

# Antioxidative Effects of Tempol on Mitochondrial Dysfunction in Diabetic Nephropathy

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**Introduction.** Oxidative stress has a well-known role in diabetic nephropathy, and mitochondria are the major source of reactive oxygen species production. This study aimed to assess the effect of tempol, a superoxide dismutase mimetic agent, on mitochondrial antioxidant enzymes and cell viability in diabetic nephropathy.

**Materials and Methods.** Adult male Wistar rats were divided into 4 groups of 7 animals. Diabetes mellitus was induced by injection of streptozotocin in 2 groups, the rat in one of which were also treated with tempol for 4 weeks. Another group without diabetes mellitus received tempol, and the last group was the control. At the end of the treatment period, the kidney mitochondria were isolated and their antioxidant enzymes, including superoxide dismutase, glutathione peroxidase, and catalase were assessed. Malondialdehyde, total antioxidant capacity, and kidney cells viability were studied, as well.

**Results.** The diabetic group was significantly different compared with the control group in malondialdehyde, catalase, and glutathione peroxidase activities. Superoxide dismutase and total antioxidative capacity did not show any significant differences among the four groups. Moreover, the diabetic group treated with tempol had significantly different glutathione peroxidase level and kidney cells viability, compared to the other diabetic group ( $P < .05$ )

**Conclusions.** Diabetic nephropathy induces changes in mitochondrial antioxidative biomarkers and cells viability, some of which can be modified by tempol administration in rats.

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## INTRODUCTION

Oxidative stress is a well-known phenomenon in the pathogenesis of diabetic vascular complications, as well as nephropathy.<sup>1</sup> On the other hand, mitochondria are known as the main sources of reactive oxygen species (ROS) creation in diabetes mellitus,<sup>2</sup> and they are injured by the ROS as well. Mitochondrion is the main energy-producing organelle in cells. It is a membrane-enclosed structure found in most eukaryotic cells. Mitochondrial dysfunction plays an important

role in diabetic nephropathy (DN).<sup>3</sup> Mitochondria produce oxygen radicals while substrates are metabolized.<sup>4</sup> This leads to oxidative injury, appearing in ways such as protein alteration,<sup>5</sup> lipid membranes peroxidation,<sup>6</sup> and mitochondrial DNA break.<sup>7</sup> A couple of enzymes may protect against ROS in mitochondria. These include manganese superoxide dismutase (SOD), catalase, glutathione peroxidase (GP), etc.<sup>8</sup>

Conversely, nitroxide 4-hydroxy-2,2',6,6'-tetramethylpiperidine 1-oxyl, also known as *tempol*,

is a well-known SOD mimetic antioxidative agent. Tempol reduces severity of DN in SOD-knockout mice during streptozotocin-induced diabetes mellitus.<sup>9</sup> Furthermore, tempol administration to obese hypertensive Zucker rats improved insulin sensitivity and reduced renal inflammation and fibrosis.<sup>10</sup> Tempol also reduced renal mesangial expansion and decreased transforming growth factor- $\beta$  in diabetic rats.<sup>11</sup>

In this study, we assessed kidney mitochondrial catalase, SOD, GP, total antioxidative capacity, lipid peroxidation (LP), and kidney cells viability, and moreover, tempol effect on these in different groups of rats including diabetic ones.

## MATERIALS AND METHODS

### Reagents and Chemicals

Chemicals used in the study were tetraethoxy propane (malondialdehyde), 2-thiobarbituric acid, trichloroacetic acid, n-butanol, sucrose, ethylenediaminetetraacetic acid, comassie blue, bovine serum albumin, 4,5(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, GP, and SOD (Ransel kit, Randox Laboratories Ltd, Crumlin, UK). All other chemicals were obtained from the Sigma.

### Animals and Interventions

The study animals were adult male Wistar rats weighing 180 g to 250 g maintained on a 12-hour light-dark cycle with free access to tap water and standard laboratory chow. The animals were randomly divided into 4 groups of 7 rats, as follows: control group, diabetic group, tempol group (tempol, 100 mg/kg/d, for 4 week), and diabetic tempol group (tempol, 100 mg/kg/d, for 4 week). Diabetes mellitus was induced using a streptozotocin injection (prepared by citrate buffer, pH 4.5; 1 dose, 60 mg/kg body weight; intraperitoneal), as validated in previous studies.<sup>12</sup> The fasting blood glucose levels were determined 3 days after streptozotocin injection using a strip-operated blood glucose sensor. The animals were considered diabetic if plasma glucose levels exceeded 250 mg/dL. At the end of the treatment, 24 hours postintervention, the animals were killed, the kidney tissue was separated and stored in liquid nitrogen, and then their mitochondria were isolated quickly and kept frozen at -80°C. All procedures for the treatment of the animals were approved

by the research ethics committee of Hamadan University of Medical Sciences.

