Original Article

Effect of tempol, a synthetic antioxidant, on renal complications of L-NAME induced preeclampsia in rat

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Abstract

Introduction: It has been suggested that oxidative stress has a crucial role in the pathophysiology of preeclampsia. In the present study, the effect of tempol, a synthetic antioxidant, on kidney injuries and oxidative stress was investigated in an experimental model of preeclampsia in rats.

Methods: Preeclampsia was induced by oral administration of L-NAME to the rats on the day 10 of pregnancy. Animals were randomly divided into six groups (10-15 rats in each group); (I) normal pregnant (II) preeclamptic (III, IV, V) preeclamptic + tempol 20, 60 and 180 mg/kg/day, respectively, (VI) preeclamptic + hydralazine 10 mg/kg/day. Urine levels of sodium, potassium, creatinine, lactate dehydrogenase and 24 h protein, blood levels of creatinine and urea, in addition to malondialdehyde concentration in blood and kidney, as well as histological glomeruli changes were assessed.

Results: L-NAME administration caused proteinuria and glomerular pathological changes. Tempol (20 and 60 mg/kg/day) significantly reduced plasma and renal ($P<0.001$) malondialdehyde levels and proteinuria (the biggest calculated P-value was less than 0.05) in preeclamptic rats. Tempol at the dose of 20 mg/kg/day improved the histological changes in preeclamptic animals, but the dose of 60 mg/kg/day restored histological findings. L-NAME did not change the other measured parameters. Hydralazine and highest dose of tempol (180 mg/kg/day) failed to affect biochemical and histological changes in experimental preeclampsia.

Conclusion: Renal complications of experimental preeclampsia such as proteinuria and glomerular injuries can be prevented by tempol. The desired effects of tempol depend on its dose.

Introduction

Preeclampsia is a leading cause of maternal, fetal morbidity and mortality. This disorder is characterized by hypertension, proteinuria and edema (Noris et al., 2005). Proteinuria is a chief hallmark of this syndrome and directly associated with other complications such as convulsion, fetal growth retardation and fetal death (Maybury and Waugh, 2005).

The etiology of preeclampsia is still unclear

(Hoffmann et al., 2008). It is proposed that reactive oxygen species (ROS) have an important role in preeclampsia progression (Harmon et al., 2016).

Methyldopa and hydralazine are the two most prescribed agents for this syndrome (Bolte et al., 2001). These drugs decrease blood pressure, but they are not renoprotective and do not have any effect on proteinuria (Mathai, 1996; Maybury and Waugh, 2005). In practice, angiotensin inhibitors are the main antihypertensive drugs with renoprotective property, but they are contraindicated in pregnant women (Cooper et al., 2006). Hence, investing the etiology of preeclampsia and searching for agents that can effectively manage all complications seem crucial.

Tempol is a superoxide dismutase (SOD)–mimetic agent with significant antioxidant activity. It was shown to have renoprotective property in experimental diabetic nephropathy (DeRubertis et al., 2007). In a previous study, tempol at low to moderate doses, reduced hypertension and aorta hyperresponsiveness to phenylephrine, as well as preserving aortic endothelium-dependent relaxation in experimental preeclamptic rats (Talebianpoor and Mirkhani, 2012).

In the present study, the effect of tempol on renal complications of NG-nitro-L-arginine-methyl-ester (L-NAME) induced preeclampsia in rat was investigated. Also, the effect of hydralzine, as a clinically accepted drug to control preeclampsia was explored.

In this study, three different tempol doses were used to evaluate biochemical and histological changes in comparison with a standard agent, which is a new approach. Preeclampsia was induced by L-NAME, a well-known inhibitor of nitric oxide production. When it is administered in animals during their middle to late period of gestation, a syndrome resembling preeclampsia in human is induced, which is associated with increased ROS production, hypertension, proteinuria and diminished placental perfusion (Sedeek et al., 2008).

