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Effect of tumor necrosis factor alpha inhibition with etanercept on renal functions in L-NAME induced hypertensive rats: insights into the possible mechanisms

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- TNF-α,
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Abstract

Many studies suggest the dominant role of the inflammatory cytokine, tumor necrosis factor alpha (TNF-α) in the prognosis of hypertension and end stage renal disease (ESRD). The objective of this study was to investigate the effects of TNF-α inhibition on renal functions in N^{ω}-nitro-L-arginine methyl ester (L-NAME) induced hypertensive rats and the potential underlying mechanisms. Four groups of rats were used in the study for 3 weeks period; normal control group, TNF- α inhibitor (etanercept) group, L-NAME-induced hypertensive group and L-NAME + etanercept group. L-NAME group showed elevated systolic and diastolic blood pressure, plasma urea, creatinine, plasma renin activity, angiotensin II, kidney tissue nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, TNF- α and malondialdehyde (MDA) together with decreased creatinine clearance rate and renal antioxidants. Treatment with etanercept affords antihypertensive effect and ameliorates L-NAME induced renal impairment by improving renin–angiotensin system (RAS) and NADPH oxidase as well as by attenuating oxidative stress.

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INTRODUCTION

Arterial hypertension is one of the leading causes of death worldwide [1]. The worldwide prevalence of arterial hypertension by the year 2000 was 25% of adult population and expected to be around 30% by the year 2025 [2]. Hypertension is usually asymptomatic however, its progression is strongly associated with complications in many target organs, such as the brain, heart and kidneys [3]. Every year, 9.4 million people die from complications of arterial hypertension [4]. Among its complications, hypertensive nephropathy (HN), is one of the leading causes of end-stage renal disease (ESRD) around the world, secondary to diabetic nephropathy (DN). More than one million people die annually due to chronic kidney disease (CKD) [5].

Chronic administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME) is well known to induce arterial hypertension in rats although the underlying mechanisms have not been completely established. However, it appears that the increase in L-NAME induced elevated blood pressure is initiated by a reduction of nitric oxide and maintained by an involvement of both the renin–angiotensin system (RAS) and sympathetic nervous system [6].

The pathophysiological mechanisms of hypertension are multiple and complex involving interaction between RAS, oxidative stress, inflammation and endothelial dysfunction. Oxidative stress plays a fundamental role in the pathogenesis of hypertension. Reactive oxygen species (ROS) production and decrease antioxidant capacity, leading to increased oxidative stress that may adversely affect vascular function. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are one of the main sources of reactive oxygen species (ROS) in biologic systems. There are seven isoforms of NADPH oxidase (Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2). Of the Nox isoforms, Nox4, Nox1 and Nox2, are abundantly expressed in the kidney and are an important sources of renal ROS and subsequent kidney injury [7].

Inappropriate activation of the intrarenal RAS contributes to the occurrence and development of hypertension and renal injury [8]. Thus the current mainstay clinical therapy for hypertension involves targeting the RAS using angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin 1 receptor blockers. However, being ineffective alone or due to their undesirable adverse effects, potential novel treatments are needed to be identified [9].

The local and systemic inflammatory response to chronic elevated blood pressure in hypertensive patients causes degeneration in the kidney, leading to a worsening of hypertension. However, it is presently unclear whether inflammation is predominately

a cause or an effect of hypertension. Tumor necrosis factor alpha (TNF-α) is a cytokine that plays a pivotal role in the inflammatory response and is synthesized by many cells, most commonly by immune cells, mainly T-lymphocytes, vascular endothelial cells, renal tubular epithelial and mesangial cells. The apoptotic and inflammatory effects of TNF-α are mediated through two distinct receptors; apoptosis is induced when TNF receptor 1 (TNFR1) is stimulated, whereas inflammation occurs when TNFR2 is stimulated [10]. Studies have demonstrated higher plasma TNF-α levels in hypertensive patients compared to normotensive patients [11].

Etanercept (Enbrel) is a TNF-α inhibitor that functions as a decoy receptor that binds to TNF-α. It was the first soluble recombinant monoclonal antibody against TNF-α to be marketed for clinical use and is now commercially available. Etanercept reduces the inflammatory effect of TNF-α and is used clinically to treat several autoimmune diseases [12].

The present study aimed to evaluate the effect of TNF-α inhibitor, etanercept on arterial blood pressure, renal functions, RAS, renal oxidative stress and NADPH oxidase activity in L-NAME-induced hypertensive rats.