### Kidney Parameters

Blood urea nitrogen, serum creatinine, urine albumin, and urine creatinine levels were estimated using an automated biochemistry machine, according to the standard procedure of kits.

### Preparation of Kidney Mitochondria

The kidney tissues were removed and minced with small scissors in a cold manitol solution containing 0.225 M of D-manitol, 75 mM of sucrose, and 0.2 mM of ethylenediaminetetraacetic acid. The minced kidney (30 g) was gently homogenized in a glass homogenizer with a Teflon pestle and then centrifuged at 700 g for 10 minutes at 4°C to remove nuclei, unbroken cells, and other nonsubcellular tissue. The supernatants were centrifuged at 7000 g for 20 minutes. These second supernatants were pooled as the crude microsomal fraction and the pale loose upper layer of sediments, which was rich in swollen or broken mitochondria, lysosomes, and some microsomes, was washed away.

The dark packed lower layer (heavy mitochondrial fraction) was resuspended in the manitol solution and recentrifuged twice at 7000 g for 20 minutes. The heavy mitochondrial sediments were suspended in Tris solution containing 0.05 M of Tris-hydrochloride buffer (pH 7.4), 0.25 M of sucrose, 20 mM of potassium chloride, 2.0 mM of magnesium chloride, and 1.0 mM of disodium phosphate at 4°C before assay.<sup>13</sup>

### Assay of Superoxide Dismutase Activity

The activity of SOD was measured using a commercial kit (Ransod kit, Randox Laboratories Ltd, Crumlin, UK). Measurement of the enzyme was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase and reacted with 2-(4-iodophenyl)-3-(4-nitrophenol) 5-phenyltetrazolium chloride to form a red formazan dye. The formazan was read at 505 nm. One unit of SOD was defined as the amount of enzyme necessary to produce 50% inhibition in the 2-(4-iodophenyl)-3-(4-nitrophenol) 5-phenyltetrazolium chloride reduction rate.

### Assay of Glutathione Peroxidase Activity

The amount of GP was determined using a

commercially available kit (Ransel kit, Randox Laboratories Ltd, Crumlin, UK) by measuring the rate of oxidation of nicotinamide adenine dinucleotide phosphate at 340 nm. A unit of enzyme was expressed as the amount of enzyme needed to oxidize 1 nmol of nicotinamide adenine dinucleotide phosphate oxidase/minute.

#### Assay of Lipid Peroxidation

The LP product in tissues was determined by 2-thiobarbituric acid reagent, expressed as the extent of malondialdehyde production during an acid heating reaction. Briefly, the samples were diluted by 1.5 mL of trichloroacetic acid (20% w/v), added to 250  $\mu$ L of this samples, and centrifuged in 3000 g for 10 minutes. Then, the precipitation was dissolved in sulfuric acid, and 1.5 mL of the mixture was added to 1.5 mL of 2-thiobarbituric acid (0.2% w/v). The mixture was then incubated for 1 hour in a boiling water bath. Following incubation, 2 mL of n-butanol was added, the solution centrifuged, cooled, and the absorption of the supernatant was recorded in 532 nm. The calibration curve of tetraethoxypropane standard solutions was used to determine the concentrations of 2-thiobarbituric acid plus malondialdehyde adducts in samples.<sup>14</sup>

#### Assay of Catalase Activity

Catalase activity was assayed in the samples by measuring the absorbance decrease at 240 nm in a reaction medium containing hydrogen peroxide (10 mM), sodium phosphate buffer (50 mM, pH 7.0). One unit of the enzyme was defined as 1 mol of hydrogen peroxide as substrate consumed per minute, and the specific activity was reported as units per milligram of protein.<sup>15</sup>

#### Assay of Total Antioxidant Capacity

The total antioxidant capacity was measured by ferric-reducing ability of plasma method. This method is based on the ability of plasma in reducing ferric to ferrous in the presence of 2,4,6-tripyridyl-S-triazine. The reaction of ferrous and 2,4,6-tripyridyl-S-triazine gives a complex with a blue color and maximum absorbance in 593 nm.<sup>16</sup>