Materials and methods

Animals
One hundred female *Sprague-Dawley* rats weighing 250-300 g were entered into the study. The animal protocol was designed to minimize pain and discomfort. Animals were kept in individual cages under controlled temperature (25°C), in 12h light- 12h dark condition. They had free access to food and water (except after day 10 of pregnancy in which the drinking water was altered as stated below). All procedures involving animals were reviewed and approved by the "Research Ethics Committee" of Shiraz University of Medical Sciences (No. 2016-294).

Drugs
L-NAME was purchased from Alexis biochemical (USA), tempol and hydralazine were obtained from Sigma (UK). L-NAME, tempol and hydralazine were dissolved in animals' drinking water.

Experimental design and animal grouping
The *Sprague-Dawley* rats were mated at night. The day that vaginal plaque was seen, it was considered as day 0 of pregnancy. On day 10, the rats were randomly divided into six groups (10-15 rats in each): group I consist of normal pregnant animals that only received water. Rats in group II were treated with L-NAME 50 mg/kg/day; rats in groups III, IV, V, and VI were treated with L-NAME (50 mg/kg/day) plus tempol 20, 60 and 180 mg/kg/day, and hydralazine 10 mg/kg/day, respectively. The tempol administered doses were selected based on the studies in which antioxidant effects were investigated in hypertensive rats (Preti et al., 2005; Yanes et al., 2005; Elmarakby et al., 2007).

In another separate group, normal pregnant rats (n=5) were given tempol 60 mg/kg/day from day 10 of pregnancy, and only kidney and plasma malondialdehyde (MDA)*,* and 24 h urine protein levels were measured (as stated below).

Blood pressure measurement
Systolic blood pressure was measured on gestational days 10, 13, 15, 18 and 21 by an automated sphygmomanometer with a tail-cuff device (ML 125/R, AD Instruments, Australia).

Biochemical assays
Urine protein of all studied groups was assayed in days 10, 15 and 20 of gestation. To achieve this, animals were placed in metabolic cages, 24 h urine was collected and its protein content was measured according to Bradford method (Bradford, 1976).

On gestational day 22, the rats were anesthetized by ether in a closed jar under the airflow hood. Blood

samples were taken via the tail artery and then centrifuged at 3000 rpm. Plasma was collected and immediately frozen at -80°C until further analyses. Urine samples were centrifuged at 3000 rpm for 20 min. The supernatant was aspirated and kept refrigerated in sealed 1.5 ml tubes.

Urine creatinine, serum creatinine and urea were measured by commercial kits based on the Jaffe and Berthelot methods, respectively. Clearance of creatinine was calculated by the formula:

Ccr= (Ucr * V)/Pcr

Ccr is creatinine clearance, Ucr is urine creatinine concentration, V is 24 h urine volume and Pcr is plasma creatinine concentration.

To investigate renal tubules possible injuries, urine lactate dehydrogenase (LDH), sodium and potassium were measured (Naghibi et al., 2007). LDH activity was assayed using a commercial kit in which the reaction: Lactate + NAD → Pyruvate + NADH was catalysed by LDH, and the concentration of produced NADH was measured at 340 nm. Urine sodium and potassium concentrations were measured by flame photometer and the obtained results were multiplied to the 24 h urine volume to give the total excreted amount in 24 h.

At day 22 of pregnancy, both kidneys were excised and the right one was homogenized in ice cold isotonic saline for MDA assay. MDA *is* a product of lipid peroxidation and its measurement is done as a marker for oxidative stress. Homogenates of right kidney were centrifuged at 10000 g for 10 min at 4°C. The MDA content was measured by the thiobarbituric acid (TBA) method (as stated below). The results were normalized to gram kidney weight.

