Research Article

Superoxide Dismutase Mimetic Tempol Normalized the Blood Pressure and Renal Functions in L-NAME Induced Hypertension Rats: The Role of Oxidative Stress

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ABSTRACT

Increased oxidative stress has been suggested to be responsible for the development of hypertension and antioxidants are potentially useful therapeutic alternative for this disease. This study was performed to examine the role of superoxide dismutase mimetic-Tempol on hypertension and renal vasoconstriction induced by L-NAME administration. In addition, the participation of α_1-adrenoceptors and angiotensin II type I receptors have also been investigated. 32 male Sprague-Dawley rats were randomly assigned into four different groups (n=8 per group) to receive no treatment, Tempol treated, L-NAME treated and Tempol+L-NAME treated respectively for 21 days. Non-invasive blood pressure, renal functional parameters, acute renal haemodynamics, oxidative stress markers and histology studies were performed during the course of experimental period. L-NAME treatment induced hypertension, renal dysfunction and oxidative stress which were characterized by an increase of systolic blood pressure, impaired renal functional parameters, elevated plasma malondialdehyde levels and attenuated sensitivity to exogenous infused α_1-adrenergic agonists and Angiotensin II. Co-administration of Tempol+L-NAME completely abolished the abnormalities created by L-NAME which can be proven by the decreased of systolic blood pressure, improved renal functional parameters with an enhancement of antioxidant enzymes. Moreover, the magnitude of renal cortical vasoconstrictions to exogenous infused α_1-adrenergic agonists and Angiotensin II has also been ameliorated. The available data suggested that the antioxidative stress activities required the interaction or crosstalk between SOD and NO. Nevertheless, SOD enzyme is the primary O_2^- scavenger compared to NO under the NOS inhibited condition.

Keywords: α_1-adrenoceptors, angiotensin II type I receptors, hypertension, L-NAME, oxidative stress, renal dysfunction, Tempol.

INTRODUCTION

Regardless of the extensive research, widespread patient education and sheer effort on the part of health care professionals, hypertensions still a leading cause of morbidity and mortality that become an important health challenge. Increased oxidative stress has been suggested to be responsible for the development of hypertension[1]. A growing body of evidence indicates that reactive oxygen species especially superoxide anion (O_2^-) play a vital role in the pathogenesis of various patterns of hypertension and renal dysfunction[2]. Experimental models such as spontaneously hypertensive rats, deoxycorticosterone acetate salt hypertensive rats, dahl salt-sensitive rats, and angiotensin II-dependent hypertensive rats, have been shown to have elevated superoxide anion products and levels in aortic vessels which directly increase the influence of superoxide anion on arterial blood pressure and renal haemodynamics[3].

Over the past decades, numerous studies had demonstrated a wide range of biological functions served by nitric oxide which can be synthesized endogenously from the L-arginine amino acid by the action of the nitric oxide synthase enzyme. It is a very short-lived biologically active gas and free radicals that can exert a wide range of physiological actions that has multiple protective effects for regulating the cardiovascular and renal systems which include anti-inflammatory effects, anti-hypertrophic activity, most importantly in the regulation of endothelium-dependent relaxation[4]. Acute and chronic inhibition of nitric oxide synthesis induced hypertension and renal dysfunction[5,6]. Several lines of evidence have also stated that nitric oxide plays a vital role in the central nervous system and modulating the cardio-circulatory function via sympathoinhibitory effect[7]. Therefore, nitric oxide synthase inhibition may results the activation of sympathetic activity that eventually leads to the elevation of arterial pressure. Similarly, it has also been noted that nitric oxide levels are diminished in hypertensive human subjects[8]. Collectively, all these evidences providing support for the notion that derangement of nitric oxide level plays a crucial role in the pathogenesis of essential hypertension.

It is generally accepted that among the several types of hypertension, there will be a transition from acute phase to chronic stage. Usually, overshoot of angiotensin levels with a fast increase in peripheral resistance and systemic arterial blood pressure have been observed during the pathogenesis of hypertension. Moreover, adrenergic responsiveness also plays an important role in such

condition[9]. During the pathophysiological conditions, there is a shift in the functional contribution of α_1-adrenoceptor subtypes in certain vascular beds. Moreover, there could be an enhancement of adrenergically induced vasoconstriction which could be mediated by α_1- adrenoceptor[10].

In the present investigation, L-NAME induced hypertension is of particular interest. In addition, the participation or interaction between α_1-adrenoceptors and angiotensin II type I (AT_1) receptors during L-NAME induced oxidative stress condition will be further addressed. Although many clinical studies have shown those vitamins and other antioxidants possess blood pressuring lowering effects in human hypertensive subjects[11]. However till now, not much investigations have been undertaken to examine whether superoxide dismutase mimetic-Tempol could mediate the changes in blood pressure. Therefore, the effect of Tempol on L-NAME induced changes in blood pressure and renal functions will be investigated in this experiment. We hypothesized that long term administration of Tempol could ameliorate oxidative stress and inflammation caused by nitric oxide inhibition and may thereby, retard the deterioration of renal functions and blood pressure.

MATERIALS AND METHODS

Animals

Thirty two male Sprague-Dawley (SD) rats (200-250g) were procured from the Central Animal Facility of University Sains Malaysia, Penang, Malaysia. The care and use of the laboratory animals were performed in accordance with the guidelines and under the approval from the Animal Ethics Committee of the university (PPSG/07(A)/044/2010/57/187). Animals were provided with commercial rat chow (Gold Coin Sdn. Bhd., Penang, Malaysia) and clean tap water *ad-libitum*. Animals were housed in the animal care facility center (temperature, 25ºC, humidity, 60-70%) with a 12h:12h day light dark cycle[12]. All animals were randomly assigned into four different groups (n=8 per group) as following: SD control (SD-C), SD with Tempol treated (SD-T), SD with L-NAME treated (SD-L), and SD with Tempol + L-NAME treated (SD-TL) respectively.

Drug preparation and induction of nitric oxide deficiency

Tempol (Sigma-Aldrich, Steinheim, Germany) and L-NAME (Sigma-Aldrich, St. Louis, MO, USA) which were prepared at a dose of 30mg/kg/day and 15mg/kg/day respectively, dissolving in distilled water. Drugs were given to the animals via drinking water for 21 days.

