renal I/R injury on renal recovery in short-, intermediate-, and long-term aspects, and to investigate relevant signaling pathways in rats.

II. MATERIALS AND METHODS

1. Animal preparation

All experiments were approved by the committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine, and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.[25]

Male Sprague-Dawley rats (10-12 weeks old, 250-300 g) were anesthetized with Rompun (vial Korea, 10 mg/kg, intraperitoneally) plus Zoletil 50 (Virbac Korea, 30 mg/kg, intraperitoneally). The rats were intubated with a 16-gauge catheter and artificially ventilated (Harvard Apparatus 683, Holliston, MA) at 30-35 cycles/min. The right femoral artery was cannulated to monitor mean arterial pressure (MAP) and collect blood. Heart rate (HR) was monitored by subcutaneous stainless-steel electrodes connected to the power lab system (ML845 PowerLab with ML132; AD Instruments, Colorado Springs, CO). The body temperature was continuously monitored throughout the experiment and maintained around 37℃ using a heating pad.

2. Experimental models and study groups

We employed an established rodent model of renal I/R injury, which has been shown to allow the longest period of ischemia to induce an appropriate degree of renal injury while being compatible with long-term survival.[26,27] After midline incision to expose both kidneys, the left kidney was removed in all rats except the Sham group. The right renal pedicle was clamped for 45 min with atraumatic microvascular clamp and then reperfused for 24 hr, 5 days, and 8 weeks to allow short-, intermediate-, and long-term studies, respectively. Ischemia and reperfusion were confirmed by visually inspecting the kidney.

MAP and HR were continuously monitored throughout the procedures and recorded at baseline, during ischemia, and immediately after reperfusion. Kidneys were collected at the end of reperfusion.

To examine the effect of TMZ on renoprotection, TMZ (3 mg/kg, Sigma, St. Louis, MO) was administered to TMZ groups while the Sham and Control groups received equivalent amount of 0.9% saline via intraperitoneal injection. The dose of TMZ was chosen based on a preliminary study in which we found that 3 mg/kg yielded maximum protective effect on renal I/R injury.

Experiment 1: short-term study

The animals were randomly assigned to four groups: 1) Sham group (N=10); 0.9% saline only, 2) Untreated Control I/R (IRC) group (N=15); 0.9% saline + I/R, 3) TMZ pre-treatment before I/R (TMZ-pre) group (N=15); TMZ treatment 1 hr before ischemia + I/R, 4) TMZ post-treatment after I/R (TMZ-post) group (N=15); TMZ treatment upon reperfusion + I/R. All animals were euthanized 24 hr after reperfusion.

Experiment 2: intermediate- and long-term studies

The animals were randomly assigned to four groups: 1) Sham group (N=6); 0.9% saline only, 2) Untreated Control I/R (IRC) group (N=15); 0.9% saline + I/R, 3) single TMZ post-treatment after I/R (TMZ-single) group (N=15); TMZ single administration upon reperfusion + I/R, 4) daily TMZ post-treatment after I/R (TMZ-daily) group (N=15); I/R + daily TMZ administration for 5 days or 8 weeks starting upon reperfusion. Animals were euthanized 5 days or 8 weeks after reperfusion depending on the study protocol.

3. Blood urea nitrogen (BUN) and creatinine analysis

Serum samples were obtained at 24 hr, 5 days, and 8 weeks after reperfusion and assayed for BUN and creatinine levels by using the picric acid and diacetyl monoxime methods,[28] respectively, in the Department of Biochemistry, Severance Hospital.

4. TUNEL assay

Detection of apoptosis on paraffin sections from each group was examined using the terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end labeling (TUNEL).[29] Five visual fields from each sample block were randomly selected and analyzed by a blind observer using an Olympus microscope with magnification X 400. The apoptotic index was analyzed (apoptotic cells/total cells X 100%) from a total of 20 fields per sample.

5. Renal histopathology examination

Paraffin-embedded kidney tissues were cross-sectioned (5 to 6 specimens per group) through the midpoint to measure histologic damage. Periodic Acid Schiff (PAS) staining was performed and the degree of tubular damage was graded on a scale from 1 to 4.[30] The degree of cell gradation was graded as follows; 1 = no change from normal, 2 = focal area of cell degradation involving less than 5% of total field, 3 = cell degradation involving 5–30% of total field, 4 = cell gradation involving greater than 30% of total field. The degree of necrosis was graded as follows; 1 = Nil, 2 = some single-cell necrosis observed, 3 = dispersed focal necrotic areas observed, 4 = confluent necrosis in most tubules observed. The degree of neutrophil infiltration was graded as follows; 1 = Nil, 2 = 1–3 cells/field observed, 3 = 4–6 cells/field observed, 4 = heavy infiltration observed. Masson's trichrome staining was used to assess renal interstitial fibrosis.[31]

6. Immunoblot analysis

After appropriate treatments, equal amount of protein from each group underwent immunoblot assay as described previously.[32] Proteins were separated on Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti-cytochrome C, anti-VEGF (santaCruz, CA), anti-NOX (Abcam, Cambridge), anti-matrix metalloproteinase (MMP)-2, anti-MMP-9, anti- tissue inhibitor of metalloproteinase (TIMP)-1, anti-TIMP-2 (Calbiochem, USA), anti-Bcl-2, anti-Bax, anti-HIF-1α, anti-phosphorylated (P)-eNOS, anti-eNOS, anti-P-inducible nitric oxide synthase (iNOS), anti-iNOS, anti-caspase-3, anti-cleaved caspase-3, anti-P-Akt, anti-Akt, and anti-Actin (all Cell Signaling Technology, Beverly, MA). Each experiment was performed at least three times.

7. Enzyme-linked immunosorbent assay (ELISA)

Serum levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-α were determined by ELISA commercial kits (R&D System, Minneapolis, MN).

8. Statistical Analysis

All results were expressed as mean ± standard deviation. The statistical analysis was performed using one-way analysis of variance (ANOVA) or Student's t test followed by Bonferroni correction. Values of $P < 0.05$ were considered significant.

III. RESULTS

1. Hemodynamic parameters

Baseline MAP and HR were similar among the groups. In the experiment 1 and 2, MAP and HR during ischemia and after reperfusion were not significantly different compared to their corresponding baseline values and there were no significant intergroup differences in MAP and HR throughout the study period (data not shown).

2. TMZ treatment ameliorated I/R injury induced renal dysfunction

In the pilot study to investigate the optimal dose of TMZ, apoptotic cell death after renal I/R injury was minimal at 3 mg/kg of TMZ (Figure 1A). The number of apoptotic cells in ischemic renal tubules was mostly reduced by 73-75% in the 3 mg/kg of TMZ group compared to those of the IRC group and 1 and 5 mg/kg of TMZ groups (Figure 1B). Therefore, TMZ at a dose of 3 mg/kg was selected for further studies.

Renal I/R injury produced a significant increase in serum BUN and creatinine levels, which were significantly attenuated in the TMZ groups. Mean serum levels of BUN were decreased by 21% (87 ± 13 mg/dL, $P < 0.05$) and 19% (89 ± 24 mg/dL, $P < 0.05$) in the TMZ-pre and TMZ-post groups compared to the IRC group (110 ± 19 mg/dL), respectively (Figure 2A). Mean serum levels of creatinine were decreased by 28% (2.5 ± 0.8 mg/dL, $P < 0.05$) and 30% (2.4 ± 1.2 mg/dL, $P < 0.05$) in the TMZ-pre and TMZ-post groups compared to the IRC group (3.4 ± 1.0 mg/dL), respectively (Figure 2B). There were no significant differences in serum BUN and creatinine levels between the TMZ-pre and TMZ-post groups.

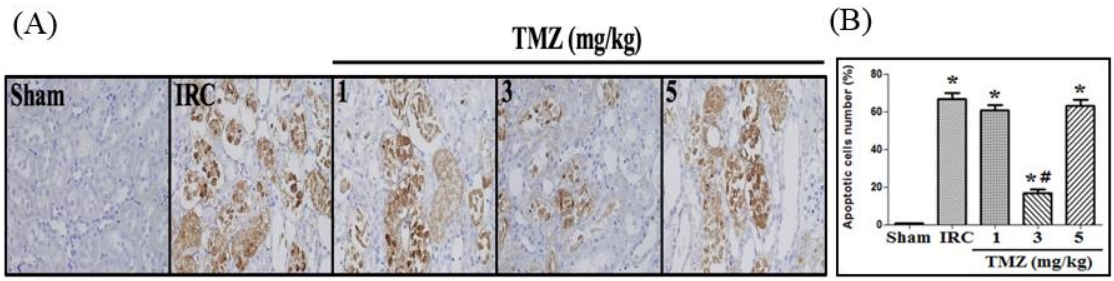

Figure 1. TMZ treatment decreased renal tubular apoptotic cell death 24 hr after renal ischemia-reperfusion (I/R) injury. (A) Representative histology of renal tubules after TUNEL assay in the Sham, IRC and 1, 3, and 5 mg/kg of TMZ pre-treatment groups. (B) The degree of apoptosis was expressed as a percentage of apoptotic cells (apoptotic cells/total cells). TMZ = trimetazidine administered at 1 hr before I/R; TUNEL = terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end labeling. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (24 hr). N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

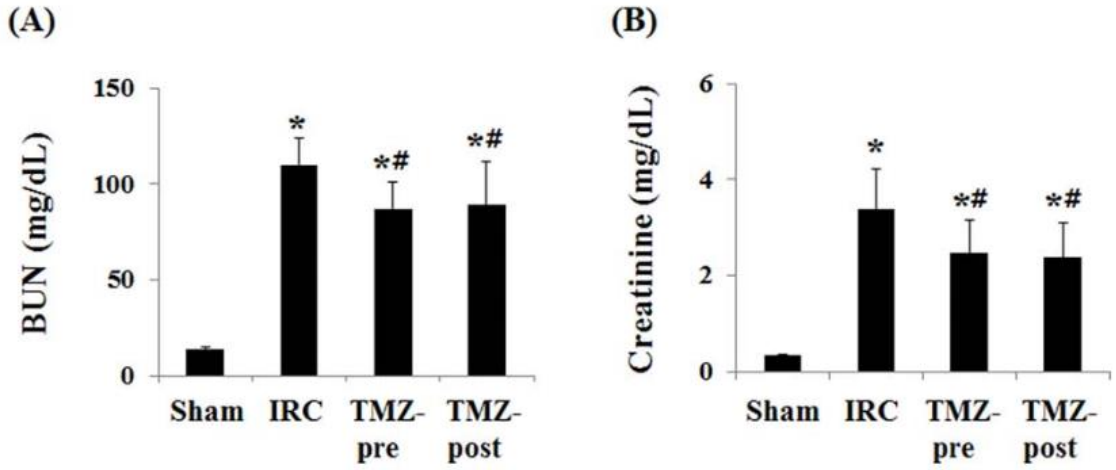

Figure 2. TMZ treatment attenuated renal dysfunction 24 hr after renal ischemia-reperfusion (I/R) injury. (A) Mean serum levels of BUN were decreased in the TMZ-pre and TMZ-post groups compared to the IRC group. (B) Mean serum levels of creatinine were decreased in the TMZ-pre and TMZ-post groups compared to the IRC group. Serum samples were assayed for BUN and creatinine by using the picric acid and diacetyl monoxime methods. Values are mean ± standard deviation from 5 independent experiments. TMZ = trimetazidine; BUN = blood urea nitrogen. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (24 hr); TMZ-pre = rats treated with TMZ (3 mg/kg) 1 h before I/R; TMZ-post = rats treated with TMZ (3 mg/kg) upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

3. TMZ treatment reduced the tubular cell damage

Renal I/R resulted in significant apoptotic cell deaths in the IRC, TMZ-pre, and TMZ-post groups compared to the Sham group 24 hr after I/R injury (Figure 3A). TUNEL-positive cells in the kidney tissue were 63% and 59% less in the TMZ-pre and TMZ-post groups than in the IRC group, respectively without any intergroup difference between the TMZ-pre and TMZ-post groups (Figure 3B).