Both plasma and tissue MDA concentrations were determined by comparing it to a standard curve of 1, 1, 3, 3 tetra ethoxy propane (TEP). Standard curve was made using serial dilutions of TEP (0, 1, 2, 2.5, 5, 10 μM). The 0.5 ml of supernatant or standard solutions was taken in a test tube and 2 ml of the TBA- trichloroacetic acid (TCA) reagent (0.375 % w/v TBA; 15 % w/v TCA and 0.25 N HCl) solution were added. The mixture was heated in a water bath (90-100°C) for 15 min, cooled in a cold water bath for 10 min. Subsequently, it was centrifuged at 2000 g for 15 min. The absorbance of solution was read spectrophotometrically at 535 nm.

Histological study

The left kidney was removed from each animal and was fixed in 10% neutral formalin solution. The 5 micron sections were stained with hematoxylin and eosin (H&E). The microscopic sections were evaluated with light microscope. Cortex and medulla, including glomeruli, tubules, blood vessels and interstitium were studied. Then, glomerular size, glomerular capillary lumen diameter, swelling of endothelial cells, number of red blood cells (RBCs) in the capillary lumen and number of mesangial cells were evaluated.

Statistical analysis

Data were analyzed using unpaired Student's t-test or analyses of variance (ANOVA) followed by a post-hoc Dunnett test, when appropriate. Differences were considered to be statistically significant when *P*-value was less than 0.05. The statistical analysis was done using the SPSS statistical software version 16.

Results

Hydralazine significantly reduced systolic blood pressure in days 13, 15, 18 and 21 of gestation in preeclamptic animals (data not shown). Tempol at doses of 20 and 60 mg/kg also showed this effect in days 15, 18 and 21 of gestation. Tempol at the dose of 180 mg/kg had no effect on blood pressure.

Twenty four h urine protein did not differ between normal and preeclamptic groups before the administration of L-NAME. In days 15 and 20 of gestation, the urine protein was significantly higher (*P*<0.001) in the L-NAME treated group compared to the control group (*P*<0.001, Table 1).

Tempol at 20 and 60 mg/kg doses significantly lowered urine protein in preeclamptic rats (the biggest calculated *P*-value was less than 0.05), so that the excreted protein amount in these groups did not show any significant difference with normal pregnant rats. In contrast, hydralazine and the highest dose of tempol i. e. 180 mg/kg did not lower this parameter significantly. Administration of tempol (60 mg/kg) to normal pregnant rats (no L-NAME) had no effect on 24 h urine protein (Table 1).

Kidney and plasma levels of MDA as indices of oxidative stress were higher in preeclamptic rats (*P*<0.001). Tempol treatment with doses of 20 and 60 mg/kg/day significantly reduced kidney and plasma concentration of MDA, comparable to that of normal

Table 1: Urine protein in days 10 (before initiation of treatments), 15 and 20 of gestation among control and treated groups.

Groups	Urine protein (mg/day) in Day 10 (before initiation of treatments)	Urine protein (mg/day) in Day 15	Urine protein (mg/day) in Day 20
Normal pregnant	3.09 ± 0.18	3.25 ± 0.25[#]	3.66 ± 0.27[†]
L-NAME (50mg/kg/day)	2.97 ± 0.19	5.36 ± 0.56	5.29 ± 0.28
L-NAME + Tempol (20mg/kg/day)	2.96 ± 0.36	3.22 ± 0.39[*]	3.61 ± 0.29[#]
L-NAME + Tempol (60mg/kg/day)	3.54 ± 0.44	3.04 ± 0.43[*]	3.29 ± 0.35[†]
L- NAME + Tempol (180mg/kg/day)	2.68 ± 0.28	4.66 ± 0.62	4.57 ± 0.45
L-NAME + Hydralazine (10mg/kg)	3.76 ± 0.31	4.69 ± 0.32	5.73 ± 0.29

Data represents mean ± SEM, (N= 10-15).
[*] $P<0.05$; [#] $P<0.01$; [†] $P<0.001$ compared to preeclamptic (L-NAME treated) group.

group. Tempol 180 mg/kg/day had no significant effect on this parameter (Fig. 1). Hydralzine just lowered plasma MDA level significantly, and its effect was less than those of tempol 20 and 60 mg/kg/day ($P<0.001$). The plasma and kidney levels of MDA of tempol (60 mg/kg/day)–treated pregnant rats (no L-NAME) showed no significant difference with normal pregnant rats, and were significantly lower than those of preeclamptic rats ($P<0.001$).

