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Effectiveness of a single versus repeated administration of trimetazidine in the protection against warm ischemia/reperfusion injury of rat liver

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Background/aim: The aim of this study was to compare the effects of single and repeated trimetazidine (TMZ) administration against warm hepatic ischemia/reperfusion (I/R) injury and to explore the possible mechanisms affected by TMZ.

Materials and methods: Wistar rats were divided into 4 groups (n = 6). Sham: rats were subjected to dissection. I/R: rats were subjected to 60 min of partial hepatic ischemia followed by 24 h of reperfusion. TMZ1: Same as I/R group but rats were pretreated with a single dose of TMZ (10 mg/kg, intraperitoneal injection) 30 min before warm ischemia. TMZ3: Same as I/R but rats were treated with 10 mg/kg TMZ for 3 successive days.

Results: TMZ treatment decreased liver injury, lipid peroxidation, and apoptosis. The repeated administration of TMZ conferred more protection than the single dose treatment concerning all studied parameters. In parallel, we noted a significant increase in phosphorylated adenosine monophosphate activated protein kinase (p-AMPK) and endothelial nitric oxide synthase (eNOS) levels in TMZ3 as compared to TMZ1.

Conclusion: Repeated administration of TMZ for 3 days was more efficient than a single dose of TMZ in protecting the liver against I/R induced apoptosis and lipid peroxidation. These effects implicate AMPK and eNOS activation.

Key words: Ischemia/reperfusion injury, liver, trimetazidine, adenosine monophosphate activated protein kinase, endothelial nitric oxide synthase, apoptosis

1. Introduction

Hepatic ischemia/reperfusion (I/R) injury is a pivotal clinical problem occurring in many conditions such as liver transplantation, major hepatectomy, trauma, and hemorrhagic shock (1). The prevention of liver vulnerability against I/R damage is a determinant in preserving hepatic function and accelerating the organ's recovery (2). Pharmacological preconditioning has been widely explored as an effective strategy to overcome the pathophysiological changes caused by hepatic warm I/R injury (3). Trimetazidine (1-2(2,3,4- trimethoxibenzyl)-piperazine) (TMZ), an antiischemic drug, decreases fatty acid oxidation and stimulates glucose utilization leading to the production of adenosine triphosphate (ATP) with less oxygen consumption (1,3). In addition, experimental and clinical results have shown that TMZ has a number of potentially useful cytoprotective impacts. It has been reported that it limits intracellular acidosis, reduces sodium and calcium accumulation into cells (4), and decreases cytolysis and membrane injury caused by oxygen free radicals (5,6). In addition, TMZ conserves mitochondrial function and energy metabolism (7).

Several routes, times, and doses of administration of this antioxidant agent have been proposed in the literature (5,8,9). Tsimoyiannis et al. (10) demonstrated that a single dose of TMZ (2.5 mg/kg) given before or after the ischemic episode gave significant protection to hepatocytes, but pretreatment twice for 5 days was more effective. Settaf et al. (11) demonstrated that TMZ, administered at 80 mg per day during 5 days before the procedure until the day of surgery, reduced postoperative transaminase levels when compared to the placebo group. Furthermore, the

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hospital stay was significantly shorter in the TMZ treated group. Regarding the encouraging results found, further trials evaluating the optimal dose response relationship are required. The objective of our study was to compare the effects of single dose and repeated TMZ administration against warm hepatic I/R injury and to explore the possible mechanisms implicated in these effects.

2. Materials and methods

2.1. Experimental design

Male Wistar rats weighing 200–250 g were used in this study. All procedures were carried out in accordance with the European Union Regulations (Directive 86/609/CEE) for animal experiments. All animals were housed under standard conditions with a 12 h light/dark routine and free access to water and food. They were anesthetized with an intraperitoneal (ip) injection of pentobarbital (5%).