2. Materials and Method:

2.1. Chemicals:

N^{ω}-nitro-L-arginine methyl ester (L-NAME) was obtained from *(Sigma Aldrich, St. Louis, MO, USA).* Etanercept was purchased from *(Pfizer Pharmaceuticals Limited, New York, USA).*

2.2. Animals:

Thirty two adult male albino Wistar rats of 10 – 12 weeks old and weighing between 220 – 250g were purchased from the animal house of Faculty of Veterinary Medicine, Benha University, Egypt. The animals were kept in cages with free access to both water and standard rat chow *(El-Nasr Company, Cairo, Egypt)* under controlled temperature (22–24°C), humidity (40–60%) and light-dark cycle (12–12 h). The experiment was conducted in accordance with the guide for the care and use of laboratory animals and efforts were made to minimize the animals' suffering. All procedures were approved by the Institutional Ethical Committee for Animal Care and Use of the Faculty of Medicine, Benha University. After 2 weeks acclimatization period, the animals were randomly assigned into four equal groups (n=8 rats per each group):

Group I (Control group): Rats in this group were given free access to food and water.

Group II (TNF- α inhibitor, etanercept group): Rats in this group received TNF- α inhibitor, etanercept subcutaneously (s.c.) at a dose of 1.25 mg/kg/day for 3 weeks as described by *Elmarakby et al.* [13].

Group III (L-NAME induced hypertensive group): Rats in this group received L-NAME only by oral gavage as dissolved in the

drinking tap water at a dose of 40 mg/kg/day for 3 weeks as described by *Sung et al.* [14].

Group IV (L-NAME + Etanercept group): Rats in this group received entanrecept s.c. at a dose of 1.25 mg/kg/day as described by Elmarakby *et al.* [13] in combination with oral L-NAME at a dose of 40 mg/kg/day as described by *Sung et al.* for 3 weeks [14].

2.3. Blood pressure measurements

Blood pressures (systolic and diastolic) were measured in calm, conscious rat at the start of the experiment and at the end of each week using a tail-cuff transducer connected to a Power lab system running Chart 8 software *(Powerlab, AD Instruments, Mountain View, CA, USA).* Before each measurement, the rats were pre-warmed to 34°C for 15 minutes in a heated chamber. The mean of five readings was taken as the final value [15].

2.4. Sample collection

At the end of the study period, rats were individually housed in metabolic cages and 24-h urine samples were collected. A midline abdominal incision was performed to the rats under ether anesthesia and blood samples were collected from the abdominal aorta. Samples were collected in clean and dry centrifuge tubes and then centrifuged at 3000 rpm for 15 min to separate the plasma for biochemical analysis. Kidneys were removed, washed with physiological saline then homogenized in ice cold 100 mmol/l phosphate buffer (pH 7.4). Homogenate was centrifuged and the resulting supernatant was used for biochemical analysis of renal oxidative stress, TNF-α and NADPH oxidase activity.

2.5. Assessment of renal function

Determination of plasma urea, creatinine and urinary creatinine were estimated colorimetrically by using commercially available diagnostic kits from *(Biodiagnostic Company, Giza, Egypt)* as previously described by *Fawcett and Scott* [16] and *Bowers and Wong* [17] respectively. Values of plasma urea and creatinine were expressed in mg/dl. Creatinine clearance (Cr Cl) rate was calculated and expressed as milliliters per minute using the standard formula (U x V) / P, where U is the concentration in urine, V is the urine flow rate, and P is the plasma concentration.

2.6. Plasma renin activity measurement

Plasma renin activity (PRA) was measured using the gamma coat PRA radioimmunoassay kit *(SRL, Tokyo, Japan).* Plasma renin activity was measured in ng/ml/h [18].

2.7. Quantification of plasma angiotensin II (Ang II) levels

Plasma Ang II level was examined with an Ang II enzyme immunoassay kit (EIA) *(Cayman chemical Company, Ann Arbor, MI, USA).* Ang II level was read at a wavelength of 405 nm [19].

2.8. Assessment of renal oxidative stress

Malondialdehyde (MDA), renal lipid peroxidation product, was measured in renal tissue homogenates in nmol/mg protein by the method described by *Placer et al.* [20]. The activities of enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were assayed by the methods of *Kakkar et al.* [21]; *Sinha* [22] *and Rotruck et al.* [23] respectively.