Metabolic and renal functional studies

Metabolic data were collected weekly starting from day 0 of the experiment using specific metabolic cages (Nalgene®, Thermo Scientific, Philadelphia, USA) by which the animals were kept for 24 hours. Body weight, water intake and urine output was recorded. Blood and urine samples were collected and centrifuged at 3500 rpm for 10 minutes before being stored at -30ºC for further biochemical analysis. Creatinine clearance (CrCl), fractional sodium excretion (FE_{Na+}) and urinary protein excretion (UPE) was calculated using standard equations[13]. Biochemical analysis for creatinine and urinary protein was performed using spectrophotometric method (Power Wave X340, Bio.Tek Instrument Inc., USA) while sodium and potassium was measured using flame photometry method (Jenway, PFP-7, England, UK).

Conscious blood pressure measurement

Non-invasive blood pressure and heart rate (HR) for conscious rats were measured weekly using tail cuff plethysmography (NIBP Controller, AD Instruments®, Sydney, Australia) to obtain the systolic (SBP), diastolic (DBP) and mean arterial blood pressure (MAP) respectively. The rats were trained at least twice before starting the actual measurements by putting them into the restrainer and leave for 30 minutes. Special care was made to lessen the stress of the animals while in the restrainer. The blood pressure recording procedure was performed at the same time of the day in a dark and calm room which was specially prepared for this purpose. A total of ten consecutive readings were selected for each rat and average values were calculated.

Heamodynamic Study

General preparation and surgical procedure

The surgical procedure was performed according to an established procedure from this laboratory[13,14]. All treated SD rats were subjected to overnight fasting but were allowed free access to drinking water. Before the surgery, rats were anesthetized with 60 mg/kg sodium pentobarbitone (Nembutal®, Ceva, Santé Animale, Libourne, France) intraperitoneally. Tracheostomy was performed to provide clear air passage throughout the entire experiment. The left jugular vein was cannulated (PE 50, Portex, Kent, UK) for the delivery of supplemental anesthesia (15 mg/kg/ml) when necessary. Following this, the left carotid artery was cannulated (PE 50, Portex, Kent, UK) to allow continuous blood pressure recording via a pressure transducer (P23 ID Gould, Statham Instrument, London, UK) which was connected to a computerized data acquisition system (PowerLab®, AD Instrumentation, Sydney, Australia). A midline incision in the abdominal region was performed to expose the left kidney and iliac artery for cannulation (PE 50, Portex, Kent, UK) to the level of the bifurcation area close to renal artery such that its beveled tip faced to the entrance of the renal artery to ensure that exogenous infused drugs are directly entered into the left kidney. A continuous infusion of saline (9g/L NaCl) at a rate of 6 ml/kg/h using a perfusion pump (PerfusorSecura FT 50 ml, B. Braun Medical AG, Sempach, Switzerland) was operated as the vehicle to deliver the infused drugs into the kidney.

Biochemical analysis for oxidative stress markers

After the above procedure, the animals were allowed to stabilize for 15 minutes and then blood sample (1 ml) was withdrawn via the cannula from carotid artery and was centrifuged at 4500 rpm for 10 minutes to obtain plasma. Plasma sample was then stored at -30°C for the further analysis of oxidative stress marker such as thiobarbituric acid reactive substances-malondialdehyde (TBARS-MDA), total superoxide dismutase (T-SOD), nitric oxide (NO) and total anti-oxidative stress capacity (T-AOC). All these kits were procured from NJJC Bio Inc., Nanjing, China. Immediately after plasma was withdrawn, the precipitated cells were resuspended in 0.5 ml of saline and reinfused intravenously to maintain the blood volume. Rats were further stabilized for another 30 minutes before the commencement of acute vasoconstriction study.

Acute vasoconstriction experiment

Acute renal vasoconstriction study was carried out using vasoactive agents to investigate the differences in the participation of α_1-adrenergic receptor and angiotensin II type I (AT_1) receptor during the pathological state and following the administration of noradrenaline (NA; 25, 50, 100 and 200 ng), phenylephrine (PE; 0.25, 0.5, 1 and 2 µg), methoxamine (ME; 0.5, 1, 2 and 4 µg) and angiotensin II (Ang II; 25, 50, 100 and 200 ng) which were prepared in saline as stock solutions and stored at 4°C. The total volume injected into the kidney was limited to 0.1 ml to ensure no spillage into the systemic circulation. NA and PE are non-selective α_1–adrenoceptor agonists whereas ME is a selective α_{1A}–adrenoceptor agonist. All agonists were administered in ascending and descending manner to obtain two responses. Then, 10 minutes washout period was given between each infusion to allow washout after each agonist[13,14]. A laser Doppler flow probe probe (OxyFlow, ADInstruments, Australia) was then placed on the left kidney surface for renal cortical blood perfusion (RCBP) measurement. The probe was linked to a laser Doppler flow meter (ADInstruments, Australia) which was directly connected with the data acquisition system (PowerLab®, ADInstruments, Sydney, Australia) using Hewlett Packard Centrino core 2 duo computerized system with windows XP operating system.

Histology study

Upon completion of the acute vasoconstriction studies, the animals were euthanized with over dose of anesthesia (Nembutal®, Ceva, Santé Animale, Libourne, France) and disposed humanely. The contralateral kidney which was not exposed to any agonists was carefully isolated from the surrounding adipose and connective tissues. Then, the kidney was excised and blotted dry on a piece of laboratory filter paper (Kottermann–Iprolab, Sdn. Bhd., Malaysia) and preserved in 10% formalin for histological examination. The kidney weight was measured to calculate the kidney index using the following standard equation (kidney weight/body weight x 100)[13].