In PAS staining, the degree of cell degradation and necrosis 24 hr after I/R injury were significantly less in both the TMZ-pre and TMZ-post groups than in the IRC group (Figure 3C, Table 1).

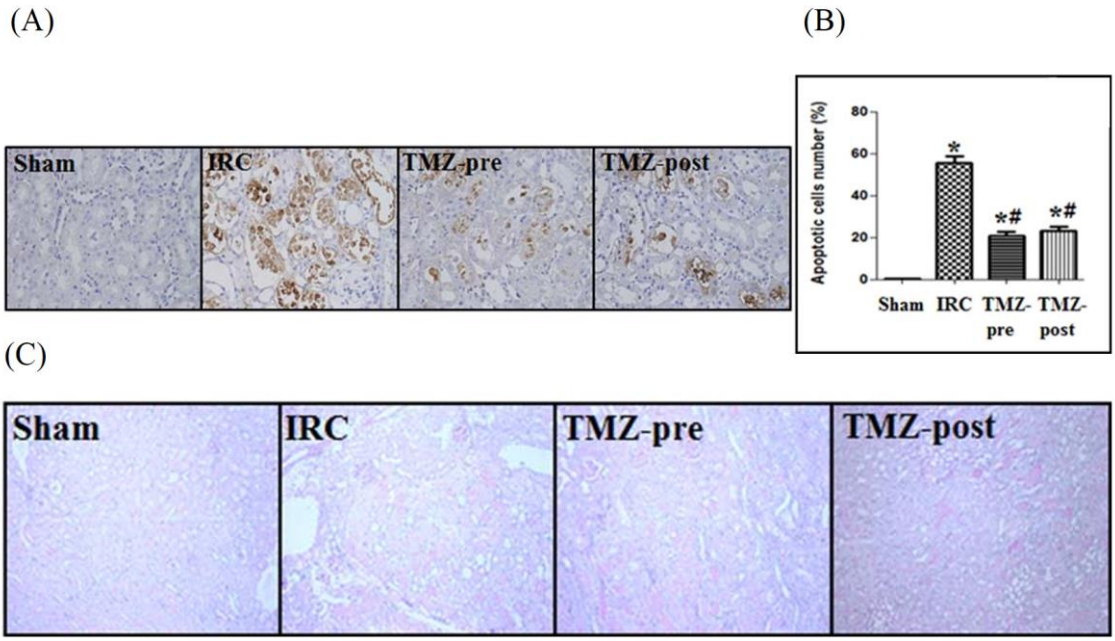

Figure 3. TMZ treatment decreased tubular apoptotic cell death, and the degree of cell degradation and necrosis 24 hr after renal ischemia-reperfusion (I/R) injury. (A) Representative histology of renal tubules after TUNEL assay in the Sham, IRC, TMZ-pre, and TMZ-post groups. (B) The degree of apoptosis was expressed as a percentage of apoptotic cells (apoptotic cells/total cells). (C) Representative histology of renal tubules after PAS staining in the Sham, IRC, TMZ-pre, and TMZ-post groups. TMZ = trimetazidine; PAS = Periodic Acid Schiff; TUNEL = terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end labeling. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (24 hr); TMZ-pre = rats treated with TMZ (3 mg/kg) 1 h before I/R; TMZ-post = rats treated with TMZ (3mg/kg) upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

Table 1. Renal histopathology assessed with PAS staining 24 hr after ischemia-reperfusion injury

Group	Cell degradation	Necrosis	Neutrophil infiltration
Sham	*Grade 1*	*Grade 1*	*Grade 1*
IRC	$2.6 \pm 0.8^{*}$	$3.6 \pm 0.5^{*}$	1.3 ± 0.5
TMZ-pre	$1.6 \pm 0.5^{*,\#}$	$1.7 \pm 0.8^{*,\#}$	1.1 ± 0.4
TMZ-post	$1.7 \pm 0.8^{*,\#}$	$1.9 \pm 0.7^{*,\#}$	1.1 ± 0.4

PAS = Periodic Acid Schiff; TMZ = trimetazidine. Sham = rats not underwent ischemia-reperfusion; IRC = rats underwent ischemia (45 min) – reperfusion (24 hr); TMZ-pre = rats treated with TMZ (3 mg/kg) 1 h before ischemia-reperfusion; TMZ-post = rats treated with TMZ (3 mg/kg) upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

4. TMZ attenuated the degree of I/R-induced decrease in Bcl-2 level and increases in Bax, cytochrome C, and cleaved caspase-3 levels

In conjunction to the anti-apoptotic effect of TMZ, Bcl-2 protein level was significantly decreased and Bax, cytochrome C and cleaved caspase-3 protein levels were significantly increased in the IRC group compared to those of the Sham group. Bcl-2 protein level was significantly higher, and Bax, cytochrome C, and cleaved caspase-3 protein levels were significantly lower in the TMZ-pre and TMZ-post groups than in the IRC group. There were no significant intergroup differences in the Bcl-2, Bax, cytochrome C, and cleaved caspase-3 protein levels between the TMZ-pre and TMZ-post groups (Figure 4).

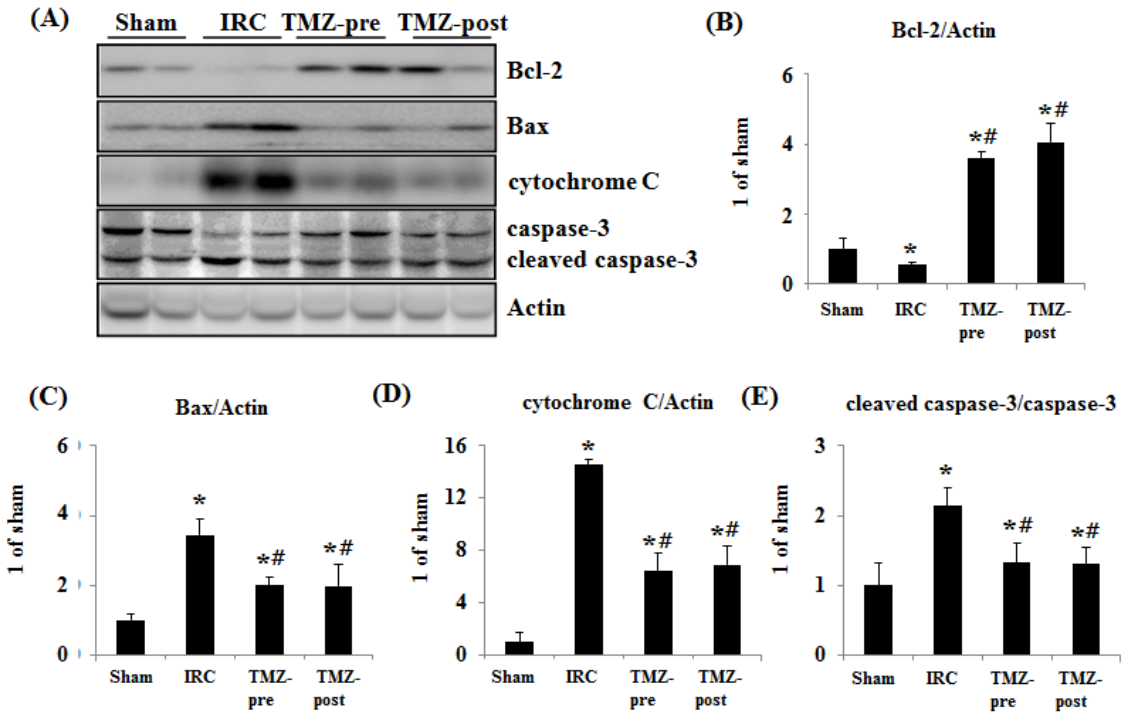

Figure 4. TMZ attenuated the degree of ischemia-reperfusion (I/R) induced decrease in Bcl-2 level and increases in Bax, cytochrome C, and cleaved caspase-3 levels 24 hr after renal I/R injury. (A) Western-blot analysis for Bcl-2, Bax, cytochrome C, and cleaved caspase-3 proteins. (B-E) Densitometric analysis of the western blot for Bcl-2, Bax, cytochrome C, and cleaved caspase-3 proteins, respectively. The kidney tissues were harvested for protein preparation. TMZ = trimetazidine; Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (24 hr); TMZ-pre = rats treated with TMZ (3 mg/kg) 1 h before I/R; TMZ-post = rats treated with TMZ (3 mg/kg) upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

5. TMZ treatment mitigated I/R-induced increases in IL-1β and TNF-α levels

The levels of IL-1β and TNF-α were significantly increased in the IRC group compared to those of the Sham group. The degrees of increases in IL-1β and TNF-α were attenuated by 49% and 56%, respectively, in the TMZ-pre group and by 53% and 40%, respectively, in the TMZ-post group compared to those of the IRC group (Figure 5A, B). The levels of IL-1β and TNF-α were similar between the TMZ-pre and TMZ-post groups.