2.9. Determination of renal tumor necrosis factor alpha (TNF-α)

The renal level of TNF-α was determined in tissue homogenate using enzyme-linked immunosorbent assay (ELISA) with a commercially available kits *(R&D Systems, Minneapolis, MN, USA).* The procedure was carried out based on the protocol of the manufacturer.

2.10. Renal NADPH oxidase activity measurement

NADPH oxidase was measured according on its capacity to reduce ferricytochrome c into ferrocytochrome (pH 7.8). Kidney homogenate (50 µg protein/experiment), NADPH (100 µM) and cytochrome c (250 µg/l final concentration) were incubated in presence of diphenyleneiodonium (100 µM). The absorbance of reduction of cytochrome c was detected at 550 nm. The difference between the absorbance of samples between 0 to 120 min was measured in nmol/mg protein [24].

2.11. Statistical analysis.

All analyses were performed using the program Statistical Package for Social Sciences version 19 (SPSS Inc, Chicago, IL, USA). The data were presented as the mean (M) ± standard deviation (SD). Comparisons between groups were analyzed by using one-way Analysis of Variance (ANOVA) and Bonferroni's Multiple Comparison Test. Probability of chance (*P* value) < 0.05 was considered statistically significant.

3. Results :

3.1. Effect of etanercept on systolic (SBP) and diastolic blood pressure (DBP) (Table 1 & 2)

At the beginning of the experiment, the baseline values of both SBP and DBP showed non- significant differences among the groups. Also non-significant differences were observed in SBP and DBP in etanercept group compared with the control group over the 3-weeks experimental period (P > 0.05). Daily oral administration of L-NAME (40 mg/kg) induced a rapid progressive increase in both systolic and diastolic blood pressures compared to the corresponding values of the control group. This increase was significant from the first week of administration till the end of the experiment. Treatment with etanercept significantly attenuated the L-NAME induced rise in SBP and DBP throughout the experimental period ($P < 0.05$).

3.1. Effect of etanercept on systolic blood pressure during the experimental period (Table 1).

Groups Systolic blood pressure (mmHg)	Group I (Control)	Group II (Etanercept)	Group III (L-NAME induced hypertension)	Group IV (L-NAME + Etanercept)
At the start of study	107 ± 4.77	109 ± 4.53	110 ± 6.74	108 ± 5.10
At the end of 1st week	104 ± 5.13	108 ± 5.62	130 ± 7.22 *	109 ± 5.55 #
At the end of 2nd week	105 ± 3.62	107 ± 3.14	160 ± 7.94 *	116 ± 6.02 #
At the end of 3rd week	103 ± 3.15	105 ± 3.55	173 ± 8.84 *	118 ± 6.43 #

Data is expressed as mean ± standard deviation, *P.* value = probability of chance, $P < 0.05$ is significant tested by using One-way analysis of variance (ANOVA) test and Bonferroni's Multiple Comparison Test.
* Significant difference *vs* the control group. # Significant difference *vs* the L-NAME induced hypertensive group.

3.2. Effect of etanercept on diastolic blood pressure during the experimental period (Table 2).

Groups Diastolic blood pressure (mmHg)	Group I (Control)	Group II (Etanercept)	Group III (L-NAME induced hypertension)	Group IV (L-NAME + Etanercept)
At the start of study	77 ± 3.16	79 ± 4.79	80 ± 4.24	78 ± 3.26
At the end of 1st week	75 ± 4.23	77 ± 5.10	96 ± 6.18 *	80 ± 3.17 #
At the end of 2nd week	73 ± 3.81	74 ± 3.72	100 ± 6.92 *	82 ± 4.33 #
At the end of 3rd week	74 ± 4.14	76 ± 3.46	103 ± 7.96 *	85 ± 5.47 #

Data is expressed as mean ± standard deviation, *P.* value = probability of chance, $P < 0.05$ is significant tested by using One-way analysis of variance (ANOVA) test and Bonferroni's Multiple Comparison Test.
* Significant difference *vs* the control group. # Significant difference *vs* the L-NAME induced hypertensive group.