A model of segmental (70%) warm hepatic I/R was used (1). Briefly, after a midline laparotomy, a nontraumatic vascular clamp was used to interrupt the arterial and portal venous blood supply to the left and median liver lobes for 60 min. Reperfusion was initiated by removal of the clamp. During the surgery, animals were placed onto a thermostatically controlled warm board to maintain body temperature at 37 °C. The rats were sacrificed after 24 h of reperfusion, and liver and serum samples were collected for analyses.

2.2. Experimental groups

Animals were randomly assigned into the following experimental groups, each containing 6 rats. In the sham group, the rats were subjected to laparotomy and hepatic triad vessels were only dissected. In the I/R group, rats were subjected to 60 min of partial (70%) ischemia followed by 24 h of reperfusion (12). The rats of the TMZ1 group were treated with 10 mg/kg (ip injection) of TMZ (Sigma Chemical, St. Louis, MO, USA) 30 min before warm ischemia (13) with the TMZ dissolved in NaCl 9‰. Finally, the rats of the TMZ3 group were treated with 10 mg/kg (ip injection) of TMZ for 3 successive days. On the third day, TMZ was injected 30 min before warm ischemia.

2.3. Biochemical assays

2.3.1 Liver injury

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma glutamyltransferase (GGT) activities in serum were measured by commercial DiaSys kits (Diagnostic System, Germany) following supplier instructions.

2.3.2. Lipid peroxidation assessment

Malondialdehyde (MDA) is an end product of peroxidation of cell membrane lipids caused by oxygen derived free radicals. It was measured in liver tissue using the thiobarbiturate reaction at 530 nm (14).

2.3.3 Western blots analysis

Liver tissue was homogenized in Hepes buffer (40 mM NaCl, 1 mM EDTA, Triton-X 0.1%, glycerol 5%, NaP_2O_7 10 mM, β-glycerophosphate 10 mM, Na3VO4 1.5 mM, NaF 50 mM, 1 complete tablet/100 mL, Hepes-KOH pH = 7.4 50 mM) using a Teflon homogenizer. Then the homogenates were centrifuged at 15,000 rpm for 20 min at 4 °C. Protein concentrations were determined according to the Bradford method. Protein extracts (50 μg/lane) were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinyldene fluoride (PVDF) membranes. Membranes were immunoblotted overnight at 4 °C with antibodies directed against total and phosphorylated AMPK, cytochrome C, cleaved caspase 9, cleaved caspase 3 (Cell Signaling Technology Inc., Beverly, MA, USA), eNOS, and β actin (Sigma Chemical, St. Louis, MO, USA). The bands were detected using chemiluminescent kit (Bio-Rad Laboratories, Hercules, CA, USA) and quantified using the Quantity One software for image analysis. Results were expressed as densitometric ratios between the protein of interest and the loading control.

2.3.4. Statistical analysis

The data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance, followed by Newman–Keuls multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of TMZ treatment on liver injury

Liver damage was assessed by transaminases (ALT and AST) activities. As revealed by Figures 1a and 1b, hepatic I/R resulted in a significant ($P < 0.05$) increase in plasma ALT and AST activities when compared to the sham operated group. The use of TMZ treatments significantly ($P < 0.05$) decreased cell injury compare with the I/R group. Interestingly, the largest reduction in ALT and AST levels was observed in the TMZ3 group as compared to TMZ1 group. The difference was statistically significant ($P < 0.05$) between both groups.

In parallel, we examined the impact of TMZ administration on ALP activity. As revealed by Figure 1c, hepatic I/R induced a significant ($P < 0.05$) increase in ALP activity in the sham group. TMZ pretreatment resulted in a significant ($P < 0.05$) reduction in this parameter when compared to the I/R group. Interestingly, the lowest activity of ALP was observed in the TMZ3 group when compared to the TMZ1 group. The difference was statistically significant ($P < 0.05$) between both groups.