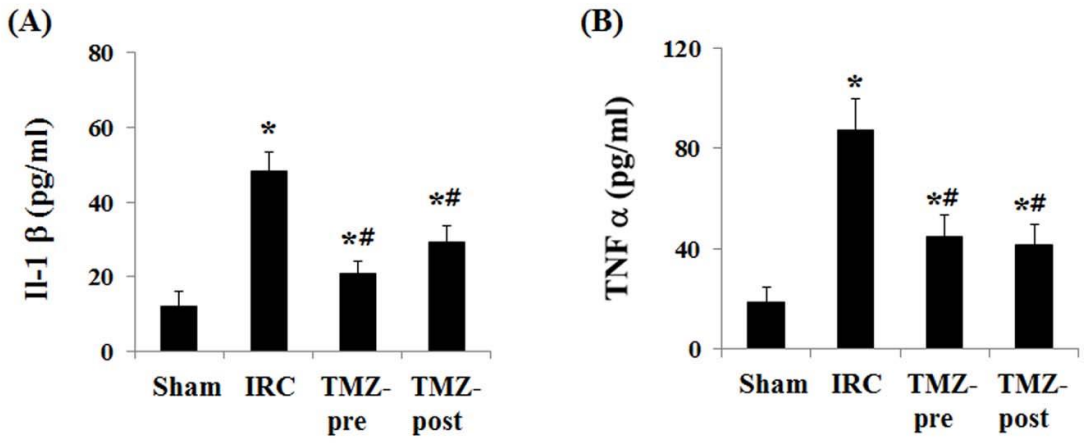

Figure 5. TMZ treatment mitigated ischemia-reperfusion (I/R) induced increases in IL-1β and TNF-α levels. (A) Mean serum level of IL-1β was significantly lower in the TMZ-pre and TMZ-post groups than in the IRC group. (B) Mean serum level of TNF-α was significantly lower in the TMZ-pre and TMZ-post groups than in the IRC group. Serum samples were assayed for IL-1β and TNF-α by using rat IL-1β and TNF-α ELISA kits. Values are mean ± standard deviation from 5 independent experiments. TMZ = trimetazidine; IL-1β = interleukin-1β; TNF-α = tumor necrosis factor-α. Sham = rats not underwent I/R; IRC= rats underwent ischemia (45 min) – reperfusion (24 hr); TMZ-pre = rats treated with TMZ (3 mg/kg) 1 h before I/R; TMZ-post = rats treated with TMZ (3 mg/kg) upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

6. TMZ treatment further enhanced the expression levels of HIF-1α, VEGF, and P-Akt

The expression levels of HIF-1α and VEGF were significantly increased and decreased, respectively, in the IRC group compared to those of the Sham group. The levels of HIF-1α and VEGF were significantly higher in the TMZ-pre and TMZ-post groups than in the IRC group. The P-Akt level was significantly increased in the IRC group compared to that of the Sham group while it was significantly higher in the TMZ treated groups than in the IRC group. The levels of HIF-1α, VEGF, and P- Akt were similar between the TMZ-pre and TMZ-post groups (Figure 6).

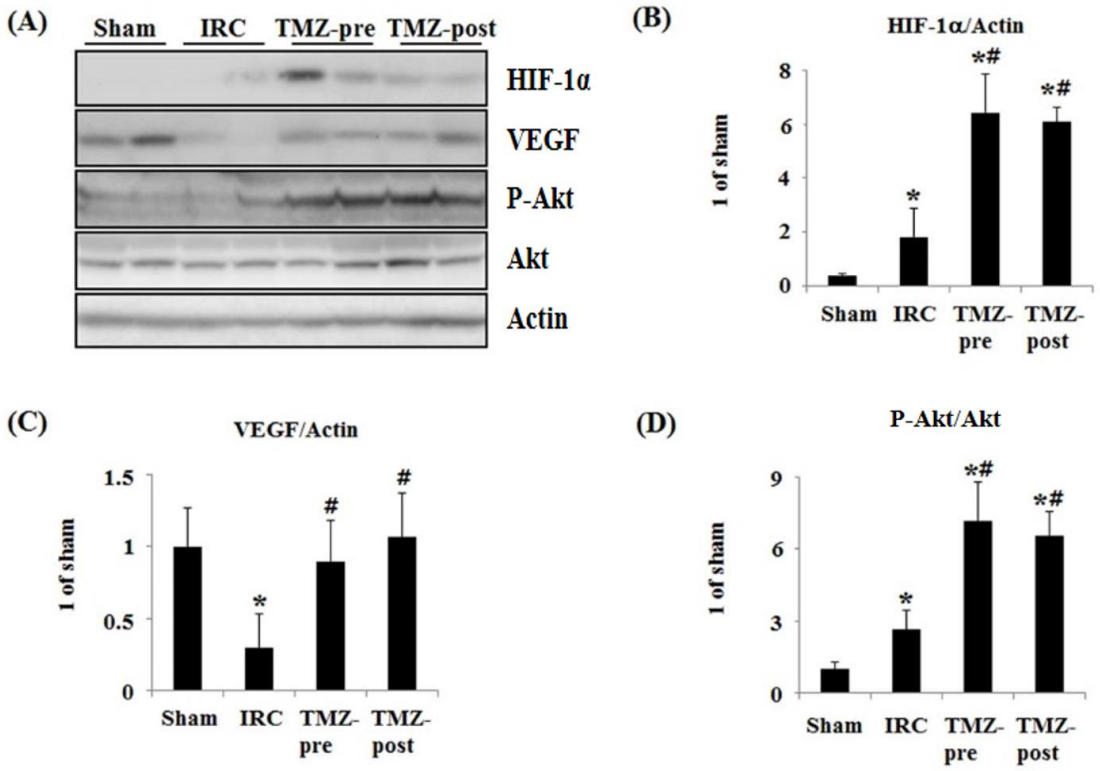

Figure 6. TMZ treatment further enhanced the expression levels of HIF-1α, VEGF, and P-Akt 24hr after renal ischemia-reperfusion (I/R) injury. (A) Western-blot analysis for HIF-1α, VEGF, and P-Akt. (B-D) Densitometric analysis of the western blot for HIF-1α, VEGF, and P-Akt, respectively. The kidney tissues were harvested for protein preparation. The protein levels of HIF-1α, VEGF, and P-Akt were determined by immunoblot analysis with specific antibodies. TMZ = trimetazidine; HIF-1α = hypoxia inducible factor-1α; VEGF = vascular endothelial growth factor; P-Akt = phosphorylated-Akt. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (24 hr); TMZ-pre = rats treated with TMZ (3 mg/kg) 1 h before I/R; TMZ-post = rats treated with TMZ (3 mg/kg) upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

7. TMZ treatment further increased the level of P-eNOS while attenuating the degree of increases in P-iNOS and NOX4 levels after renal I/R injury

The expressions of P-eNOS, P-iNOS, and NOX4 were significantly increased in the IRC group compared to those of the Sham group. The expression of P-eNOS was significantly increased, and the expressions of P-iNOS and NOX4 were significantly decreased in the TMZ-pre and TMZ-post groups than in the IRC group. The expressions of P-eNOS, P-iNOS, and NOX4 were comparable between the TMZ-pre and TMZ-post groups (Figure 7).

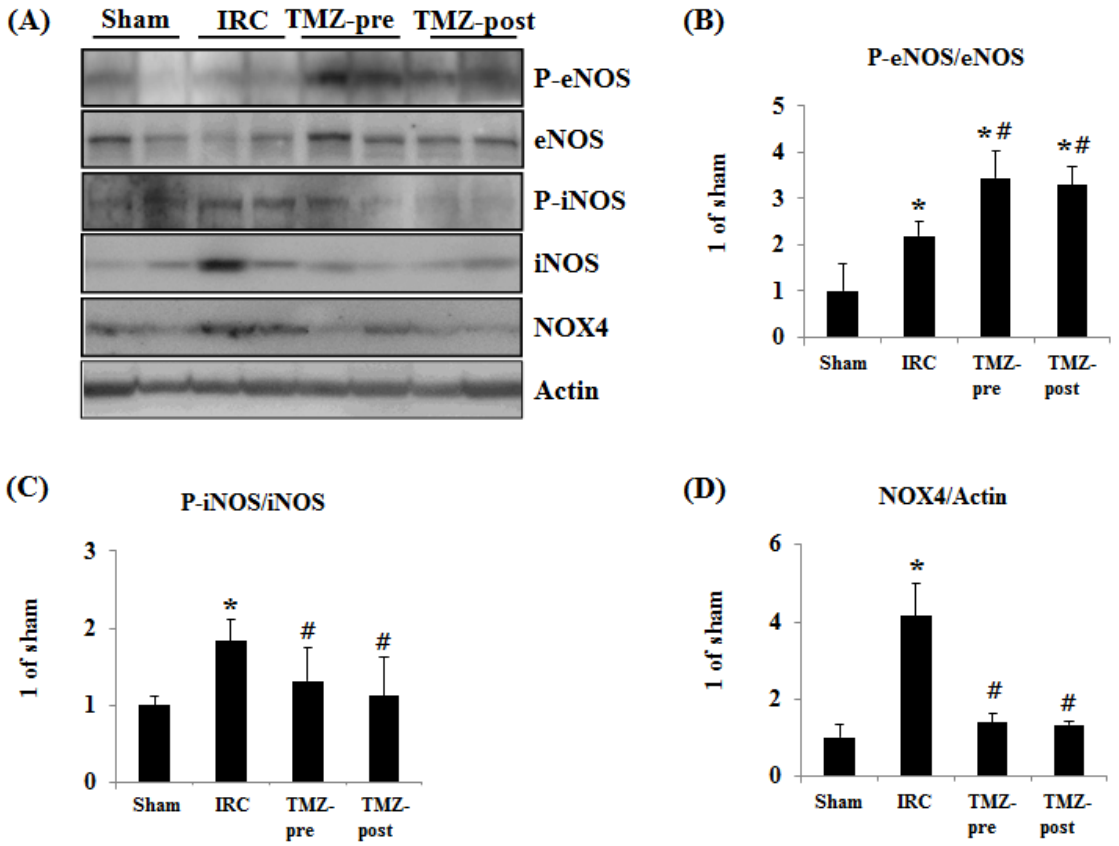

Figure 7. TMZ treatment further increased the level of P-eNOS while attenuating the degree of increases in P-iNOS and NOX4 levels 24 hr after renal ischemia-reperfusion (I/R) injury. (A) Western-blot analysis for P-eNOS, eNOS, P-iNOS, iNOS, and NOX4. (B-D) Densitometric analysis of the western blot for P-eNOS/eNOS, P-iNOS/iNOS, and NOX4. The kidney tissues were harvested for protein preparation. The protein levels of P-eNOS, eNOS, P-iNOS, iNOS, and NOX4 were determined by immunoblot analysis with specific antibodies. TMZ = trimetazidine; P-eNOS = phosphorylated-endothelial nitric-oxide synthase; eNOS = endothelial nitric-oxide synthase; P-iNOS = phosphorylated-inducible nitric oxide synthase; iNOS = inducible nitric oxide synthase. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (24 hr); TMZ-pre = rats treated with TMZ (3 mg/kg) 1 h before I/R; TMZ-post = rats treated with TMZ (3 mg/kg) upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

8. Renal function recovered at 5 days and 8 weeks after renal I/R injury

Further investigations for the intermediate- and long-term effects of post I/R treatment with TMZ on renal recovery were performed.