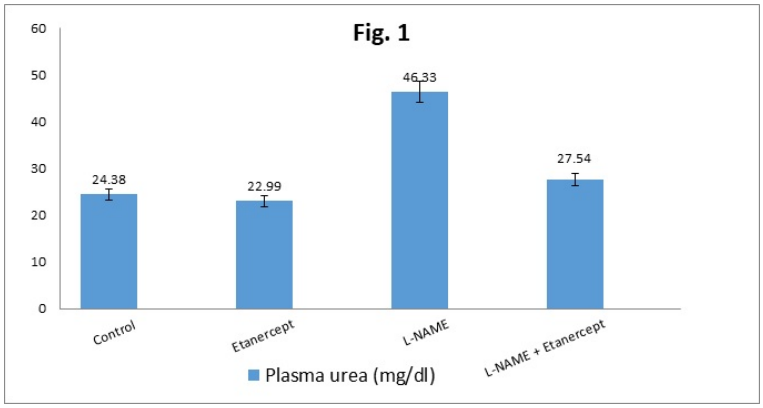
3.2. Effect of etanercept on renal functions in hypertensive rats (Table 3 and fig. 1 & 2)

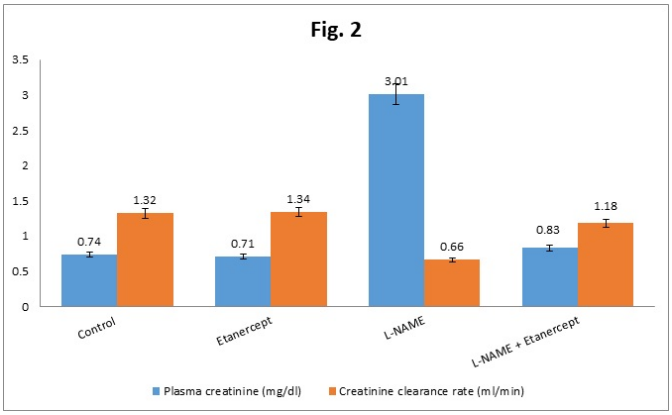
There was non-significant change in plasma urea, creatinine and creatinine clearance rate in etanercept group compared with the control group ($P > 0.05$). Treatment with L-NAME resulted in significant increase in plasma urea and creatinine ($P < 0.05$) and significant decrease in creatinine clearance rate when compared with the control rats ($P < 0.05$). Etanercept produced a significant reduction in plasma urea and creatinine ($P < 0.05$) and significant increase in creatinine clearance rate ($P < 0.05$) as compared with L-NAME induced hypertensive rats.

3.3. Effect of etanercept on renal function parameters in rats at the end of the experimental period (Table 3).

Group I (Control) Parameter	Group I (Control)	Group II (Etanercept)	Group III (L-NAME induced hypertension)	Group IV (L-NAME + Etanercept)
Plasma urea (mg/dl)	24.38 ± 2.18	22.99 ± 2.19	46.33 ± 4.42 *	27.54 ± 2.22 #
Plasma creatinine (mg/dl)	0.74 ± 0.05	0.71 ± 0.06	3.01 ± 0.21*	0.83 ± 0.07 #
Creatinine clearance rate (ml/min)	1.32 ± 0.02	1.34 ± 0.01	0.66 ± 0.03*	1.18 ± 0.02 #

Data is expressed as mean ± standard deviation, *P.* value = probability of chance, $P < 0.05$ is significant tested by using One-way analysis of variance (ANOVA) test and Bonferroni's Multiple Comparison Test.
* Significant difference *vs* the control group. # Significant difference *vs* the L-NAME induced hypertensive group.





3.3. Effect of etanercept on renin angiotensin system in hypertensive rats (Table 4 and fig 3)

Non-significant change in plasma renin activity and plasma Ang. II was observed between etanercept group and the corresponding control group ($P > 0.05$). Induction of hypertension with L-NAME resulted in significant increase in plasma renin activity and Ang. II ($P < 0.05$) as compared with the control group. Treatment of with etanercept resulted in a significant decrease in plasma renin activity and plasma angiotensin II as compared with L-NAME induced hypertensive rats ($P < 0.05$).