Similarly, upon I/R injury, serum GGT activity was significantly increased in the I/R group as compared to the sham group (Figure 1d). This rise was then significantly (P

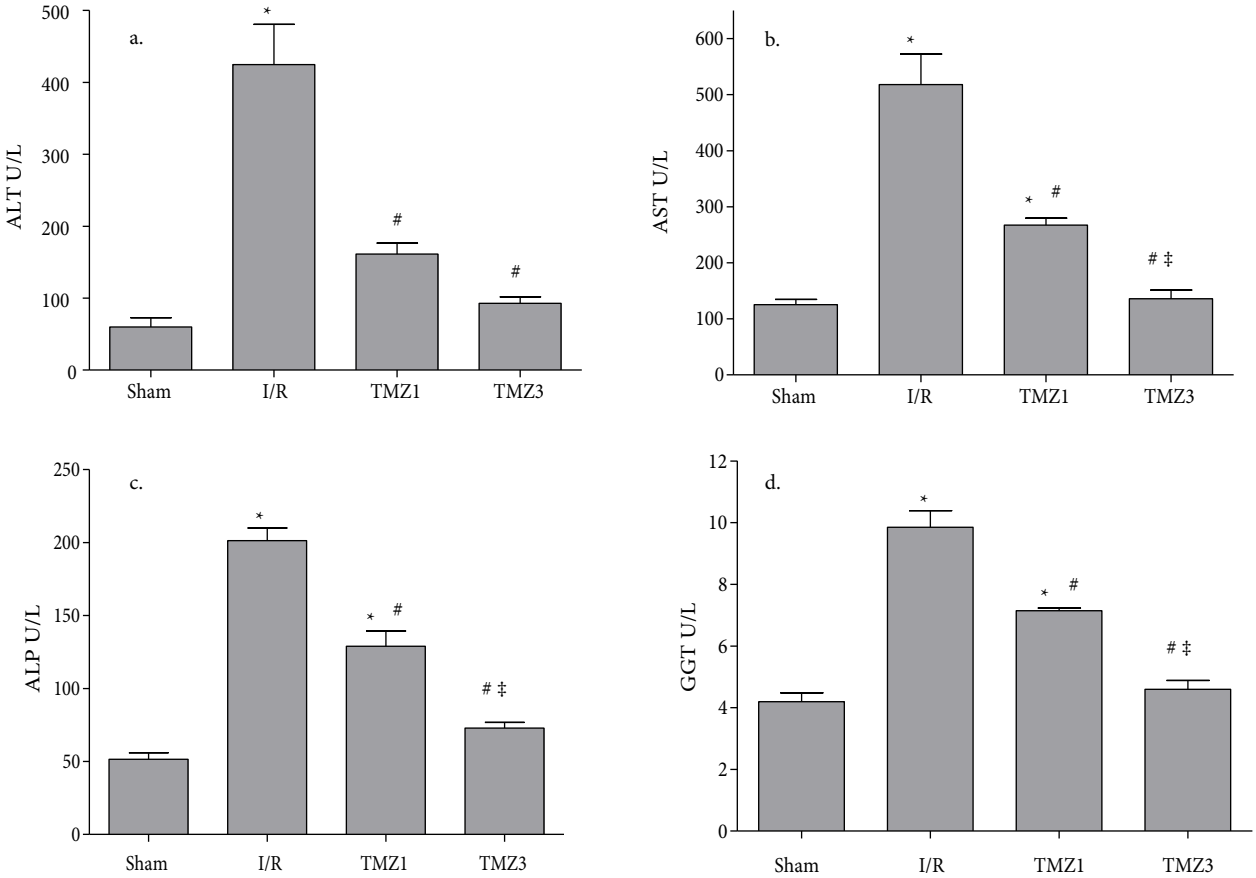

Figure 1. Evaluation of alanine amino transaminase (ALT) (a), aspartate amino transaminase (AST) (b), alkaline phosphatase (ALP) (c), and gamma glutamyltransferase (GGT) (d) activities. Results are presented as mean ± standard error of the mean (n = 6 in each group). * P < 0.05 vs. sham group. # P < 0.05 vs. I/R group. ‡ P < 0.05 vs. TMZ1 group.