#### Total Protein

The protein content was quantified by the method

of Bradford. Concentrated Coomassie blue was diluted in 250  $\mu$ L of distilled water, and then 750  $\mu$ L of this diluted dye was added to 50  $\mu$ L of the sample. The mixture was incubated at room temperature for 10 minutes and an absorbance measurement was taken at 595 nm by a spectrophotometer. A standard curve was constructed by using bovine serum albumin ranging between 0.25 mg/mL and 1 mg/mL.<sup>17</sup>

#### Assay of Cell Viability

The cell viability assay is a quantitative colorimetric method to determine cell viability. It utilizes the yellow tetrazolium salt, which is metabolized by mitochondrial dehydrogenase enzyme from viable cells to yield a purple formazan reaction product that was determined spectrophotometrically at wavelength of 570 nm. The percentage of cell viability of each test sample was calculated.<sup>18</sup>

#### Statistical Analysis

The mean and standard error values were determined for all the parameters and the results were expressed as mean  $\pm$  standard deviation. Data normality was accessed using the Kolmogorov-Smirnov test and the 1-way analysis of variance was used to determine if there was any differences between the four groups. Differences were considered significant when the *P* value was less than .05. The SPSS software (Statistical Package for the Social Sciences, version 18.0, SPSS Inc, Chicago, IL, USA).

## RESULTS

### Diabetes Induction

After 3 days of follow-up, diabetes mellitus (fasting blood glucose > 250 mg/dL) was induced in the rats which received streptozotocin intraperitoneally. Diabetic nephropathy was confirmed by significant rising of albumin-creatinine ratio after 4 weeks ( $258 \pm 28$  in the control group versus  $2000 \pm 26$  in the diabetic groups). Blood urea nitrogen also increased ( $44 \pm 3.39$  mg/dL versus  $60.4 \pm 2.8$  mg/dL, respectively), but not serum creatinine ( $0.61 \pm 0.06$  mg/dL versus  $0.66 \pm 0.04$  mg/dL, respectively).

### Superoxide Dismutase Activity

One unit of SOD was defined as the amount

of enzyme necessary to produce 50% inhibition in the 2-(4-iodophenyl)-3-(4-nitrophenol) 5-phenyltetrazolium chloride reduction rate. It increased in the diabetic group, compared with the control group and decreased by tempol administration in the diabetic tempol group. However, there was no significant difference among the four groups.

### Catalase Activity

One unit of the enzyme was defined as 1 mol of hydrogen peroxide as substrate consumed per minutes. It was significantly higher in the diabetic group, compared with the control group. Tempol administration, however, decreased catalase activity in the diabetic rats insignificantly (Figure 1).

### Glutathione Peroxidase Activity

One unit of enzyme was expressed as the amount of enzyme needed to oxidize 1 nmol of nicotinamide adenine dinucleotide phosphate oxidase per minute. Unlike the other enzymes, GP was lower in the diabetic versus control rats ( $P < .05$ ) and raised in the diabetic tempol group significantly (Figure 1).