Data analysis

Data obtained from metabolic, functional, oxidative stress markers and blood pressure were analyzed with repeated measure one-way ANOVA followed by *Bonferroni post hoc* test. The data obtained from acute vasoconstriction studies was determined by the change in RCBP and was expressed as percentage of the baseline value. Baseline RCBP was also analyzed with repeated measure one-way ANOVA followed by *Bonferroni post hoc* test. The response in RCBP to exogenous agonists was taken as the mean difference between the baseline values for both ascending and descending doses. Statistical analysis of the renal vasoconstrictor experiments were done by two-way ANOVA followed by *Bonferroni post hoc* test. All data were presented as mean±S.E.M. The differences between the mean were considered significant at 5% level. The statistical analysis was done using GraphPad Prism version 5.01 (GraphPad Software Inc, San Diego, California, USA).

RESULTS

Effect of Tempol on metabolic and conscious blood pressure parameters

Data of metabolic and non-invasive blood pressure were presented in (Table 1). Animals that were subjected to different treatment had steady body weight gain throughout study period, only SD-C and SD-T animals showed significant ($P<0.05$) body weight gained at the end of treatment period as compared to day 0. In term of water intake, SD-L animals experienced significant ($P<0.05$) reduction of water intake started from day 14 onwards. However, the rest of the experimental groups have no significant change in their water intake behavior. There was no significant difference in the urine output parameter during the entire study period in all treatment groups. L-NAME treatment for 21 days significantly ($P<0.05$) increased the systolic, diastolic and mean arterial blood pressure. This phenomenon was started in the early day 7 of the study period. L-NAME treated animals experienced significant ($P<0.05$) decrease of heart rate started from day 14 onwards. Treatment with Tempol alone has no effect on blood pressure or heart rate. However, co-administration of Tempol+L-NAME significantly ($P<0.05$) ameliorate the anomalous conscious blood pressure and heart rate parameters effectively at the end of the treatment period.

Effect of Tempol on renal functional and baseline renal haemodynamic parameters

Changes in baseline renal functional and haemodynamic parameters were presented in (Table 2). NO blockade via L-NAME in the drinking water for continuous 21 days caused a significant ($P<0.05$) reduction in creatinine clearance started from day 7 onwards. Animals treated with Tempol experienced significant ($P<0.05$) increased in creatinine clearance. However, animals co-treated with Tempol+L-NAME has normal creatinine clearance such that manifested in control animals. In addition, 21 days of

continuous L-NAME blockade caused a significant (P<0.05) increase of urinary protein excretion started from day 14 onwards associated with a significant (P<0.05) increase of urinary protein to urinary creatinine ratio. However, animals treated with Tempol alone did not experience any change in these parameters. Concurrent administration of Tempol+L-NAME was able to prevent the deterioration of urinary protein excretion and ameliorate urinary protein to creatinine ratio indexes. The fractional excretion of sodium and urinary sodium to urinary potassium ratio parameters were not affected in control and Tempol treated animals. In contrary, L-NAME treated animals had significant (P<0.05) reduction of fractional excretion of sodium started from day 7 onwards. Similar observation was also manifested in urinary sodium to potassium ratio which started to onset from day 14 onwards. Co-administration of Tempol+L-NAME significantly (P<0.05) increased the fractional excretion of sodium which in turn normalized the urinary sodium to potassium ratio significantly (P<0.05). There was a significant (P<0.05) increase of kidney index observed in L-NAME treated animals but not in control or Tempol treated animals. However, Tempol administration together with L-NAME was able to normalized the kidney index significantly (P<0.05). As expected, the baseline renal cortical blood perfusion was not significantly different between the control and Tempol treated animals. However, the baseline renal cortical blood perfusion was significantly (P<0.05) reduced as compared to control and Tempol treated animals by approximately 35%. Interestingly, co-treatment of Tempol+L-NAME was able to restore the baseline renal cortical blood perfusion significantly (P<0.05) by approximately 24% with respect to their L-NAME treated animals.

Table 1: Metabolic and non-invasive blood pressure parameters of SD-C, SD-T, SD-L and SD-TL groups

Parameter	Group	n	Data				Overall
			0	7	14	21	
Body Weight (g)	SD-C	8	221±8	238±7	251±8	261±8*	
	SD-T	8	215±5	238±3	253±8	261±6*	
	SD-L	8	217±3	220±7	222±10	240±8	
	SD-TL	8	214±3	232±4	232±5	238±4	
Water Intake (ml)	SD-C	8	36±1	33±2	34±2	35±2	
	SD-T	8	33±1	33±2	37±2	38±2	
	SD-L	8	35±3	26±2*	27±1*	26±2*	S
	SD-TL	8	34±2	36±3	31±2	33±3	
Urine Output (ml)	SD-C	8	12±1	14±1	15±1	16±1	
	SD-T	8	14±1	15±1	16±1	16±1	
	SD-L	8	15±1	12±1	11±0	12±1	
	SD-TL	8	15±1	15±1	16±1	17±1	
SBP (mmHg)	SD-C	8	118±5	117±2	117±2	116±2	
	SD-T	8	106±1	106±2	108±2	107±2	
	SD-L	8	111±2	133±2*	145±4*	154±1*	S L
	SD-TL	8	107±2	120±3	123±1	123±0	
DBP (mmHg)	SD-C	8	90±2	96±1	97±3	93±3	
	SD-T	8	85±2	90±2	90±2	90±1	
	SD-L	8	96±3	112±2	125±3*	135±2*	S L
	SD-TL	8	88±1	94±2	100±3	100±3	
MAP (mmHg)	SD-C	8	100±2	103±1	104±3	101±2	
	SD-T	8	92±2	95±2	96±1	96±1	
	SD-L	8	98±2	119±2*	132±3*	141±1*	S L
	SD-TL	8	94±1	103±2	107±2	108±1	
Heart Rate (BPM)	SD-C	8	369±10	367±4	368±17	370±8	
	SD-T	8	369±5	371±4	367±5	369±4	
	SD-L	8	364±5	344±4	337±2*	325±4*	S L
	SD-TL	8	369±3	363±9	359±3	366±7	

Data were presented as mean±S.E.M (n=8) and were analyzed by repeated measure one-way ANOVA followed by *Bonferroni post hoc* test. "*" indicates significant difference (P<0.05) compared to Day 0. "S" indicates significant difference (P<0.05) compared to SD-C group on Day 21. "L" indicates significant difference (P<0.05) between SD-L to SD-TL group respectively. Comparison was performed among Day 21 only.