< 0.05) attenuated by TMZ pretreatments when compared to the I/R group. A significant difference was detected among both TMZ groups.

3.2. Effect of TMZ treatment on lipid peroxidation

We examined the protective effect of TMZ on lipid peroxidation (Figure 2). Hepatic I/R caused a significant (P < 0.05) rise in tissue MDA level compared with the sham group. After the TMZ treatment, we observed a significant drop in this parameter compared to the I/R group. However, no statistical difference was obtained between TMZ groups.

3.3. Effect of TMZ treatment on apoptotic proteins levels

We also determined whether TMZ was able to reduce apoptosis of liver cells. The relevance of apoptosis was assessed by tissue cytochrome C, cleaved caspases 9, and cleaved caspases 3 proteins levels (Figure 3). A unique administration of TMZ decreased the cleaved caspase 3 and cleaved caspase 9 protein levels (P < 0.05 versus I/R, respectively) but no significant difference was observed concerning cytochrome C when compared to the I/R

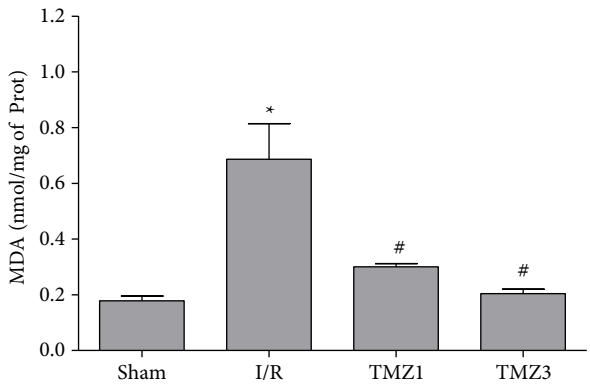

Figure 2. Evaluation of malondialdehyde concentration in tissue. Results are presented as mean ± standard error of the mean (n=6 in each group). * P < 0.05 vs. sham group. # P < 0.05 vs. I/R group. ‡ P < 0.05 vs. TMZ1 group.

group. However, when TMZ was administered for 3 days, we noted an important decrease in the amount of all studied parameters regarding I/R. In addition, there was