### Lipid Peroxidation Activity

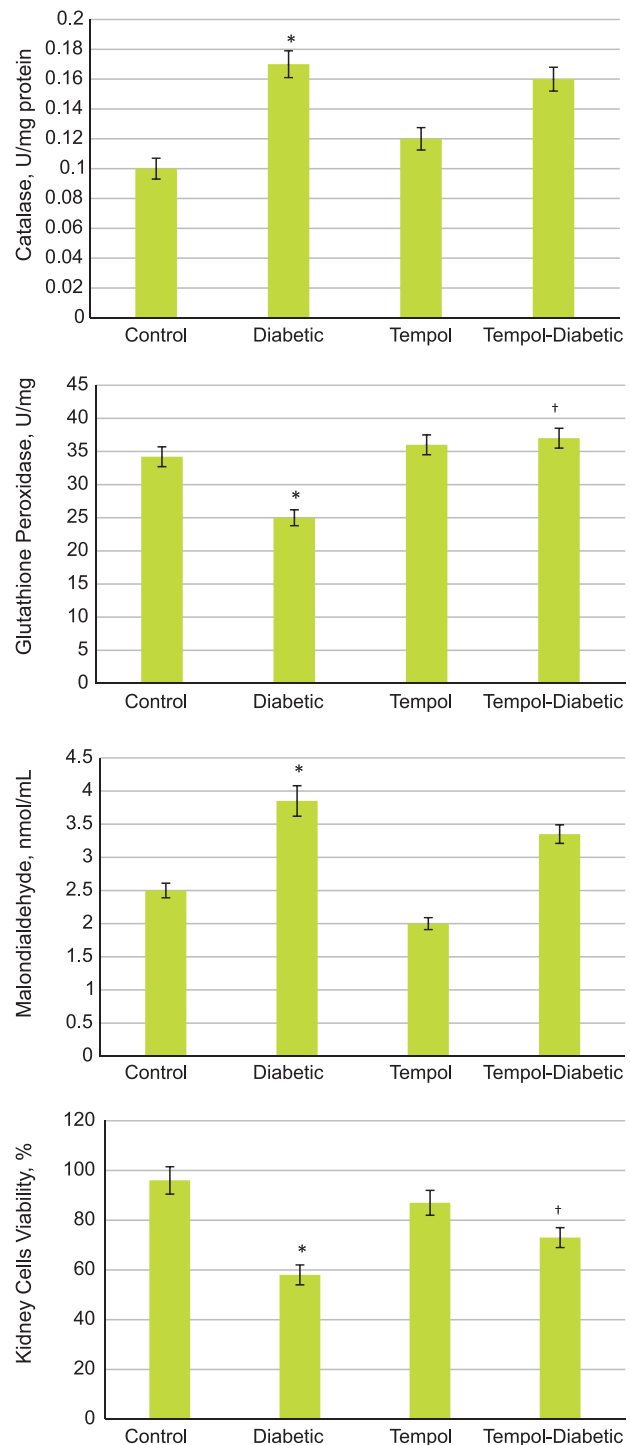
Lipid peroxidation production was determined by the extent of malondialdehyde creation during an acid heating reaction. Compare to control group, malondialdehyde increased in the diabetic group notably, but was not affected by tempol administration (Figure 1).

### Total Antioxidative Capacity

The total antioxidative capacity was not significantly different between the four animal groups.

### Kidney Cells Viability

Diabetes mellitus induction decreased viability of cells in comparison to the controls, and on the other hand, tempol administration significantly increased it ( $P < .05$ ; Figure 1). Moreover, correlations between kidney cells viability and superoxide dismutase, catalase, and malondialdehyde activities were moderate, indirect, and significant ( $P < .05$ ; Figure 2), while the correlation with glutathione peroxidase was weak ( $r = 0.33$ ) and nonsignificant ( $P > .05$ ).



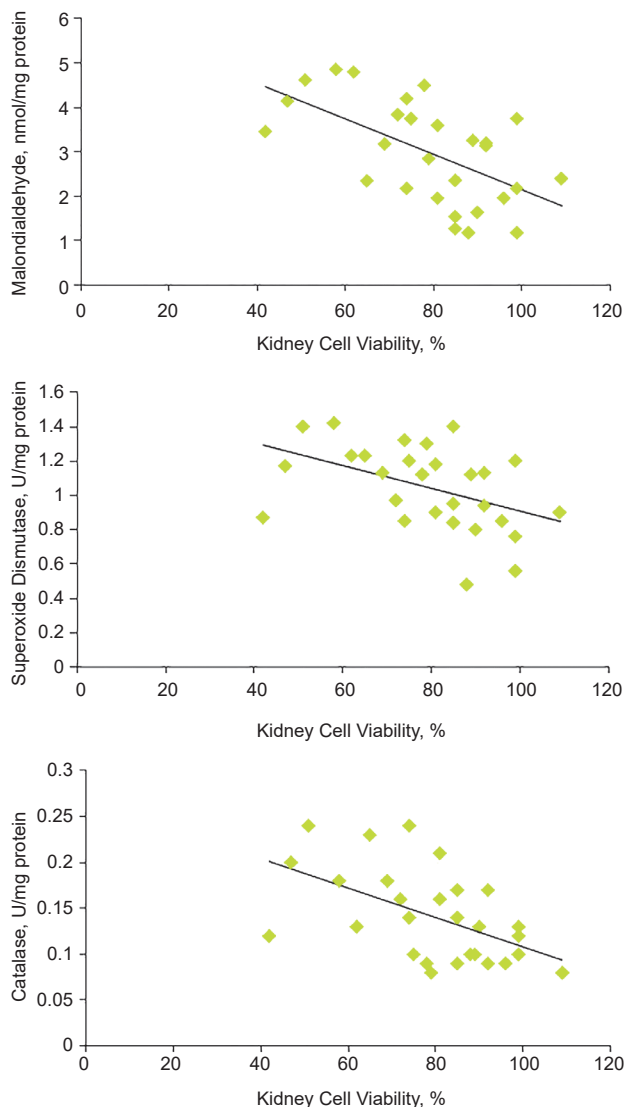
**Figure 1.** Changes of kidney mitochondrial antioxidative enzymes and kidney cells viability in diabetic and nondiabetic rats and the impact of tempol.