Urine sodium, potassium, LDH, plasma creatinine and creatinine clearance did not show any significant differences among groups (Table 2).

The initial body weight (day 11 of pregnancy) and final body weight (day 22 of pregnancy) are shown in Table 3. As it can be seen, preeclamptic rats that had not received any treatment, tempol 180 mg/kg and hydralazine 10 mg/kg gained about 10% lower weight during pregnancy. The final body weight) of other groups were significantly greater than the preeclamptic control group and showed no statistically difference with that of normal pregnant rats (Table 3).

There were marked pathological changes in kidney glomeruli of L-NAME- treated animals (Fig. 2). In preeclamptic animals, endothelial cell swelling and about 10-15% increase in the size of glomeruli, narrowing of the lumen in about 75% of glomerular capillaries, lack of RBCs in about 50% of capillaries and increase of the number of mesangial cells in some part of glomeruli were observed. In the normal

group, the capillary lumen was patent and the normal circulating RBCs were present. Preeclamptic animals treated with tempol at dose of 20 mg/kg/day showed normal glomeruli size and mesangial cells number. Also, some capillaries had normal lumen size and RBCs were present in some of them. The dose of 60 mg/kg/day restored the light microscopic findings almost to the normal situation (Fig. 2). Tempol at high dose (180 mg/kg/day) and hydralazine had no remarkable effect on glomerular changes and the observation of a few RBCs in capillaries was the only difference with that of preeclamptic animals (Fig. 2).