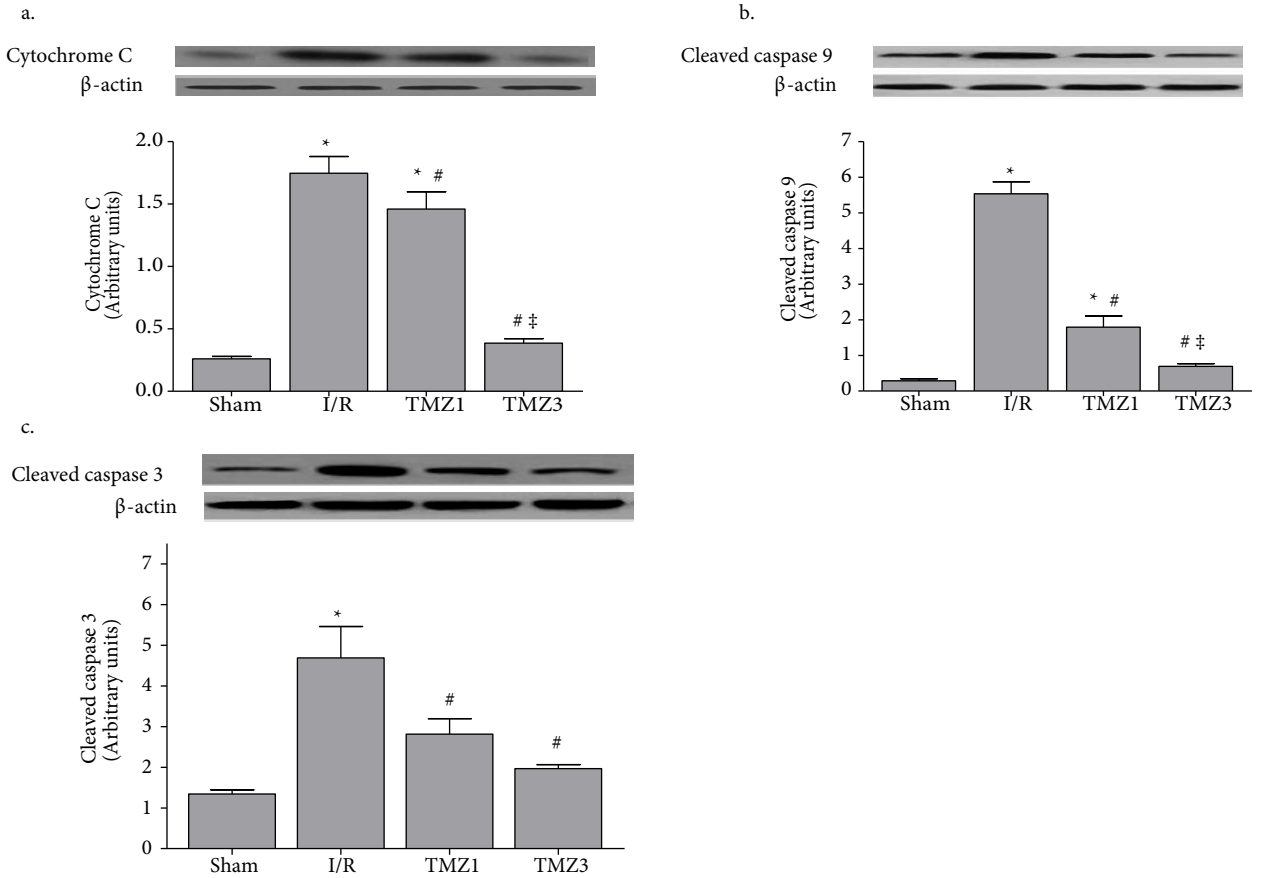

Figure 3. Western blot of cytochrome C (a), cleaved caspase 9 (b), and cleaved caspase 3 (c) levels. One representative blot of 6 independent experiments is shown at the top whereas densitometric analysis is shown at the bottom. Results are presented as mean ± standard error of the mean (n = 6 in each group). * P < 0.05 vs. sham group. # P <0.05 vs. I/R group. ‡ P < 0.05 vs. TMZ1 group.

a significant difference between TMZ groups regarding cytochrome C and cleaved caspase 9 (P < 0.05, respectively) except for cleaved caspase 3 (P > 0.05).

3.4. Effects of TMZ treatment on the p-AMPK and eNOS levels

As indicated in Figure 4, hepatic warm I/R decreased p-AMPK protein level (P < 0.05) when compared to sham tissue, but TMZ administration augmented the p-AMPK level. This increase was statistically significant compared to the I/R group (P < 0.05). The AMPK phosphorylation was furthermore enhanced by the repetitive TMZ administration when compared to the TMZ1 group. The difference between the 2 groups was statistically significant (P < 0.05).

In parallel, Western blot analysis denoted that the immediate administration of TMZ before warm hepatic I/R did not affect the amount of eNOS compared to the I/R group. However, the repetitive administration of TMZ for 3 days induced an important rise in the eNOS level. The difference was statistically significant (P < 0.05) between the I/R and TMZ1 groups.

4. Discussion

The objective of our study was to investigate the protective effect of TMZ preconditioning in hepatic I/R injury using 2 dosing protocols and its effects in the signaling mechanism involved in the physiopathology of liver ischemia.