\* $P < .05$  compared with the control group

† $P < .05$  compared with the diabetic group

## DISCUSSION

Manganese SOD, which is an important antioxidative enzyme, mainly regulates ROS



**Figure 2.** Correlation between kidney cells viability and mitochondrial antioxidative enzymes.

metabolism in the mitochondria. Consequently, its activity might be reduced with ROS exposure.<sup>19</sup> Circumstances that direct to manganese SOD dysfunction lead to tissue damage coupled with DN. On the other hand, resveratrol, a well-known antioxidant agent, improved kidney injury and mitochondrial biogenesis in the kidney of db/db mice, via normalization of manganese SOD function.<sup>20</sup> Conversely, Munusamy and MacMillan-Crow showed that manganese SOD activity was not altered in hyperglycemic milieu in the early stages of diabetes mellitus.<sup>21</sup> Our study showed similar results with tempol administration. Conversely, kidney cells viability diminishes in hyperglycemic milieu, as we showed and the relationship between

SOD and kidney cells viability was indirect and significant. Therefore, it seems manganese SOD compensates by rising its activity per remaining cells.

Catalase has classically been considered a cytoplasmic enzyme. However, recent studies have documented its activity in heart mitochondria.<sup>22</sup> Furthermore, catalase was significantly upregulated in heart mitochondria of diabetic rats.<sup>23</sup> We showed the same modification in kidney mitochondria. Again, augmentation per cells, presumably, pays off diminishing viable cells. Nevertheless, it did not change significantly following tempol administration.

In contrast to Moreira and colleagues,<sup>24</sup> we showed downregulation of glutathione peroxidase in kidney mitochondria of the diabetic rats, which improved significantly by tempol administration. It may be explained by different periods of diabetes mellitus induction of rats (4 weeks versus 12 weeks).

Malondialdehyde is one of the well-known end products of lipid peroxidation and is an indicator of oxidative stress injury.<sup>25</sup> Mitochondrial malondialdehyde was increased significantly in the DN groups. Moreover, we showed that kidney cells viability decreased significantly in DN, which could be improved by tempol administration. Yu and colleagues<sup>26</sup> showed that viability of glomerular mesangial cell and SOD were decreased in high glucose milieu. Conversely, malondialdehyde was increased, which could be modified by a different dosage of a kind of ginseng sapogenin (PPD). Our study showed a significant correlation between kidney cells viability and malondialdehyde.

All mitochondrial antioxidative enzymes, with the exception of GP, had moderate, significant, and of course indirect correlation with kidney cells viability. It indicates that in oxidative stress circumstance, these enzymes activity increases to compensate reduced cell viability. However, this is not the case about GP. It gives the impression that this enzyme activity remains invariable per cell, still in diabetic oxidative stress. It is well known that GP activity depends on glucose-6-phosphate dehydrogenase action, which already lessens in diabetic milieu, and it perhaps explains why GP is an exception. The mechanisms responsible for the decrease of glucose-6-phosphate dehydrogenase activity are not fully known. Xu and coworkers<sup>27</sup> proposed that decreased expression and posttranslational



modification played a role equally. Hence, this study give us an idea about different mechanism of regulating antioxidant enzymes, which should be evaluated deliberately by further studies. To the best of our knowledge, this is the first report describing correlation of kidney cells viability with mitochondrial antioxidative enzymes in DN.

## CONCLUSIONS

Oxidative stress severely impacts DN. Mitochondria on the other hand, have major role in generating and also are injured by ROS. We studied changes of a couple kidney mitochondrial enzymes and tempol effects on them for the first time. Moreover, we showed that kidney cells viability was altered by tempol in DN.

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## CONFLICT OF INTEREST

None declared.

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