Discussion

During pregnancy, preeclampsia is a threatening disorder and is a leading cause of maternal and fetal morbidity and mortality. This disorder is characterized by hypertension, proteinuria and edema. The etiology of preeclampsia is unclear, but it seems that ROS have an important role in preeclampsia development (Mathai, 1996; Harmon et al., 2016). In our previous study, tempol at mild to moderate doses (20 and 60 mg/kg/day) showed favorable effects on systolic blood pressure and vascular responsiveness of preeclamptic rats (Talebianpoor and Mirkhani, 2012). In the present study, the effect of tempol on renal complications was investigated in experimental preeclampsia rat model. Moreover, the effect of hydralazine, as a reference drug, was studied and
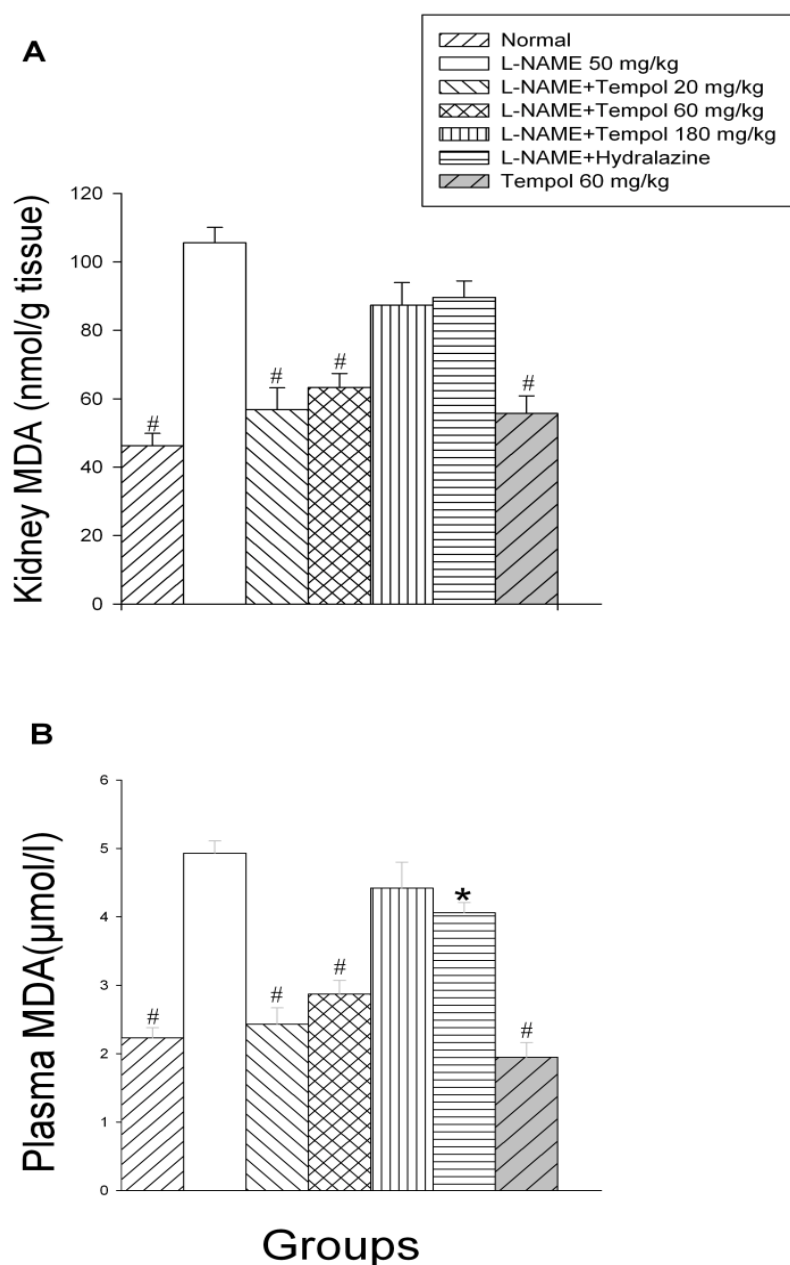
Fig.1. Concentration of MDA in kidney (A) and plasma (B) of normal pregnant rats, preeclamptic rats (received L-NAME), preeclamptic rats received tempol (20, 60 and 180 mg/kg/day) and hydralazine (10 mg/kg/day), and normal pregnant rats received tempol (60 mg/kg/day). Symbols represent means ± SEM of 10-15 experiments on gestational day 20. $^*P<0.01$, $^\#P<0.001$ in comparison to preeclamptic group.

compared with that of tempol.

Preeclampsia was induced through oral administration of L-NAME on the day 10 of gestation till the last day of pregnancy. It is worth mentioning that no animal model including L-NAME administration can mimics all aspects of preeclampsia in human (Sones and Davisson, 2016). However, L-NAME administration resulted in hypertension and proteinuria, two hallmark symptoms of human preeclampsia, suggesting that it was a useful model (Table 1). L-NAME administration was also associated with increased levels of plasma and kidney MDA, which is an indicator of oxidative stress (Fig. 1). In accordance with this functional/ biochemical changes, glomerular endothelial cell swelling, narrowing of capillary lumen, decrease number of RBCs in the capillary lumen and mesangial cell proliferation were seen in histological studies on preeclamptic animals (Fig. 2). However, it seems that the deleterious effect of L-NAME administration on kidney was confined to glomeruli of pregnant animals and no evidence of tubular injury (LDH level, excreted sodium and potassium) was observed (Table 2). In human, serum LDH level

Table 2: Effect of tempol and hydralazine on serum creatinine, creatinine clearance, serum urea, lactate dehydrogenase activity, amount of sodium and potassium in urine in different groups.