The elevated serum BUN and creatinine levels returned to baseline values in all groups at 5 days after renal I/R injury and were maintained at similar levels until 8 weeks after renal I/R injury without any intergroup differences (Figure 8A, B). Post I/R treatment with TMZ did not affect the recovery of renal function in both the TMZ-single and TMZ-daily groups.

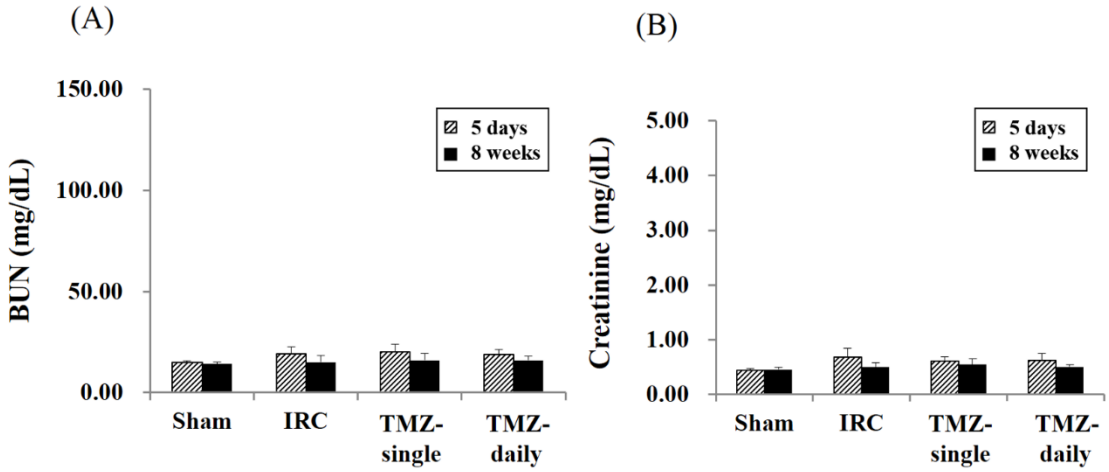

Figure 8. Renal function recovered at 5 days and 8 weeks after renal ischemia-reperfusion (I/R) injury. (A) Serum BUN levels were normalized at 5 days and 8 weeks after renal I/R injury and there were no significant differences among the groups. (B) Serum creatinine levels were normalized at 5 days and 8 weeks after renal I/R injury and there were no significant differences among the groups. Serum samples were assayed for BUN and creatinine by using the picric acid and diacetyl monoxime methods. Values are mean ± standard deviation from 5 independent experiments. TMZ = trimetazidine; BUN = blood urea nitrogen. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (5 days or 8 weeks); TMZ-single = rats treated with TMZ (3 mg/kg) upon reperfusion; TMZ-daily = rats treated with TMZ (3 mg/kg) once daily for 5 days or 8 weeks starting upon reperfusion. N = 5 in each group.

9. Post I/R treatment with TMZ attenuated renal tubular cell necrosis and apoptosis at 5 days after renal I/R injury

The percentage of renal apoptotic cells was significantly greater in the IRC group than in the Sham group (1% *vs* 19%) at 5 days after renal I/R injury. The percentages of renal apoptotic cells were significantly less in the TMZ-single (6%) and TMZ-daily groups (6%) than in the IRC group (19%) at 5 days after renal I/R injury (Figure 9A, B). In PAS staining, the degrees of cell necrosis were significantly less in the TMZ-single and TMZ-daily groups than in the IRC group at 5 days after renal I/R injury (Figure 9C, Table 2). The degrees of tubular apoptotic cell death and cell necrosis were comparable between the TMZ-single and TMZ-daily groups.

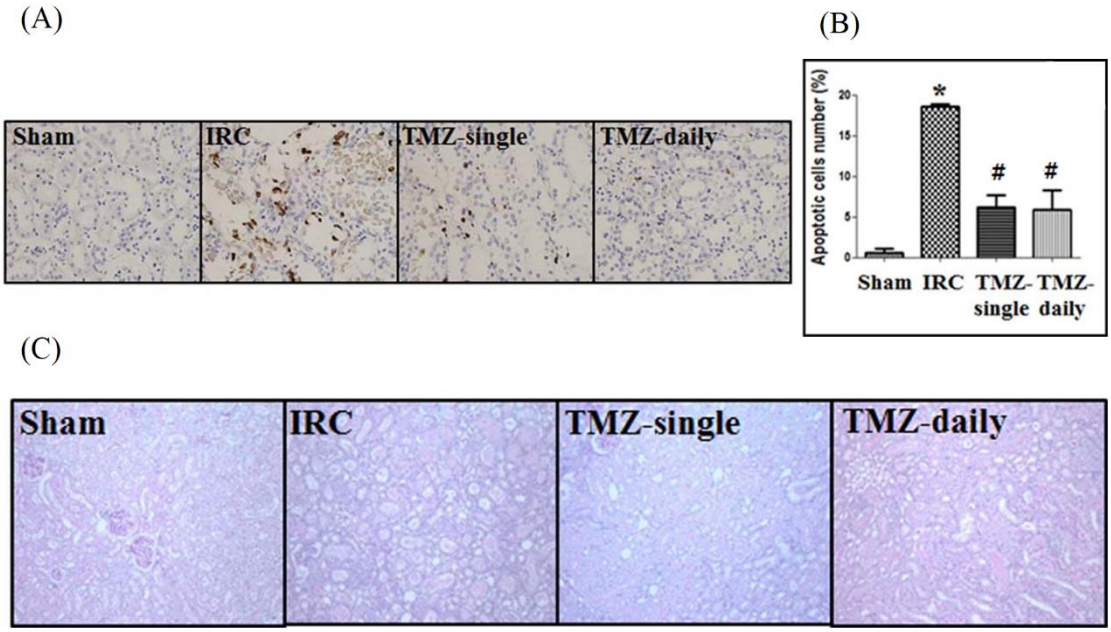

Figure 9. Post ischemia-reperfusion (I/R) treatment with TMZ attenuated renal tubular cell necrosis and apoptosis at 5 days after renal I/R injury. (A) Representative histology of renal tubules after TUNEL assay in the Sham, IRC, TMZ-single and TMZ-daily groups. (B) Degree of apoptosis was expressed as a percentage of apoptotic cells (apoptotic cells/total cells). (C) Representative histology of renal tubules after PAS staining in the Sham, IRC, TMZ-single, and TMZ-daily groups. TMZ = trimetazidine; TUNEL = terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end labeling; PAS = Periodic Acid Schiff. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (5 days); TMZ-single = rats treated with TMZ (3 mg/kg) upon reperfusion; TMZ-daily = rats treated with TMZ (3 mg/kg) once daily for 5 days starting upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

Table 2. Renal histopathology assessed with PAS staining at 5 days after ischemia-reperfusion injury

Group	Cell degradation	Necrosis	Neutrophil infiltration
Sham	*Grade 1*	*Grade 1*	*Grade 1*
IRC	3.1 ± 0.9*	2.9 ± 0.9*	1.9 ± 1.2
TMZ-single	2.1 ± 0.7	1.3 ± 0.5$^{\#}$	1.1 ± 0.4
TMZ-daily	2.1 ± 0.7	1.6 ± 0.8$^{\#}$	*Grade 1*

PAS = Periodic Acid Schiff; TMZ = trimetazidine. Sham = rats not underwent ischemia-reperfusion; IRC = rats underwent ischemia (45 min) – reperfusion (5 days); TMZ-single = rats treated with TMZ (3 mg/kg) upon reperfusion; TMZ-daily = rats treated with TMZ (3 mg/kg) once daily for 5 days starting upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

10. TMZ attenuated the degree of I/R-induced decrease in Bcl-2 level and increases in Bax, cytochrome C, and cleaved caspase-3 levels at 5 days after renal I/R injury

Bcl-2 level was significantly reduced in the IRC group compared to the Sham group, while it was significantly increased in the TMZ-single and TMZ-daily groups compared to the IRC group at 5 days after renal I/R injury. Bax, cytochrome C, and cleaved casepase-3 levels rose in the IRC group compared to those of the Sham group. The increases in Bax, cytochrome C, and cleaved caspase-3 levels were significantly attenuated in the TMZ-single and TMZ-daily groups compared to those of the IRC group at 5 days after renal I/R injury (Figure 10).

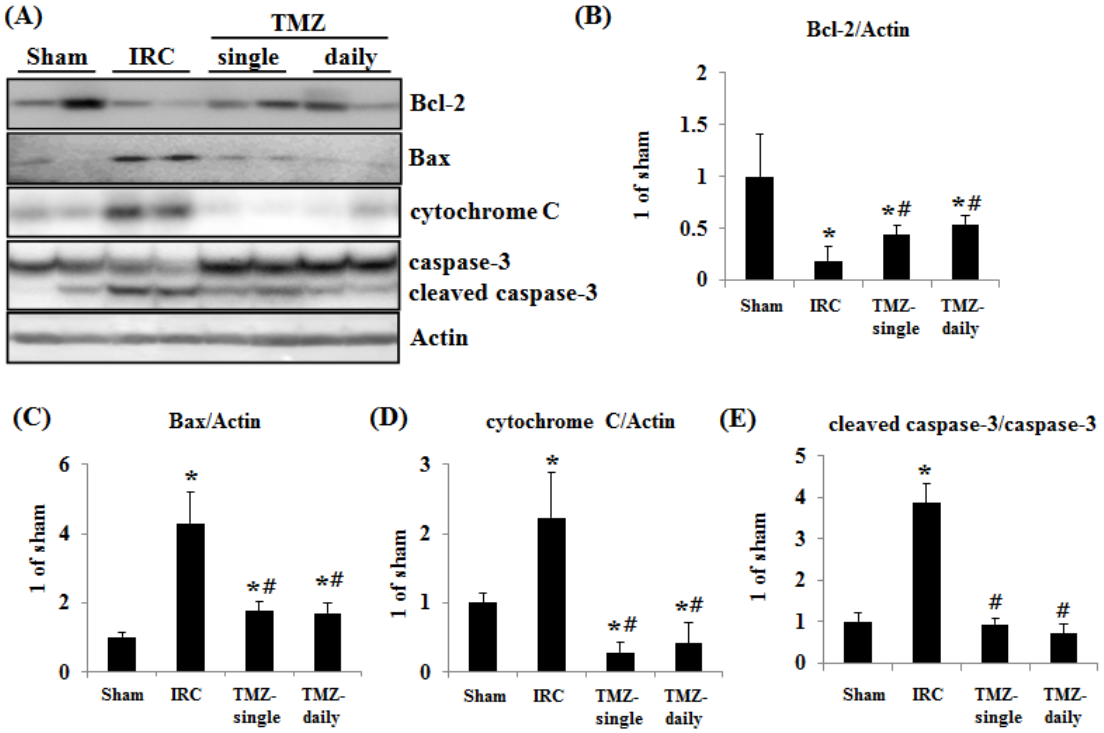

Figure 10. TMZ attenuated the degree of ischemia-reperfusion (I/R) induced decrease in Bcl-2 level and increases in Bax, cytochrome C, and cleaved caspase-3 levels at 5 days after renal I/R injury. (A) Western-blot analysis for Bcl-2, cytochrome C, caspase-3, and cleaved caspase-3. (B-E) Densitometric analysis of the western blot for Bcl-2, Bax, cytochrome C, caspase-3, and cleaved caspase-3. The kidney tissues were harvested for protein preparation. The protein levels of Bcl-2, cytochrome C, caspase-3, and cleaved caspase-3 were determined by immunoblot analysis with specific antibodies. TMZ = trimetazidine. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (5 days); TMZ-single = rats treated with TMZ (3 mg/kg) upon reperfusion; TMZ-daily = rats treated with TMZ (3 mg/kg) once daily for 5 days starting upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

11. Post I/R treatment with TMZ enhanced HIF-1α, VEGF and P-Akt expressions at 5 days after renal I/R injury

Renal I/R induced increases in HIF-1α and P-Akt levels in the IRC group compared to those of the Sham group. The levels of HIF-1α and P-Akt were further increased in the TMZ-single and TMZ-daily groups than in the IRC group at 5 days after renal I/R injury. I/R induced decrease in VEGF level in the IRC group compared to that of the Sham group. VEGF level was significantly increased in the TMZ-single and TMZ-daily groups compared to that of the IRC group at 5 days after renal I/R injury (Figure 11). There were no significant differences in the levels of HIF-1α, VEGF, and P- Akt between the TMZ-single and TMZ-daily groups.