Table 2: Renal functional and baseline haemodynamic parameters of SD-C, SD-T, SD-L and SD-TL groups

Parameter	Group	n	Day				Overall
			0	7	14	21	
CrCl (ml/min/kg)	SD-C	8	1.28±0.14	1.49±0.54	1.46±0.46	1.34±0.15	
	SD-T	8	1.38±0.24	2.37±0.50*	2.23±0.25*	2.26±0.38*	S
	SD-L	8	1.55±0.27	0.73±0.37*	0.62±0.06*	0.83±0.09*	S L
	SD-TL	8	1.62±0.20	1.73±0.21	2.01±0.20	1.95±0.16	
UPE (mg/kg/day)	SD-C	8	27.92±0.53	27.08±0.49	28.25±0.50	25.83±0.48	
	SD-T	8	26.26±0.49	26.43±0.48	26.19±0.50	26.69±0.52	
	SD-L	8	27.63±0.51	27.06±0.53	34.13±0.71*	37.01±0.77*	S L
	SD-TL	8	27.77±0.50	27.14±0.42	27.20±0.40	28.87±0.41	
FE_{Na+} (%)	SD-C	8	0.69±0.11	0.67±0.09	0.64±0.09	0.67±0.04	
	SD-T	8	0.63±0.09	0.65±0.29	0.60±0.26	0.64±0.10	
	SD-L	8	0.63±0.08	0.30±0.03*	0.30±0.04*	0.22±0.02*	S L
	SD-TL	8	0.62±0.07	0.57±0.05	0.50±0.06	0.48±0.04	
Upr : Ucr ratio	SD-C	8	0.51±0.05	0.59±0.09	0.51±0.05	0.52±0.10	
	SD-T	8	0.49±0.07	0.48±0.02	0.46±0.05	0.46±0.01	
	SD-L	8	0.46±0.04	0.57±0.04	0.60±0.05*	0.61±0.03*	S L
	SD-TL	8	0.45±0.03	0.41±0.03	0.42±0.03	0.41±0.03	
$U_{Na}^{+} : U_{K}^{+}$ ratio	SD-C	8	0.24±0.01	0.27±0.01	0.27±0.02	0.23±0.01	
	SD-T	8	0.24±0.02	0.25±0.02	0.23±0.01	0.22±0.01	
	SD-L	8	0.28±0.01	0.23±0.01	0.20±0.01*	0.19±0.01*	S
	SD-TL	8	0.24±0.00	0.27±0.01	0.26±0.06	0.24±0.01	
Kidney Index (%)	SD-C	8	-	-	-	0.33±0.01	
	SD-T	8	-	-	-	0.35±0.01	
	SD-L	8	-	-	-	0.42±0.01	SL
	SD-TL	8	-	-	-	0.33±0.01	
Baseline RCBP (BPU)	SD-C	8	-	-	-	270±3.81	
	SD-T	8	-	-	-	263±5.25	T
	SD-L	8	-	-	-	176±4.81	S L
	SD-TL	8	-	-	-	240±1.92	S

Data were presented as mean±S.E.M (n=8) and were analyzed by repeated measure one-way ANOVA followed by *Bonferroni post hoc* test. "*" indicates significant difference (P<0.05) compared to Day 0. "S" indicates significant difference (P<0.05) compared to SD-C group on Day 21. "T" indicates significant difference (P<0.05) between SD-T to SD-TL group. "L" indicates significant difference (P<0.05) between SD-L to SD-TL group respectively. Comparison was performed among Day 21 only.

Effect of Tempol on renal cortical vasoconstrictor response

Intra-renal bolus injection of exogenous noradrenaline, phenylephrine, methoxamine and angiotensin II had produced dose-dependent renal cortical vasoconstrictions in all the experimental groups which can be observed from the dose response curves showed in(Figure 1A), (Figure 1B), (Figure 1C) and (Figure 1D) respectively. The overall percentage drop of renal cortical vasoconstriction was presented in (Figure 1E).

Noradrenaline

The magnitude of renal cortical vasoconstriction in response to exogenous infused noradrenaline in Tempol treated animals had no significant difference as compared to their control counterparts. However, L-NAME treated animals experienced an attenuated renal cortical vasoconstriction significantly (P<0.05) as compared to control animals. Concurrent treatment of Tempol+L-NAME was able to recover the magnitude of renal cortical vasoconstriction significantly (P<0.05) as compared to L-NAME treated animals.

Phenylephrine

In subsequent phase of the experiment, the magnitude of renal vasoconstrictor responses induced by phenylephrine in all the experimental animals showed similar pattern as in noradrenaline phase. There was no significant difference in renal cortical vasoconstriction between control and Tempol treated animals but L-NAME treated animals had significant (P<0.05) lower renal cortical vasoconstriction as compared to control animals. Co-administration of Tempol+L-NAME was able to improve the sensitivity of the kidney to exogenous infused phenylephrine significantly (P<0.05).