Our results consolidate previous reports demonstrating that TMZ protects rat liver against I/R injury (13). Rats pretreated with TMZ showed reduced activity of AST, PAL, and GGT. We noted that repeated administration of TMZ for 3 days was more efficient in reducing liver damage than a single dose. A growing body of evidence has proven that TMZ has antioxidant effects and its role on lipid peroxidation inhibition has been well established in different organs (8,9). Lipid peroxidation disrupts membrane fluidity and cell compartmentalization, which results in cell lysis (3). The antiischemic effect of TMZ is usually attributed to its impact on the balance between fatty acid and glucose oxidation. TMZ favors a shift from fatty acid oxidation to glucose oxidation, via the inhibition of the mitochondrial long-chain 3-ketoacyl CoA thiolase, and thereby attenuates the detrimental effects of I/R (8).

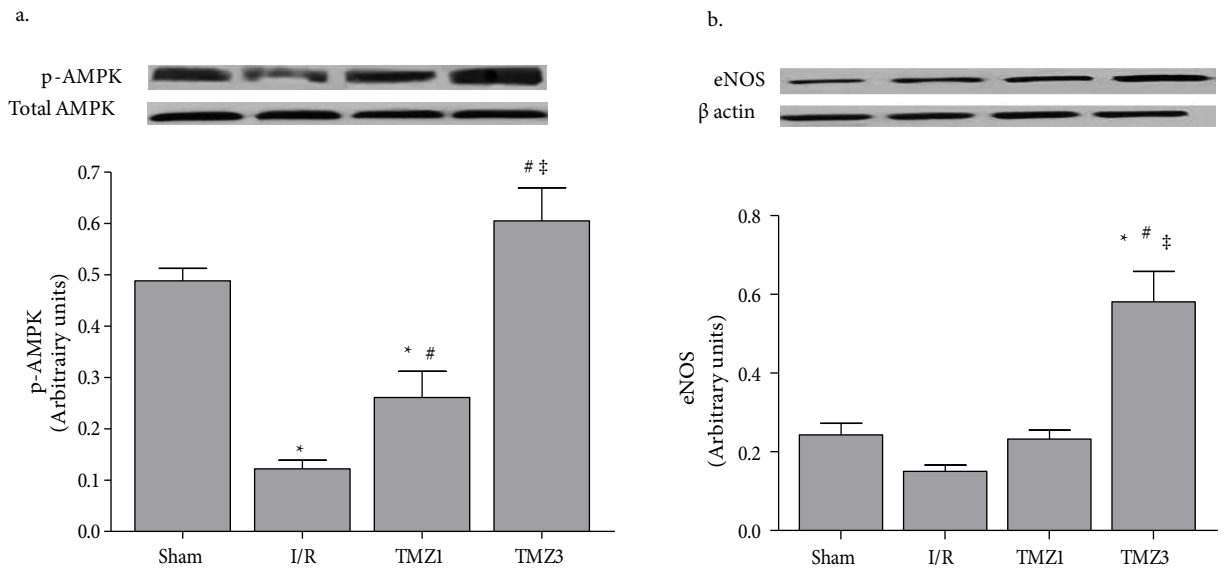

Figure 4. Western blot of total and phosphorylated-AMPK (a), and eNOS (b) levels. One representative blot of 6 independent experiments is shown at the top whereas densitometric analysis is shown at the bottom. Results are presented as mean ± standard error of the mean (n = 6 in each group). * P < 0.05 vs. sham group. # P < 0.05 vs. I/R group. ‡ P < 0.05 vs. TMZ1 group.

Elimadi et al. (14) reported that TMZ produced some metabolites that might possess antioxidant capacity when it is metabolized by the liver, the major site of drug metabolism.

In line with this, our current results revealed that the pretreatment of rats with TMZ before the induction of liver ischemia decreased the leakage of hepatocyte enzymes and prevented lipid peroxidation. We noted that repeated administration of TMZ for 3 days was more efficient in reducing liver damage than a single dose. However, no significant change was noted between the 2 models of TMZ administration concerning MDA concentration in tissues.