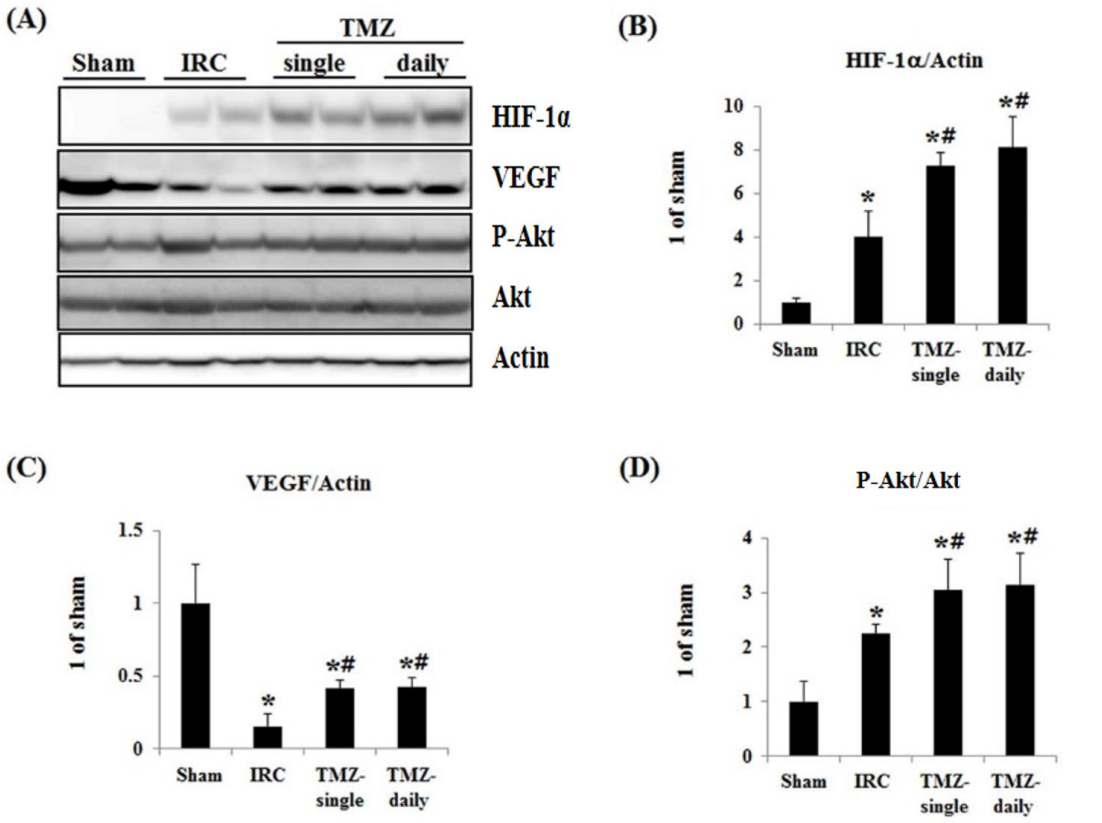

Figure 11. Post ischemia-reperfusion (I/R) treatment with TMZ enhanced HIF-1α, VEGF, and P-Akt expressions at 5 days after renal I/R injury. (A) Western-blot analysis for HIF-1α, VEGF, and P-Akt. (B-D) Densitometric analysis of the western blot for HIF-1α, VEGF, and P-Akt. The kidney tissues were harvested for protein preparation. The protein levels of HIF-1α, VEGF, and P-Akt were determined by immunoblot analysis with specific antibodies. TMZ = trimetazidine; HIF-1α = hypoxia-inducible factor-1α; VEGF = vascular endothelial growth factor; P-Akt = phosphorylated-Akt. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (5 days), TMZ-single = rats treated with TMZ (3 mg/kg) upon reperfusion, TMZ-daily = rats treated with TMZ (3 mg/kg) once daily for 5 days starting upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

12. Post I/R treatment with TMZ enhanced P-eNOS expression, and attenuated P-iNOS and NOX4 expressions at 5 days after renal I/R injury

The expression of P-eNOS, which was weakly detected in the Sham and IRC groups, was significantly enhanced in the TMZ-single and TMZ-daily groups at 5 days after renal I/R injury. I/R-induced increases in P-iNOS and NOX4 levels were significantly attenuated in the TMZ-single and TMZ-daily groups than in the IRC group. The degrees of expressions of P-eNOS, P-iNOS, and NOX4 were comparable between the TMZ-single and TMZ-daily groups (Figure 12).

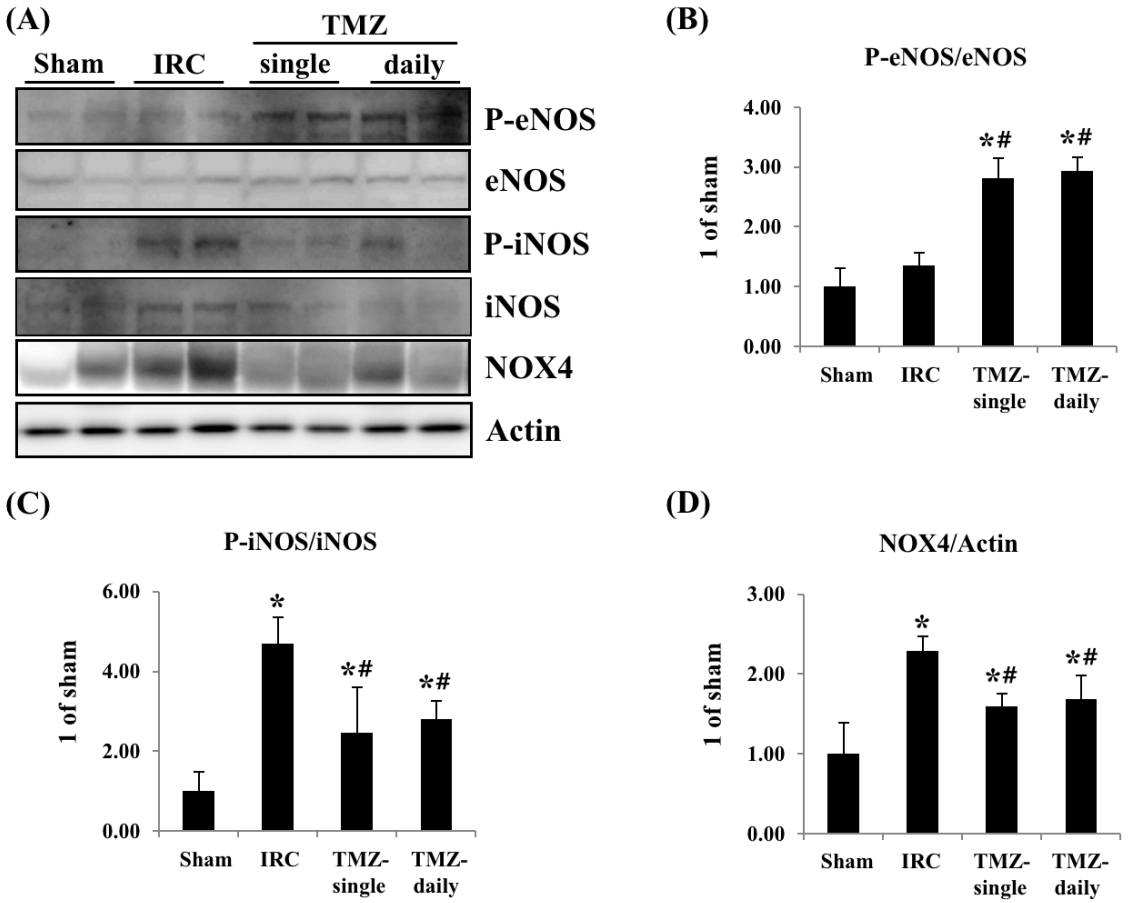

Figure 12. Post ischemia-reperfusion (I/R) treatment with TMZ enhanced P-eNOS expression, and attenuated P-iNOS and NOX4 expressions at 5 days after renal I/R injury. (A) Western-blot analysis for P-eNOS, eNOS, P-iNOS, iNOS, and NOX4. (B-D) Densitometric analysis of the western blot for P-eNOS/eNOS, P-iNOS/iNOS, and NOX4. The kidney tissues were harvested for protein preparation. The protein levels of P-eNOS, eNOS, P-iNOS, iNOS, and NOX4 were determined by immunoblot analysis with specific antibodies. TMZ = trimetazidine; P-eNOS = phosphorylated-endothelial nitric-oxide synthase; eNOS = endothelial nitric-oxide synthase; P-iNOS = phosphorylated-inducible nitric oxide synthase; iNOS = inducible nitric oxide synthase. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (5 days); TMZ-single = rats treated with TMZ (3 mg/kg) upon reperfusion; TMZ-daily = rats treated with TMZ (3 mg/kg) once daily for 5 days starting upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

13. Post I/R treatment with TMZ attenuated the degree of I/R-induced increases in MMPs and further increased the levels of TIMPs at 5 days after renal I/R injury

MMP-2 and MMP-9 levels were significantly increased in the IRC group compared to those of the Sham group. MMP-2 and MMP-9 levels were significantly lower in the TMZ-single and TMZ-daily groups than in the IRC group. TIMP-1 and TIMP-2 levels were significantly increased in the IRC group compared to those of the Sham group, while they were further significantly increased in the TMZ-single and TMZ-daily groups than in the IRC group. There were no significant differences in the levels of those proteins between the TMZ-single and TMZ-daily groups (Figure 13).