Groups	Serum creatinine (mg/dl)	Creatinine clearance (ml/minute)	Serum urea (mg/dl)	LDH (IU/gr urine creatinine)	Amount of excreted sodium (meq/24h)	Amount of excreted potassium (meq/24h)
Normal pregnant	0.67 ± 0.09	0.35 ± 0.07	47.02 ± 3.45*	35.29 ± 7.51	0.43 ± 0.05	1.74 ± 0.22
L-NAME (50mg/kg/day)	0.56 ± 0.06	0.38 ± 0.05	57.35 ± 2.69	43.85 ± 5.11	0.37 ± 0.04	2.03 ± 0.16
L-NAME + Tempol (20mg/kg/day)	0.62 ± 0.09	0.35 ± 0.08	50.51 ± 3.67	54.12 ± 6.76	0.43 ± 0.05	1.84 ± 0.20
L-NAME + Tempol (60mg/kg/day)	0.69 ± 0.19	0.47 ± 0.11	50.11 ± 3.67	54.37 ± 4.07	0.40 ± 0.05	1.84 ± 0.16
L- NAME + Tempol (180mg/kg/day)	0.60 ± 0.12	0.39 ± 0.10	54.85 ± 7.98	49.43 ± 7.72	0.47 ± 0.04	2.23 ± 0.27
L-NAME + Hydralazine (10mg/kg)	0.47 ± 0.10	0.4 ± 0.14	56.41 ± 3.17	39.4 ± 9.18	0.39 ± 0.07	1.78 ± 0.20

Data represents mean ± SEM, (n= 10-15).

*$P<0.05$ compared to preeclamptic (L-NAME treated) group.
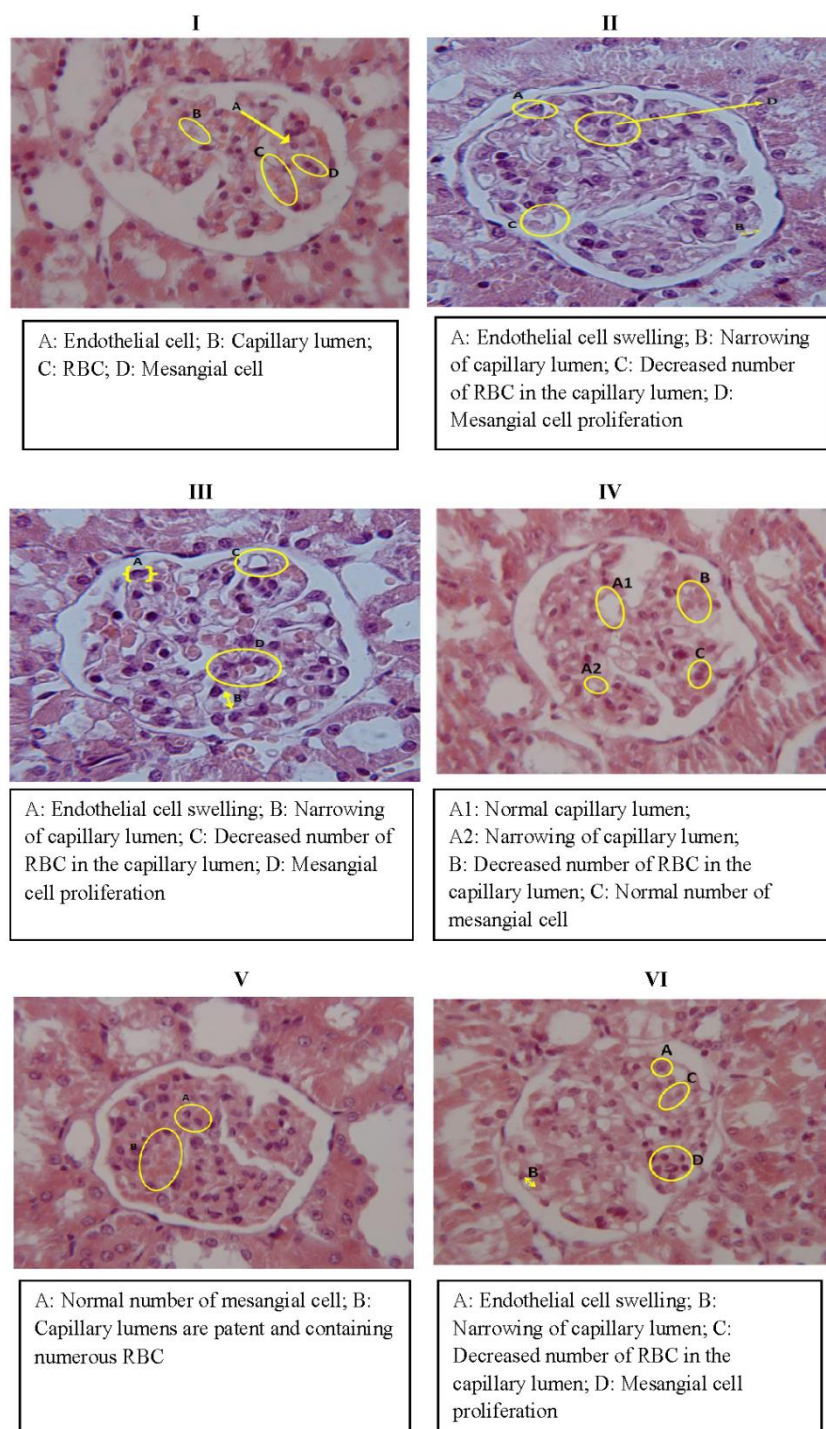
Fig.2. Light microscopy (H & E staining, ×400) of kidney glomeruli in normal group (I), preeclamptic group (II), preeclamptic rats treated with hydralazine 10 mg/kg/day (III), preeclamptic rats treated with tempol 20 mg/kg/day (IV), preeclamptic rats treated with tempol 60 mg/kg/day(V), preeclamptic rats treated with tempol 180 mg/kg/day (V).