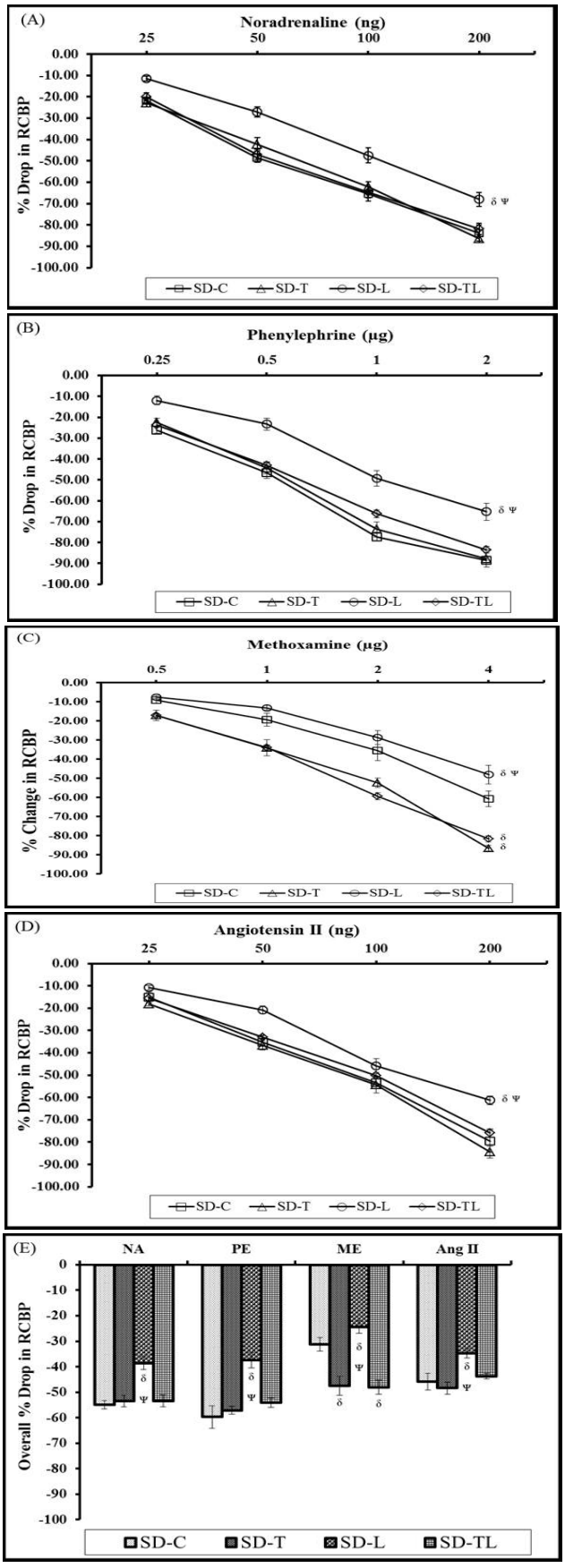

Figure 1: Line graph showed the dose response curve of the acute renal vasoconstriction studies to graded doses of (A) Noradrenaline, (B) Phenylephrine, (C) Methoxamine (D) Angiotensin II and (E) Overall % drop RCBP in all treatment groups (n=8). Values were showed in mean±S.E.M. "δ" indicates significant difference (P<0.05) compared to SD-C group. "ψ" indicates significant difference (P<0.05) between SD-L to SD-TL group.

Methoxamine

The magnitude of renal cortical vasoconstriction in response to methoxamine was significantly (P<0.05) higher in Tempol treated animals as compared to control animals. In contrary, L-NAME treated animals had attenuated renal cortical vasoconstriction by approximately 36% significantly (P<0.05) as compared to control animals. However, this situation was improved significantly (P<0.05) in animals co-treated with Tempol+L-NAME.

Angiotensin II

As shown in adrenergic agonists section, the renal cortical vasoconstrictor response due to exogenous infused angiotensin II in Tempol treated animals had no significant (P>0.05) difference as compared to control animals. In contrary, the magnitude of the renal vasoconstriction in the L-NAME treated animals induced by angiotensin II was significantly (P<0.05) lower as compared to control animals. Interestingly, co-administration of Tempol+L-NAME was able to ameliorate the response to exogenous infused angiotensin II significantly (P<0.05) as compared to those animals treated with L-NAME alone.

Effect of Tempol on oxidative stress parameters

As depicted in (Figure 2A), the plasma MDA levels in Tempol treated animals was no significantly different (P>0.05) as compared to control animals. In opposite, the plasma MDA level in L-NAME treated animals was significantly (P<0.05) higher as compared to control counterparts. Concurrent administration of Tempol+L-NAME was able to lower the plasma MDA level significantly (P<0.05) as compared to L-NAME treated animals but was not returnable to the similar level as shown in control and Tempol treated animals.

The result of plasma T-SOD levels was demonstrated in (Figure 2B). There was no significant difference in the plasma T-SOD levels between Tempol treated and control animals. Animals with chronic L-NAME administration for 21 days had significant (P<0.05) lower plasma T-SOD as compared to control animals. Co-administration of Tempol+L-NAME was able to heighten the plasma T-SOD level significantly (P<0.05) as compared to L-NAME treated animals but the plasma T-SOD level was slightly lower than Tempol treated animals.

The results for plasma NO level was shown in (Figure 2C). The plasma NO level in Tempol treated animals were significantly (p<0.05) higher than control animals. Similar observation has also manifested in animals co-treated with Tempol+L-NAME. In contrary, L-NAME treated animals had significant (P<0.05) lower plasma NO level than control animals. Co-administration of Tempol+L-NAME was able to raise the plasma NO level significantly (P<0.05) where the plasma NO level was higher than L-NAME treated animals but was not returnable to similar level as shown in control and Tempol treated animals.