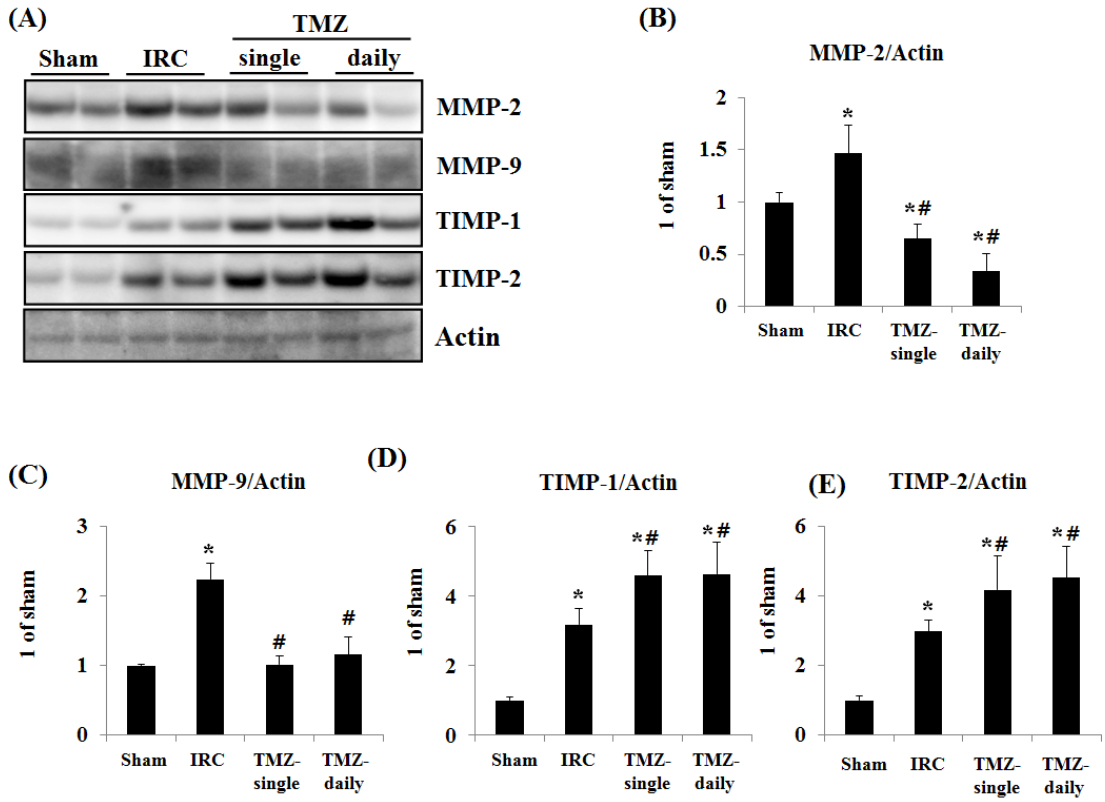

Figure 13. Post ischemia-reperfusion (I/R) treatment with TMZ attenuated the degree of I/R-induced increases in MMPs and further increased the levels of TIMPs at 5 days after renal I/R injury. (A) Western-blot analysis for MMP-2, MMP-9, TIMP-1, and TIMP-2. (B-E) Densitometric analysis of the western blot for MMP-2, MMP-9, TIMP-1, and TIMP-2. The kidney tissues were harvested for protein preparation. The protein levels of MMPs and TIMPs were determined by immunoblot analysis with specific antibodies. TMZ = trimetazidine; MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (5 days); TMZ-single = rats treated with TMZ (3 mg/kg) upon reperfusion; TMZ-daily = rats treated with TMZ (3 mg/kg) once daily for 5 days starting upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham, $^{\#}P < 0.05$ versus IRC.

14. Renal histopathologies were recovered at 8 weeks after renal I/R injury regardless of TMZ treatment

TUNEL assay and PAS staining of renal tissues demonstrated almost full recovery at 8 weeks after renal I/R injury in the IRC group as well as in the TMZ treated groups. No differences in apoptotic change and renal necrosis were found among the groups (Figure 14, Table 3).

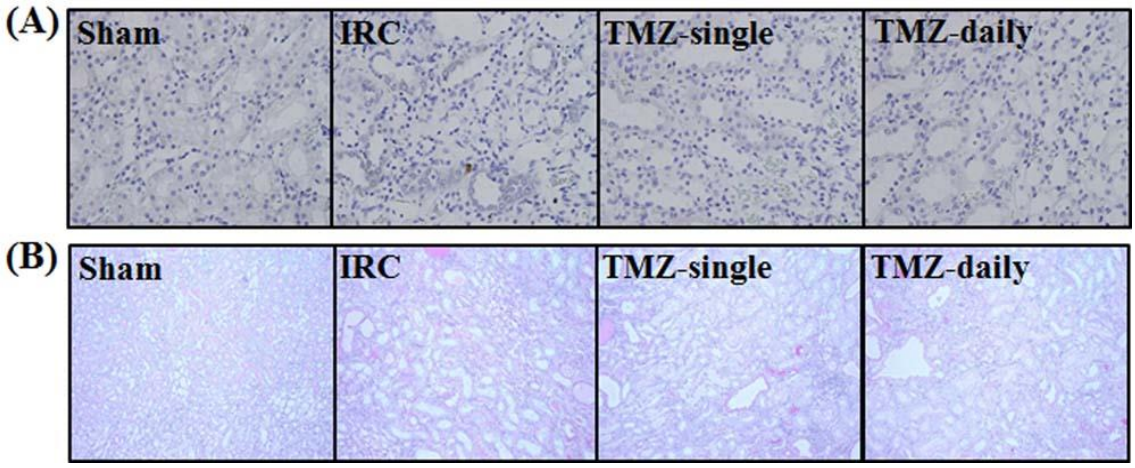

Figure 14. Renal histopathologies were almost fully recovered at 8 weeks after renal ischemia-reperfusion (I/R) injury regardless of TMZ treatment. (A) Representative histology of renal tubules after TUNEL assay in the Sham, IRC, TMZ-single, and TMZ-daily groups. (B) Representative histology of rental tubules after PAS staining in the Sham, IRC, TMZ-single, and TMZ-daily groups. TUNEL = terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end labeling; TMZ = trimetazidine; PAS = Periodic Acid Schiff. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (8 weeks); TMZ-single = rats treated with TMZ (3 mg/kg) upon reperfusion; TMZ-daily = rats treated with TMZ (3 mg/kg) once daily for 8 weeks starting upon reperfusion. N = 5 in each group.

Table 3. Renal histopathology assessed with PAS staining at 8 weeks after ischemia-reperfusion injury

Group	Cell degradation	Necrosis	Neutrophil infiltration
Sham	*Grade 1*	*Grade 1*	*Grade 1*
IRC	1.9 ± 0.4	1.1 ± 0.4	*Grade 1*
TMZ-single	1.4 ± 0.5	*Grade 1*	*Grade 1*
TMZ-daily	1.1 ± 0.4	*Grade 1*	*Grade 1*

PAS = Periodic Acid Schiff; TMZ = trimetazidine. Sham = rats not underwent ischemia-reperfusion; IRC = rats underwent ischemia (45 min) – reperfusion (8 weeks); TMZ-single = rats treated with TMZ (3 mg/kg) upon reperfusion; TMZ-daily = rats treated with TMZ (3 mg/kg) once daily for 8 weeks starting upon reperfusion. N = 5 in each group.

15. Post I/R treatment with TMZ did not change the Bcl-2 and Bax levels at 8 weeks after renal I/R injury

I/R injury significantly increased Bcl-2 and Bax levels in the IRC group compared to those of the Sham group at 8 weeks after renal I/R injury. Bcl-2 and Bax levels were not attenuated by TMZ treatment. They were comparable among the TMZ-single, TMZ-daily, and IRC groups (Figure 15).

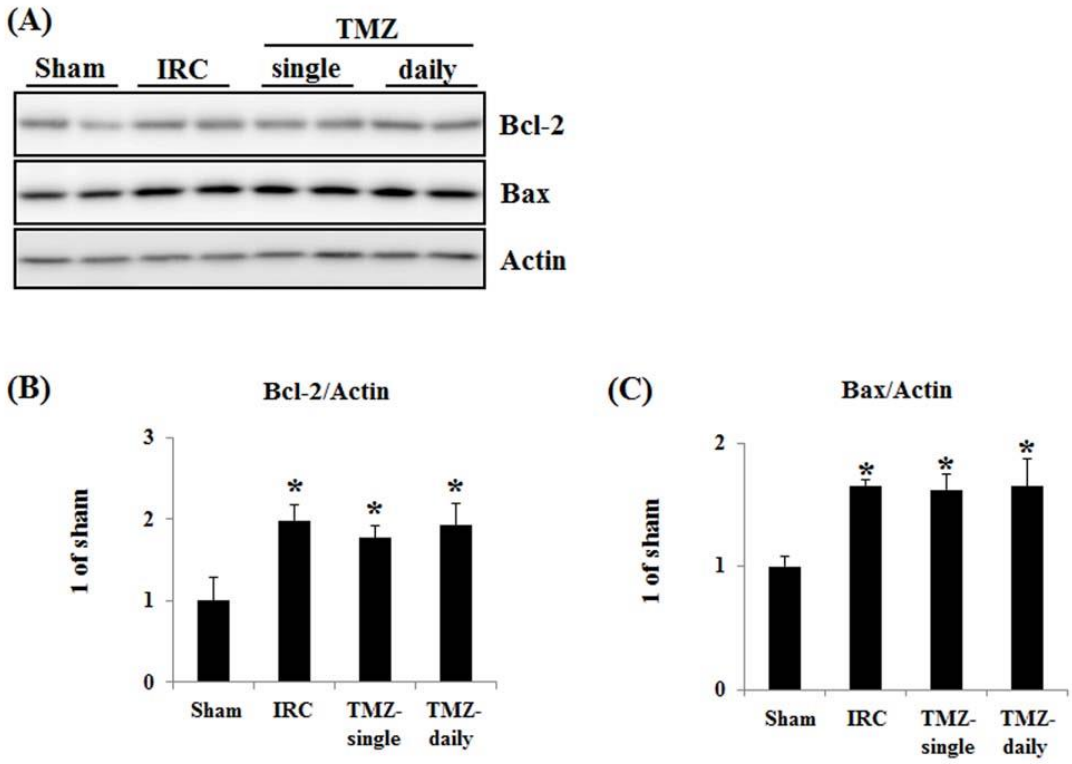

Figure 15. Post ischemia-reperfusion (I/R) treatment with TMZ did not attenuate I/R-induced increase in Bcl-2 and Bax levels at 8 weeks after renal I/R injury. (A) Western-blot analysis for Bcl-2 and Bax. (B, C) Densitometric analysis of the western blot for Bcl-2 and Bax. The kidney tissues were harvested for protein preparation. The protein levels of Bcl-2 and Bax were determined by immunoblot analysis with specific antibodies. TMZ = trimetazidine. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (8 weeks); TMZ-single = rats treated with TMZ (3 mg/kg) upon reperfusion; TMZ-daily = rats treated with TMZ (3 mg/kg) once daily for 8 weeks starting upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham.