We further showed that TMZ reduced apoptosis. Indeed, Western blot results revealed that TMZ pretreatment significantly reduced the cleaved caspase 9, the cleaved caspase 3 levels, and cytochrome C release when compared to the I/R group. Previous studies demonstrated that TMZ could limit apoptotic death in different experimental models (15,16). These apoptotic proteins are known to have a pivotal role in I/R pathophysiology (15). In fact, TMZ might bind to the inner mitochondrial membrane and inhibit the activation of the permeability transition pore and subsequent cytochrome C release (17). When released, cytochrome C forms a complex with Apaf 1 and procaspase 9, resulting in the activation of caspase 9, which induces caspase 3 activation and apoptosis. Data from the present study provide strong evidence that TMZ preconditioning preserves mitochondrial integrity and reduces mitochondria mediated apoptosis. It seems that the administration of TMZ for 3 days was more effective in reducing apoptosis.

The hepatoprotective mechanisms of TMZ are complex (9,16). Khan et al. (9) reported that TMZ is cardioprotective when administered before reperfusion and that this protection appears to be mediated by activation of p38 mitogen-activated protein kinase and Akt signaling pathway. Moreover, Wu et al. (18) demonstrated that TMZ induced the activation of Akt/eNOS signaling pathways to mitigate H_2O_2 induced oxidative stress and cell death. To gain further insight into the mechanisms involved in the hepatoprotective effect of TMZ and the influence of the protocol of administration, we investigated the activation of AMPK and eNOS signaling pathways, which play a critical role in protecting various organs against I/R injury including the heart (19) and kidney (20). It also has a protective role against hepatic I/R injury in the liver (21). Bouma et al. (22) demonstrated that the addition of 5-amino 4 imidazole carboxamide riboside, an AMPK activator, to University of Wisconsin solution improved liver graft preservation. AMPK promotes ATP generating pathways, while inhibiting energy consuming anabolic ones (23). AMPK also appears to function as a sensor for other signaling pathways. A relationship between AMPK and nitric oxide (NO) has previously been described. Zaouali et al. (24) demonstrated that increased levels of eNOS and NO generation are in part due to AMPK phosphorylation after cold I/R injury in rat liver. It is well established that NO plays a crucial role in defending organs against I/R injury through its antioxidant, antiinflammatory,

and antiapoptotic capacities (25). Moreover, Jayle et al. have demonstrated that the TMZ protective effect was associated with NO generation in a pig kidney model of I/R (6). Our results revealed that repeated TMZ treatment enhanced AMPK phosphorylation and eNOS activation. However, single dose administration activated AMPK but not eNOS.

Taken together, all these data suggested that the pretreatment of rats with TMZ before the induction of liver I/R decreased liver injury and oxidative stress. The underlying protective mechanism was the activation of AMPK/eNOS cell signaling pathway and the inhibition of apoptosis. Furthermore, we demonstrated that dosing time of TMZ determines the mechanisms and the degree of protection. Elimadi et al. (14) investigated the effect of an intramuscular injection of different doses of TMZ on hepatic warm I/R injury. They demonstrated that 10 mg/kg per day for 7 days was the optimal TMZ dosage that gave the maximal protective effects at both cellular and mitochondrial level. However, Cau et al. (17) demonstrated that intravenous administration of TMZ is protective against renal I/R injury at a dose of 5 and 10 mg/kg in an in vivo pig model of warm I/R and that the dose of 10 mg/kg did not increase TMZ efficiency. The discrepancies observed between results may implicate, at least partially, the routes, timing, and doses of TMZ administration, as well as I/R protocols. Further investigations to evaluate the optimal time and dose of administration of TMZ are required to have more insight into clinical translation.

In light of these findings, we suggest that repeated administration of TMZ for 3 days was more efficient than a single dose of TMZ in protecting the liver against I/R induced apoptosis and lipid peroxidation. These protective effects implicate AMPK and eNOS activation.

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