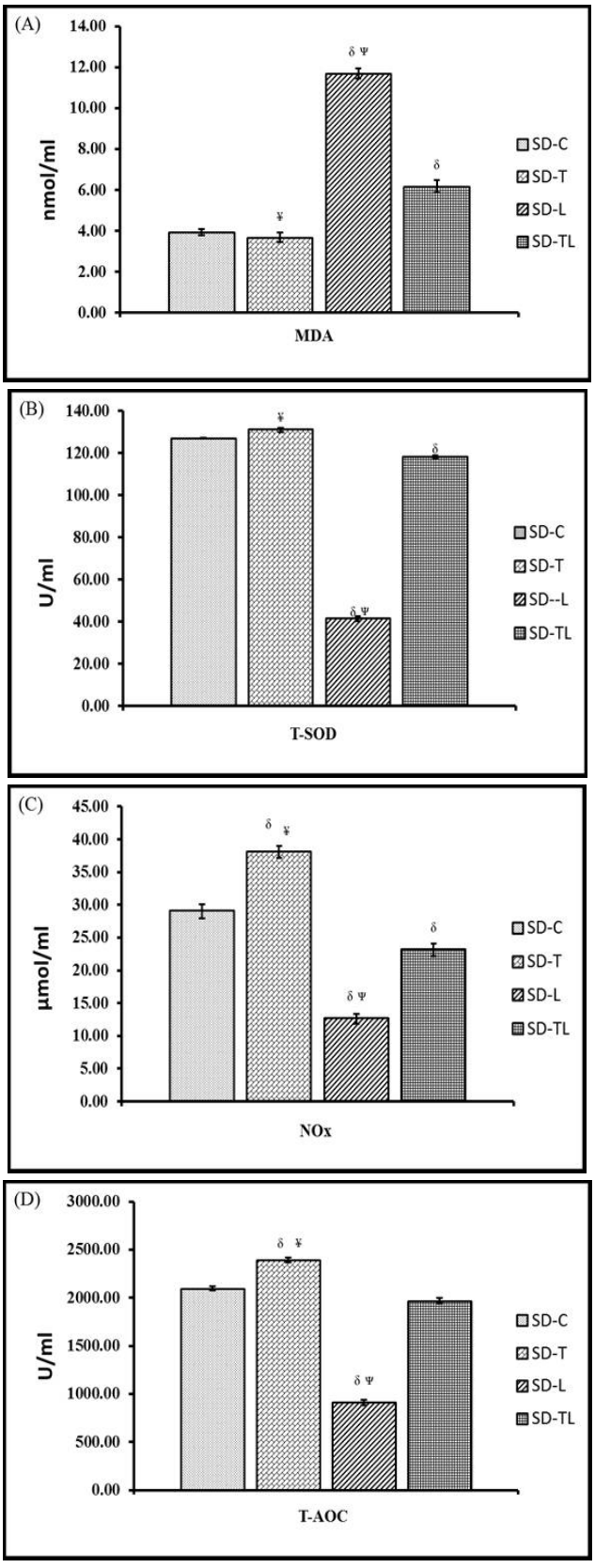

Figure 2: Bar Graph showed the biochemical analysis of oxidative stress markers (A) MDA, (B) T-SOD, (C) NOx and (D) T-AOC. Values were showed in mean±S.E.M (n=8). "δ" indicates significant difference (P<0.05) compared to Cx group. "¥" indicates significant difference (P<0.05) between SD-T to SD-TL group and "ψ" indicates significant difference (P<0.05) between SD-L to SD-TL group respectively.

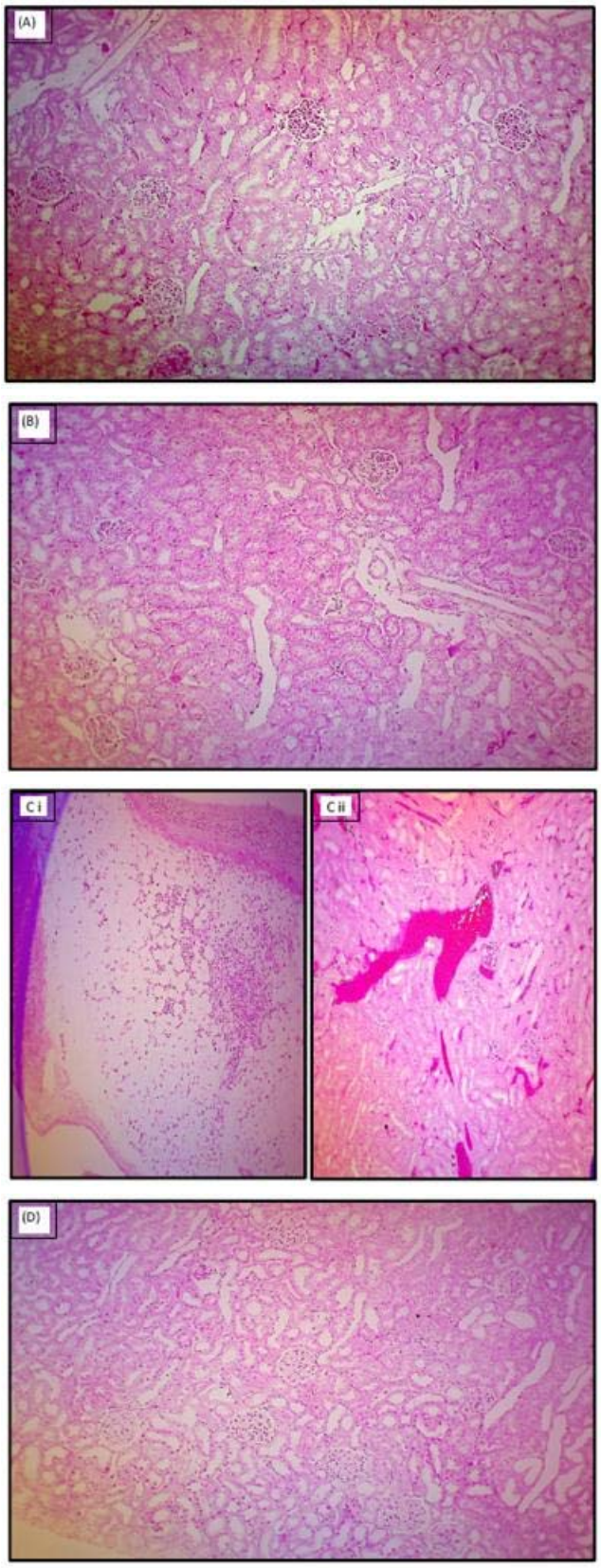

Figure 3: Histopathology studies of rat kidneys. (A) SD-C rat, (B) SD-T rat, (C) SD-L rat and (D) SD-TL rat. SD-L rat treated with L-NAME showed minor protein casts in glomerular region and mild ischaemic damage in tubular area. This picture was captured near to the renal capsular region by which there was a prominent focal chronic inflammation observed in the renal interstitium area (i). There was also a mild arterioles congestion seen in this rat's renal tissue (ii). The rest of the groups had normal architecture on their renal tissues.
(Haematoxylin and eosin stain; original magnification 100x).

The result of T-AOC analysis was depicted in (Figure 2D). Tempol treated animals had significant (P<0.05) higher plasma T-AOC level compared to control animals but animals with L-NAME treated had significant (P<0.05) lower plasma T-AOC level than control animals. Animals co-treated with Tempol+L-NAME did not show any significant difference in plasma T-AOC level as compared to control animals but was able to emanate the plasma T-AOC level significantly (P<0.05) compared to L-NAME treated animals. However, the T-AOC level was slightly lower than Tempol treated animals.