16. Post I/R treatment with TMZ did not inhibit renal fibrotic change at 8 weeks after renal I/R injury

In Masson's trichrome staining, renal fibrosis was significantly more prominent in the IRC, TMZ-single, and TMZ-daily groups than in the sham group at 8 weeks after renal I/R injury (Figure 16). TMZ-daily group exhibited less renal fibrosis compared to the IRC and TMZ-single groups without any statistical significance.

HIF-1α levels were not detected in all groups (data not shown). Compared to the Sham group, VEGF, MMPs, and TIMPs levels were significantly higher in the IRC, TMZ-single, and TMZ-daily groups. There were no significant differences in those proteins levels among the IRC, TMZ-single, and TMZ-daily groups (Figure 17).

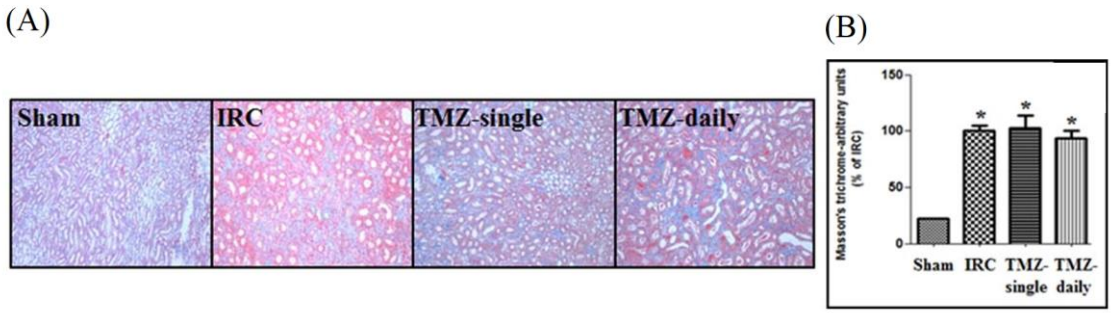

Figure 16. Post ischemia-reperfusion (I/R) treatment with TMZ did not inhibit renal fibrotic change at 8 weeks after renal I/R injury. (A) Tissues stained with Masson's trichrome showed similar degree of renal fibrosis in the IRC, TMZ-single, and TMZ-daily groups, which were all significantly more prominent in those groups compared to the Sham group. Blue staining indicates the presence of collagen. (B) Quantitative analysis of tubulointerstitial fibrosis. TMZ = trimetazidine. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (8 weeks); TMZ-single = rats treated with TMZ (3 mg/kg) upon reperfusion; TMZ-daily = rats treated with TMZ (3 mg/kg) once daily for 8 weeks starting upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham.

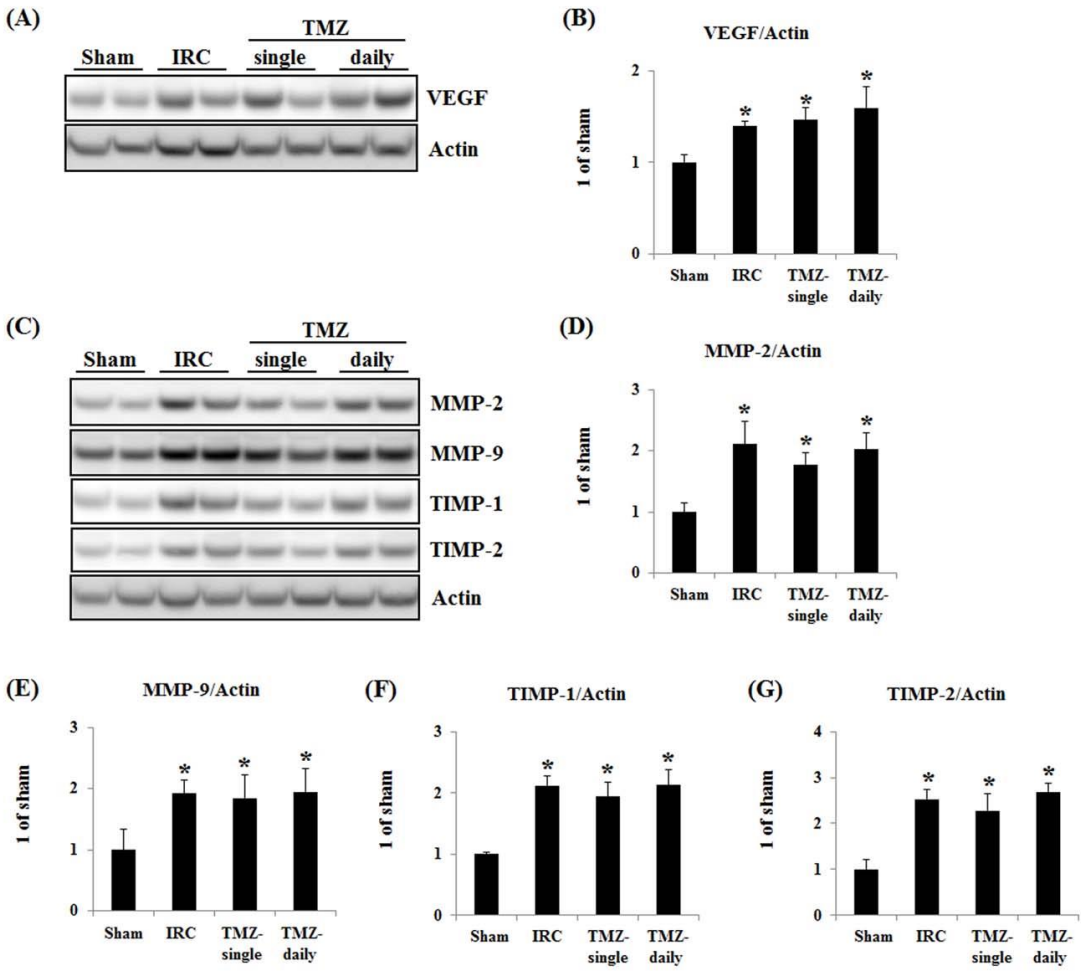

Figure 17. Post ischemia-reperfusion (I/R) treatment with TMZ did not modulate the I/R-induced changes in VEGF, MMPs and TIMPs expressions at 8 weeks after renal I/R injury. (A) Western-blot analysis for VEGF. (B) Densitometric analysis of the western blot for VEGF. (C) Western-blot analysis for MMP-2, MMP-9, TIMP-1, and TIMP-2. (D) Densitometric analysis of the western blot for MMP-2, MMP-9, TIMP-1, and TIMP-2. The kidney tissues were harvested for protein preparation. The protein levels of VEGF, MMPs, and TIMPs were determined by immunoblot analysis with specific antibodies. TMZ = trimetazidine; VEGF = vascular endothelial growth factor; MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase. Sham = rats not underwent I/R; IRC = rats underwent ischemia (45 min) – reperfusion (8 weeks); TMZ-single = rats treated with TMZ (3 mg/kg) upon reperfusion; TMZ-

daily = rats treated with TMZ (3 mg/kg) once daily for 8 weeks starting upon reperfusion. N = 5 in each group. $^{*}P < 0.05$ versus Sham.

IV. DISCUSSION

In the current study investigating the impact of post I/R TMZ treatment on a short (24hr)-, intermediate (5 days)-, and long (8 weeks)-term renal recovery against renal I/R injury in a rat model, we could observe significant attenuation of renal injury by TMZ at 24 hr as well as 5 days after renal I/R injury. Post I/R treatment with TMZ inhibited pro-inflammatory response, apoptotic cell death and oxidative stress accompanied by up-regulation of HIF-1α and VEGF, activation of P-Akt, alteration of the ratio of Bax to Bcl-2, down regulation of IL-1β and TNF-α, and MMPs, and up-regulation of TIMPs. Contrary to its short- and intermediate-term renoprotective effects, however, post I/R treatment with TMZ, either given once or daily for 8 weeks, could not further suppress the progression of long-term renal fibrosis and expressions of the related signaling pathways when compared to the IRC group.

AKI is a major risk factor associated with increased morbidity and mortality in hospitalized patients. AKI is largely attributable to I/R injury in association with kidney transplantation, cardiac surgery, hypovolemic shock and/or sepsis. Despite of several favorable experimental results to attenuate renal I/R injury,[8,32,33] a definite preventive or treatment strategy has not yet been elucidated. A major drawback limiting the clinical application of these favorable experimental results is the inability to provide treatment measures before the renal I/R injury, which is not feasible in most clinical situations. Still, just two experimental studies had elucidated the effects of post-treatment with drugs other than immunosuppressants on renal I/R injury; one study reported a moderate effect of post I/R treatment with hydrogen sulfide to reduce renal damage,[34] and the other study reported no renoprotective effect of cerivastatin administrated after the renal I/R injury.[35] In addition, researches addressing the long-term impact of a treatment on renal function after renal I/R injury are scanty as well, while renal deterioration would be gradually aggravated even after complete clinical recovery from AKI.[5,36]

TMZ is a commonly used anti-anginal drug. Due to its well-known cardioprotective effect and its hemodynamic stability, treatment indications of TMZ have extended in clinical fields.[37,38] Renoprotective effect of TMZ against renal I/R injury mediated through its anti-oxidative, anti-inflammatory, and anti-apoptotic effects have also been reported.[21,23] Reduced apoptosis accompanied with down-regulated caspase-3 activity,[27,39] and attenuated cellular inflammation were observed in an experimental model of cold renal I/R injury treated with TMZ.[40] Of particular interest, TMZ inhibited renal fibrosis through up-regulation of HIF-1α in a study evaluating its long-term effects in pigs.[11] In most of the studies, however, TMZ was administered as a single dose before the I/R insult, while TMZ is used as a chronic medication in clinical practice. Thus, based on its theoretical advantages and a high possibility for clinical application, the short-, intermediate-, and long-term effects of a single and repeated administration of TMZ after renal I/R injury were examined in the present experiment.