correlated with the severity of preeclampsia (Dave et al., 2016), but renal tubular injury is not usually observed unless the acute renal failure complicates the syndrome.

Both hydralazine and tempol at doses of 20 and 60 mg/kg significantly reduced preeclamptic rats' systolic blood pressure. The beneficial effect of hydralazine on blood pressure was produced earlier than tempol. These observations were compatible with the proposed mechanisms of these two agents, namely, direct vasodilation vs. potent antioxidant action, respectively.

In addition to its favorable effect on blood pressure, tempol at doses of 20 and 60 mg/kg/day significantly

Table 3: Weight of pregnant rats at Day 11 and 22 of pregnancy.

Groups	Body weight at 11 days of pregnancy	Body weight at 22 days of pregnancy
Normal pregnant	277 ± 5	353 ± 3[#]
L-NAME (50mg/kg/day)	273 ± 5	320 ± 5
L-NAME + Tempol (20mg/kg/day)	266 ± 5	343 ± 5[*]
L-NAME + Tempol (60mg/kg/day)	270 ± 3	345 ± 5[#]
L- NAME + Tempol (180mg/kg/day)	265 ± 4	314 ± 6
L-NAME + Hydralazine (10mg/kg)	260 ± 3	312 ± 5

Data represents mean ± SEM, (n= 10-15).
[*] $P<0.05$; [#] $P<0.01$ compared to preeclamptic (L-NAME treated) group.