Effect of Tempol on renal histological structure

As depicted in (Figure 3A), control animals showed normal architecture in their renal tissues. Treatment of Tempol to normal animals did not cause any damage on their renal tissues (Figure 3B). However, the renal tissues of L-NAME treated animals experienced minor protein casts with a prominent focal chronic inflammation occurred in the renal interstitium area (Figure 3Ci). In addition, a mild ischaemic arteriolar damage was also observed in tubular area (Figure 3Cii). Co-administration of Tempol+L-NAME was able to prevent the damage of renal tissues caused by the deleterious effect of L-NAME supplementation (Figure 3D).

DISCUSSION

Superoxide anion (O_2^-) and reactive oxygen species are the constant cellular metabolites produced by the living organisms during oxidative stress condition. Indeed, the development of oxidative stress is depending on the balance between their production and degradation. Under normal circumstances, O_2^- is instantly reduced by the superoxide dismutase (SOD) enzyme in living tissues. Besides that, NO, another benign antioxidant free radical can also eliminate the harmful superoxide anion from the tissue, thus helping to maintain minimal level of O_2^- in normal condition and provides a protective function against the action of O_2^-[15]. It is well known that NO plays a pivotal role in the regulation of renal function and long term maintenance of blood pressure. This can be evidenced by the fact that inhibition of intrarenal NO production increased blood pressure. Moreover, it has been suggested that reduced NO was a common denominator of many hypertensive models[16]. Therefore, it seems that the antioxidative stress defense system involved the interaction of SOD and NO. The present studies were undertaken to evaluate the effects of the administration of SOD mimetic-Tempol, on the progression of systemic hypertension and renal dysfunction caused by chronic NO synthase inhibition.

Based on this experiment, Tempol treated animals had significant body weight gained as compared to control animals, although L-NAME and Tempol+L-NAME treated animals showed normal body weight gain but the gaining rate was slower than Tempol and control animals. On the water intake parameter, L-NAME treated animals started to drink less from day 7 onwards and continued till day 21, but this phenomenon was not observed in other experimental animals. Similarly, a reduction of urine output was also observed in L-NAME treated animals as compared to the rest of the animals. These phenomena could be due to the deficiency of NO which indeed enhanced the development of oxidative stress that could indirectly influenced the food intake and drinking behavior on the NO deficient animals. Although the precise reason behind this observation was not delineated, however the similar observation has been reported in cyclosporine A and Gentamicin induced oxidative stress experiment[12,17].

Chronic administration of L-NAME causes a sustained increase of systolic, diastolic and mean arterial blood pressure. Our results are in agreement with those reported by others[18,19]. In present experiment, the scavenging action of O_2^- via chronic administration of Tempol successfully attenuated the elevation of systolic, diastolic and mean arterial blood pressure significantly. Similar observation was also manifested in animals co-treated with Tempol +L-NAME. There are evidences hypothesized that NO plays an important role in the regulation of central nervous system, modulation the cardio-circulatory function by means of a sympathoinhibitory effect. Thus, NO inhibition may lead to the activation of sympathetic activity and an additional increased in arterial blood pressure[20]. Of note, it is also well known in human hypertensive subjects, the NO levels are diminished, thus providing these supports that NO play an important role in the pathogenesis of essential hypertension. It is widely assumed that the mechanism behind the L-NAME induced hypertension involves O_2^- quenching of NO[21,22]. Our finding further validate for the notion that the vasoconstrictor effect of O_2^- due to L-NAME administration is mediated via down-regulation of NO system by which the plasma NO level was depreciated as compared to control animals. Tempol administration in nitric oxide deficient animals was able to restore the plasma NO level significantly as compared to animals treated with L- NAME alone. In fact, this further described that treatment with SOD could metabolized O_2^- that directly improves the basal release of NO in the systemic circulatory system. Moreover, Tempol is a membrane-permeable SOD mimetic that can freely crosses the blood brain barrier. Thus, it is possible that this superoxide scavenger can lower blood pressure via central sympathoinhibition pathway[23].

The baroreflex function in L-NAME induced hypertension has been extensively examined in other studies with regard to the control of heart rate. Some of them have found a decreased in heart rate[24,25]; others have reported either an increase[26]or no changes[27]. Although there were few reports in the literature addressed the heart rate variability. Nevertheless, the results from this study are consistent with those obtained by Robert *et al.*[28] and Ramchandra*et al.*[29], supporting our present findings of L-NAME induced decrease in heart rate. The most offerable explanation is that the L-NAME induced hypertension causes a decreased in central sympathetic activity

through arterial baroreceptors, resulting in decrease of heart rate. Co-administration of Tempol and L-NAME reversed the heart rate changes significantly to the level of control animals.