In the current study, we found that post I/R treatment with TMZ exhibited similar efficacy in terms of renoprotection at 24 hr after the renal I/R injury compared to pre-treatment with TMZ before the renal I/R injury (Figure 2-3). These results were consistent with those of the previous studies that showed organ protective effects of TMZ administered after ischemic insult on the heart, retina and testis.[41-43] In those studies, anti-oxidative property of post I/R treatment with TMZ were depicted as an underlying mechanism in the heart and testis.[42,43] Of note, although renal function was spontaneously recovered at 5 days after renal I/R injury (Figure 8), beneficial effects of post I/R treatment with TMZ was still evident even at 5 days after renal I/R injury (Figure 9). Post I/R treatment with TMZ significantly mitigated apoptotic cell death and cell necrosis through inhibition of apoptotic pathway, enhancement of the expressions of HIF-1α, and attenuation of decrease in VEGF level followed by the activation of Akt-eNOS signaling as well as up-regulation of TIMPs, and down-regulation of MMPs (Figure 10-13).

A complex interplay of multiple mechanisms contributes to I/R-induced organ damage. In the hypoxic kidney, HIF-1α is expressed predominantly in tubular epithelial cells and works as

a master regulator of hypoxic stress.[14,44] In that context, renal I/R injury elicited significant increase in HIF-1α level, which was increased further by post I/R treatment with TMZ both at 24hr and 5 days after renal I/R injury. Increase in HIF-1α level was accompanied by up-regulation of VEGF, which was significantly mitigated in the IRC group compared to the Sham group (Figure 6, 11). In a previous study, VEGF expression in the proximal tubules was also lost early after AKI.[45] VEGF, regulated by HIF-1α, is known to be involved in the regulation of expression of many genes that help to reduce renal injury after I/R.[36] Thus, increased expression of HIF-1α accompanied by enhanced expression of VEGF with TMZ might have contributed to the reduction of early renal injury after I/R in the present study. In addition, attenuated early renal injury with TMZ could be partly attributed to augmentation of Akt phosphorylation and P-eNOS level in this study (Figure 6, 7, 11, 12). eNOS system plays a pivotal role in various organ protection,[15,46,47] and Akt/eNOS signaling pathway has been reported to lessen I/R-induced oxidative stress in the heart and endothelial progenitor cells.[43,48]

Of note, TMZ diminished expressions of MMPs and enhanced expressions of TIMPs (Figure 13), which seemed to be associated with mitigated early renal injury in the treated groups. Early after renal I/R injury, the influx of inflammatory mediators together with degradation of necrotic cells induce up-regulation of degradative enzymes in the renal interstitium. Among the degradative enzymes, MMP-2 is particularly important. It has a high activity against collagen IV or basement membrane[49] and plays an important role in the process of vascular endothelial damage,[50] as shown that inhibition of MMPs reduced I/R-induced AKI.[51] In that context, the attenuated activation of MMPs with TMZ might have conveyed beneficial influence in mitigating the renal damage at 5 days after renal I/R injury in this study. After renal I/R injury, TIMPs, an important inhibitor of MMPs, are activated to counteract the extracellular matrix degradation by MMPs. Therefore, enhanced activation of TIMPs at 5 days after renal I/R injury in the current study could have exerted additional favorable effects by suppressing the activities of MMPs in the TMZ-treated groups.

Contrary to the protective effects observed in the short- and intermediate-term, TMZ could not prevent renal fibrosis at 8 weeks after renal I/R injury in the present study (Figure 16). I/R-induced changes in protein expressions like VEGF, MMPs, and TIMPs as well as Bcl-2 and Bax were consistent at 8 weeks after renal I/R injury, and TMZ treatment as a single or once daily for 8 weeks could not modulate the I/R-induced changes of these signal proteins (Figure 15, 17). These results are discordant with the results of previous reports showing that TMZ could reduce the fibrosis development after I/R injury in pigs,[11] and attenuate the interstitial fibrosis in cyclosporine A induced nephrotoxicity in rats.[52] In that regard, the timing of HIF-1α activation can be considered as a serious factor influencing on the lack of long-term effect of TMZ in this study. In the study reporting the beneficial effect of HIF-1α prolyl hydroxylation (PHD) inhibition in the suppression of AKI to chronic kidney disease transition, pre-ischemic but not post-ischemic PHD inhibition ameliorated fibrosis on day 12.[53] The importance of timing of HIF activation was also seen in a rat remnant kidney model in which renal fibrosis worsened after PHD inhibitor treatment at weeks 2–12 or was improved after PHD inhibitor treatment at weeks 4-12 after renal injury.[54] Additionally, in view of the fibrosis progression, the finding that VEGF expression was significantly greater in the I/R group than in the Sham group at 8 weeks after I/R injury despite the disappearance of HIF-1α expression (undetectable in all groups) in the current study seems to be meaningful. Activating signals including intercellular proteolysis were reported to activate dysangiogenic VEGF signaling independent of HIF-1α, which caused the loss of endothelial integrity,[55] and attributed to the loss of nursing function of pericytes that stabilize capillaries.[56] The offset of the balance between MMPs and TIMPs might also have played a role in the process of interstitial fibrosis in the current study. In a study observing MMPs and TIMPs levels for 24 weeks after I/R injury, after an initial phase of increased extracellular matrix turnover following I/R injury, the balance subsequently turned towards developing fibrosis.[57] Continuous activation of TIMPs rendered renal fibrosis to develop since decreased extracellular matrix degradation is the key element in the development

of renal fibrosis.[57] In conjunction, the lack of effect of TMZ on the consistently activated TIMPs activity at 8 weeks after I/R injury could have contributed to the progression of renal fibrosis in this study.

Contrary to our expectation, daily administered TMZ did not exert additional effect in the current study. It has been well-perceived that a single episode of I/R injury is associated with transition to chronic kidney disease. Therefore, further studies aimed at identifying the right timing and duration of TMZ treatment against renal I/R may provide further insights regarding the prevention of fibrosis progression. Yet, there are also reports that protective signals in the early stages promoted injury or impeded repair in the later stage in the course of AKI,[58,59] while attenuating the early injury is undoubtedly of great clinical importance as well.

Taken together, post I/R treatment with TMZ might allow ischemic kidneys to regain renal function and structure more rapidly compared to non-treated kidneys, but not enough to resolute renal fibrosis in long-term aspect. Despite that, the early and intermediate renoprotective effect of TMZ treatment after I/R injury deserves for a meaningful clinical implication since the degree of recovery after AKI would significantly be associated with chronic renal function.[6]

V. CONCLUSIONS

In conclusion, post I/R treatment with TMZ significantly attenuated the degree of short- and intermediate-term renal I/R injury, as affirmed by up-regulation of HIF-1α, VEGF, and P-Akt, and inhibition of oxidative and apoptotic pathways including eNOS system and Bax. However, post I/R treatment of TMZ failed to inhibit the long-term renal fibrotic change. Considering the clinical importance of early renal function recovery after I/R injury and TMZ's convenient clinical applicability, the result of this study could possibly be translated to patients insulted with renal I/R injury to accelerate the recovery from renal damage.

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ABSTRACT (IN KOREAN)

다양한 투여 기간의 trimetazidine이 허혈-재관류 손상에 미치는 효과 : 쥐 신장 모델에서 단기 및 장기 효과

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박진하

급성 신손상은 입원 환자들의 예후를 악화시키는 위험 요소이며 주로 허혈-재관류 손상의 결과로 발생하는 것으로 알려져 있다. Trimetazidine (TMZ, 1 - [2, 3, 4 - trimethoxybenzyl] piperazine, dihydrochloride)은 수십 년 동안 사용된 항허혈제로서, 다양한 세포 생존 경로의 조절을 통하여 허혈-재관류 손상을 감소시킬 수 있는 이론적 가능성이 있다. 그러나 임상 연구를 포함한 대부분의 연구는 허혈-재관류 손상 전에 투여한 TMZ의 단기 효과에 대한 것에 집중되어 임상적 상황과의 관련성이 낮으며, 적용에 어려움이 있다. 본 연구에서는 신장의 허혈-재관류 손상 직후 투여한 TMZ가 단기뿐만 아니라 장기간의 신장 기능 및 조직학적 손상 회복에 미치는 영향을 쥐 모델에서 관련 신호 전달 경로의 평가와 함께 알아보고자 하였다.

단기 연구에서는 쥐들을 Sham군, 허혈-재관류군, TMZ 전처치군 및 TMZ 후처치군의 네 군으로 나누어 실험을 진행하였다. TMZ (3 mg/kg)를 TMZ 전처치군에서는 허혈 1시간 전에, TMZ 후처치군에서는 재관류 직후 복강내 투여하였다. 재관류 24시간 후 쥐들을 희생시켰다. 중기 및 장기 연구에서는

쥐들을 Sham군, 허혈-재관류군, TMZ 1회 투여군, TMZ 매일 투여군의 네 군으로 나누어 실험을 진행하였다. TMZ (3 mg/kg)를 TMZ 1회 투여군에서는 재관류 직후에, TMZ 매일 투여군에서는 재관류 직후부터 매일 복강내 투여하였다. 재관류 5일 및 8주 후 쥐들을 희생시켰다. Sham을 제외한 모든 쥐에서 왼쪽 신장을 제거하고 오른쪽 신장 동맥과 정맥을 비외상성 미세 혈관 클램프로 45 분간 허혈시킨 후 재관류시켰다. TMZ 후처치에 대한 효과는 신장 기능 (serum blood urea nitrogen [BUN] 과 creatinine), 세포 사멸 및 섬유화와 같은 신장의 조직학적 변화, hypoxia inducible factor (HIF)-1α, vascular endothelial growth factor (VEGF), phosphorylated-Akt (P-Akt), endothelial nitric oxide synthase (eNOS) 및 Bcl-2, matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinase (TIMPs)와 같은 관련 신호 단백질을 측정함으로써 시행하였다.

신장의 허혈-재관류 손상 24 시간 후의 관찰 결과 TMZ 후처치는 혈청 BUN 및 creatinine 수치, 세포 사멸 정도를 감소시켰으며, 이는 HIF-1α-VEGF와 P-Akt/eNOS 및 Bcl-2 관련 세포 사멸 신호 전달 경로의 상향 조절을 통하여 이루어졌다. 혈청 BUN 및 creatinine의 농도가 신장의 허혈-재관류 손상 5 일 후에 기준치로 회복되기는 하였지만, TMZ 후처치는 치료 프로토콜과 상관 없이 HIF-1α-VEGF와 P-Akt/eNOS, TIMPs의 상향 조절, MMPs의 활성도 감소와 Bcl-2에 대한 Bax의 비율 조절을 통해 허혈-재관류 손상 5 일째의 신장 세포 괴사 및 세포 사멸을 감소시켰다. 그러나, TMZ 후처치는 신장 허혈-재관류 손상 8주 후에 관찰되는 신장 섬유화의 진행 및 관련 신호 경로의 발현을 억제하지 못하였다.

이와 같은 결과는 TMZ의 후처치가 장기적으로 신장 섬유화를 억제하지 못하지만, 단기 및 중기 신장 보호에 유익한 효과가 있음을 나타낸다.

핵심되는 말: 단기 및 장기, 세포 신호전달 체계, 신장, trimetazidine, 허혈-재관류 손상