reduced proteinuria, plasma and kidney MDA levels, comparable to that of normal pregnant rats. Indeed, increased and decreased kidney MDA was similar with proteinuria in different groups (Fig. 1, Table 1). These two administered doses, especially 60 mg/kg/day, also prevented the deleterious effects of L-NAME on renal glomeruli (Fig. 2). In contrast to tempol, hydralazine did not show significant effect on proteinuria (Fig. 1, Table 1), an observation that can be seen in human preeclampsia, which is the main weakness of hydralazine (Mathai, 1996). These findings indicated that the oxidative stress has a greater role in comparison with glomerular hypertension in glomerular injury seen in experimental preeclampsia. Tanir et al. (2005) tested the antihypertensive and renoprotective effects of Quercetine in preeclamptic animals and showed that quercetine as an antioxidant could improve proteinuria with no effect on blood pressure. Shibata et al. reported that even though hydralazine normalized blood pressure in aldosterone-induced renal injury in rats, but it had no effect on proteinuria and oxidative stress (Shibata et al., 2007). It is also reported that oxidative stress is very important in diabetic nephropathy and hydralazine has no effect on glomerular injuries in this disorder (Yoshida et al., 2009). In contrast, Tang et al. (1997) showed that antihypertensive drugs such as hydralazine could improve proteinuria in diabetic rats by controlling blood pressure.

A numerous studies have proposed that oxidative stress has a critical role in the L-NAME-induced nephrotoxicity that could be prevented by some antioxidants (Tanir et al., 2005). Tempol is a potent antioxidant with low molecular weight that has excellent cell permeability (Teke et al., 2008). In addition, it was reported that tempol has protective effects on rat renal injuries induced by renal ischemia-reperfusion, gentamicin and endotoxic shock (Leach et al., 1998; Chatterjee et al., 2000; Karataş et al., 2004). Also, tempol has renoprotective effect in a genetic mouse model of preeclampsia (Mathai, 1996). Hence, in our study the obtained results of the tempol effects on glomerular injury are acceptable and consistent with other reports (Sharma et al., 2005; Datta et al., 2006; Hoffmann et al., 2008; Knight et al., 2010; Sverrisson et al., 2014). To best of our knowledge, despite these positive experimental results no controlled clinical trial has tested the effect of antioxidants in pregnant women with preeclampsia, yet. One possible reason that no study has been designed can be due to uncertainty about the safety of therapeutic doses of antioxidants on the fetus health.

The high dose of tempol 180 mg/kg/day did not exhibit improvement in the measured parameters. The same pattern of dose-dependent action of tempol was seen in our previous study on vascular tissue of preeclamptic animals (Talebianpoor and Mirkhani, 2012). Tempol as a SOD-mimetic agent changes the

highly reactive O_2^- to H_2O_2. Catalase as an endogen antioxidant converts H_2O_2 into H_2O. It seems that increased renal formation of H_2O_2 by tempol at high dose counteracts renoprotective and antihypertensive effects, which may intensify proteinuria (Ardanaz et al., 2008; Rafikova et al., 2008; García-Redondo et al., 2009). Similar results have been reported by Lu et al. (2010).

It is worth mentioning that plasma creatinine level and creatinine clearance did not show any significant differences among studied groups, implying that in the applied model the renal blood flow did not change significantly.

Studying the initial body weight and final body weight revealed that preeclamptic animals receiving tempol 20 and 60 mg/kg gained weight during pregnancy comparable with normal group, while this was about 10 % lower in the other groups (Table 3). At least, part of this effect can be attributed to lower fetuses' weight seen in these groups (data not shown). Hydralazine treated animals had no change in urine excreted sodium and their final body weight was similar to the control preeclamptic group, implying that the drug did not induce sodium and water retention.

While the obtained results in the current study are encouraging, it must be kept in mind that experimental preeclampsia is different from those seen clinically, and it is too early to use this compound and other antioxidants in clinical setting. For the next step, study the teratogenicity of the experimentally effective antioxidants is highly recommended.

Conclusion

Our results showed that tempol at low to moderate doses (20 and 60 mg/kg/day) attenuated renal injuries such as proteinuria, oxidative stress and pathological alterations in the preeclamptic rats. Even though it was also shown that hydralazine reduced blood pressure, it did not produce any favorable effects on the renal outcomes.

Conflict of interest

The authors declare that none of them have any conflicts of interest with the contents of this article.

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