There are hypothesis indicate that the development of hypertension involves abnormal renal excretory function which is critical for the initiation, development and maintenance of hypertension[6,30]. The kidney regulates the body fluid via homeostatic feedback mechanism, which couples the long term regulation of arterial pressure to extracellular volume such as water and sodium via pressure natriuresis process. Under normal condition, the kidney response to the changes of arterial blood pressure by modulating the urinary sodium and water excretion. This obligatory requirement is crucial for the maintenance of water and sodium balance by the kidney for long term control of arterial pressure. It has been demonstrated that NO blockade induces the occurrence of oxidative stress which increases the chances of renal dysfunction[31]. The above finding was in agreement with our studies by which L-NAME treated animals experienced reduction of urine excretion. In addition to that, reduction of fractional sodium excretion has also been observed. An investigation for the participation of renin-angiotensin system in this L-NAME induced hypertension model has been done via the urinary sodium to potassium ratio. This is a very important renal functional marker for the aldosterone action on the collecting ducts in the pair of kidneys. L-NAME treated animals exhibited a reduced of urinary sodium to potassium ratio started from the early day 14 of the study period, the decreased of this ratio indicated that the activation of renin angiotensin aldosterone activities has taken place, this could be another factor that responsible for the development of hypertension. The progression of renal damage varies widely among experimental models of hypertension, while elevated blood pressure, per se, clearly plays a dominant role in the progression of renal injury in many form of hypertension. Chronic NO synthase inhibition induced renal vasoconstriction[32]. Recently, several studies have suggested that NO is involved in the maintenance of renal blood flow, urinary flow and glomerular filtration rate in rats with acute renal failure[33,34]. Similar observation has also been observed. In our study by which L-NAME treated animals showed significant lower baseline renal cortical blood perfusion. The manifestation of low urine output has signified that reduction of glomerular filtration rate which was represented by the reduction of creatinine clearance. There was an increase of urinary protein excretion found in L-NAME treated animals which indicates that the occurrence of proteinuria has occurred. This situation was further confirmed with the studies of urinary protein to creatinine ratio. Moreover, the augmentation of kidney index has also been observed in these animals. Histological studies on the renal tissues of L-NAME treated animals have exhibited focal inflammation which was represented by the abscess inflammatory macrophages associated with a mild arteriolar congestion. Now, it is well proven both functionally and histologically studies that NO deficiency induced renal organ dysfunction. NO is important for the modulation of vascular tone and the control of renal organ blood flow which plays a major role in the interglomerular dynamics regulation. However, chronic administration of Tempol ameliorated the renal damage and oxidative stress induced by NO synthase deficiency. Tempol treatment also significantly increase the creatinine clearance, fractional sodium excretion and urinary protein excretion parameters. This therapeutic effect could be due to the scavenging action of SOD on O_2^- which in turn enhanced the plasma NO level and the total antioxidant capacity of the animals.

As been mentioned earlier, NO blockade induced hypertension and renal dysfunction. Moreover, increased renal sympathetic nerve activity is known to be another factor that caused the decreasing renal excretory functions[30]. Similar observation has also been identified in hypertensive laboratory animals and human subjects[35,36]. Therefore, in the present study, we further investigate the participation of α_1-adrenoceptor and angiotensin II type I receptors in L-NAME induced nitric oxide deficient animals using noradrenaline and phenylephrine - both are α_1-specific agonist, methoxamine- an α_{1A}-specific agonist and angiotensin II- an AT_1 receptor agonist. As expected, the magnitude of renal vasoconstrictor response to exogenous bolus infused angiotensin II in L-NAME treated animals was lower than control animals. Similarly, the sensitivity of renal cortical vasculature in L-NAME treated animals in response to exogenous infused noradrenaline; phenylephrine and methoxamine was also significantly decrease as compared to their control counterparts. This observation shows that the L-NAME treated animals experienced an attenuated sensitivity in the renal cortical vasculature to exogenous AT_1 agonist and α_1-adrenergic stimuli, that directly indicates the over expression of AT_1 receptors and α_1-adrenoceptor especially with α_{1A}- subtypes are confirmed responsible for L-NAME induced hypertension. This is well agrees with the observations by others who studied the interaction of AT_1 receptors and α_1-adrenoceptors in oxidative stress induced hypertension animals[37-39]. Tempol administration abolished all these effect significantly which suggesting that the hypertensive action due to NO deficiency is impart mediated by reactive oxygen species by which angiotensin II activates NADH/NADPH oxidase and increases O_2^- production in renal vascular tissue[37,40]. Therefore, the antioxidant stress activity of Tempol happened together with the interaction or crosstalk between SOD and NO in our model. This is because under NO deficiency condition, the plasma T-SOD level was decreased together with reduction of plasma NO level in L-NAME treated animals. However, when Tempol and L-NAME were administrated together, the plasma T-SOD level was elevated together with plasma NO level. Therefore, it is reasonable to speculate that a disruption

of NO synthase activity may be critically linked to upregulation of O_2^- anion and/or other oxidative enzymes. The postulated mechanism for the interaction of SOD and NO in scavenging O_2^- using Tempol and L-NAME in the vascular system was presented in (Figure 4).

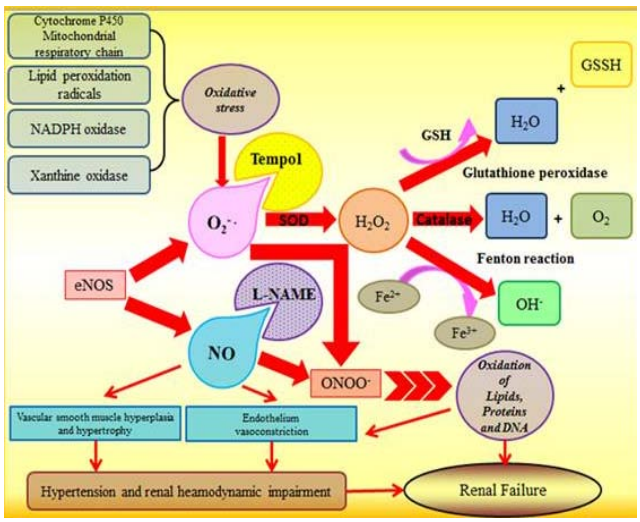

Figure 4: Reactive oxygen species in the vascular system and the proposed effect of Tempol and L-NAME in the vascular system during oxidative stress.

CONCLUSION

In summary, this study has shown for the first time that Tempol administration was able to lower high blood pressure and ameliorates the renal functions consequence by NO synthase inhibition. From the available data, we can further support the hypothesis that derangement of NO synthesis can cause the production of O_2^- in the systemic and renal vasculature that primarily determine the degree of oxidative stress, and alters the renal haemodynamics and excretory functions. Moreover, the over-expression of α_1-adrenoceptors and AT_1-receptors in L-NAME induced hypertension model has also been confirmed. Finnally, this study has demonstrated the importance of the SOD enzyme in the regulation of renal function in both normal physiological and pathophysiological condition. Despite NO is an important vasodilator and O_2^- scavenger, however, when NOS is inhibited, the T-SOD and NO were still remain in low level but this condition was fully improved when SOD mimetic-Tempol was administered; this shows that SOD enzyme is the primary O_2^- scavenger compared to NO although the interaction of SOD and NO might occur during the scavenging process.

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