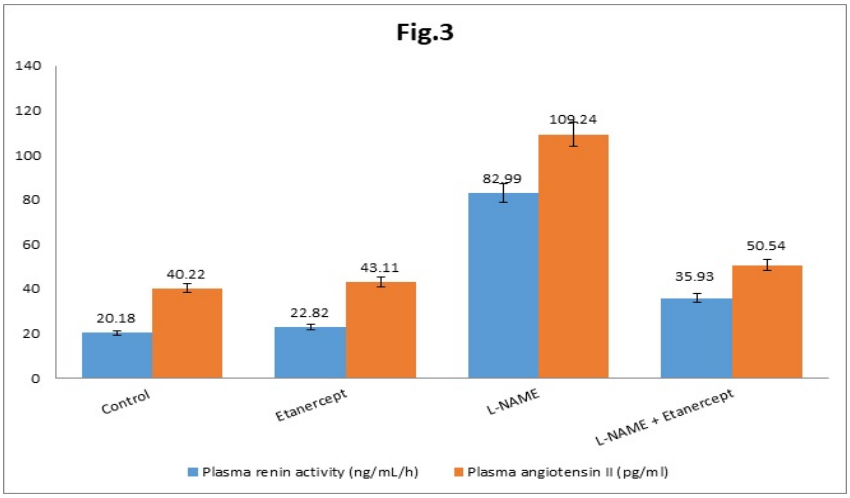
3.4. Effect of etanercept on plasma renin activity and plasma angiotensin II (Table 4).

Groups / Parameter	Group I (Control)	Group II (Etanercept)	Group III (L-NAME induced hypertension)	Group IV (L-NAME + Etanercept)
Plasma renin activity (ng/ml/h)	20.18 ± 3.31	22.82 ± 4.71	82.99 ± 9.80 *	35.93 ± 6.39 #
Plasma Angiotensin II (pg/ml)	40.22 ± 0.65	43.11 ± 0.78	109.24 ± 0.57 *	50.54 ± 0.43 #

Data is expressed as mean ± standard deviation, *P.* value = probability of chance, $P < 0.05$ is significant tested by using One-way analysis of variance (ANOVA) test and Bonferroni's Multiple Comparison Test

* Significant difference *vs* the control group

Significant difference *vs* the L-NAME induced hypertensive group.



3.4. Effect of etanercept on renal tissue oxidative stress (Table 5 and fig 4, 5 & 6)

Non-significant change in renal tissue MDA or renal antioxidant enzymes (SOD, CAT and GPx) was detected between the control group and etanercept group ($P > 0.05$). Administration of L-NAME to rats resulted in significant increase in renal tissue MDA ($P < 0.05$) together with significant decrease ($P < 0.05$) in renal antioxidants (SOD, CAT and GPx). Treatment of rats with etanercept resulted in significant decrease in renal tissue MDA and significant increase in renal antioxidant enzymes as compared with L-NAME hypertensive group ($P < 0.05$).

3.5. Effect of etanercept on renal TNF-α and NADPH oxidase (Table 6 and fig 4 & 5)

Elevated levels of TNF-α and NADPH oxidase in renal tissue homogenate were detected in L-NAME treated group as compared with the control group ($P < 0.05$). Administration of etanercept resulted in significant decrease in renal tissue levels of TNF-α and NADPH oxidase when compared to the L-NAME group ($P < 0.05$). There are non-significant changes between group I and II ($P > 0.05$).

3.5. Effect of etanercept on renal oxidative stress markers (Table 5).

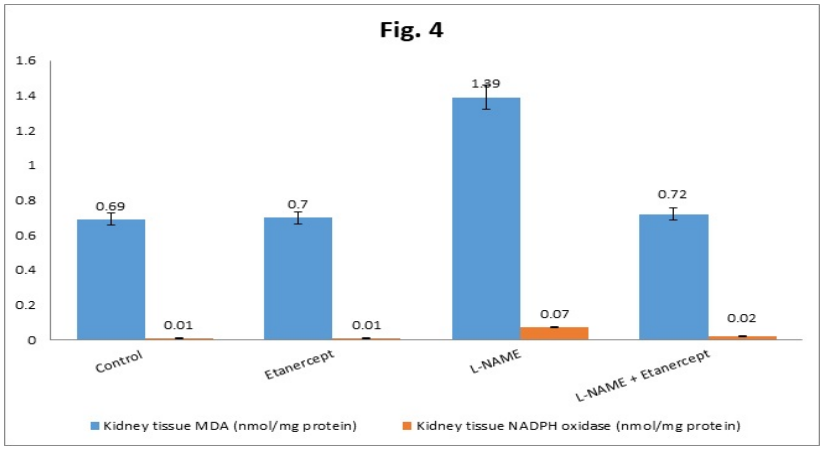
Groups / parameter	Group I (Control)	Group II (Etanercept)	Group III (L-NAME induced hypertension)	Group IV (L-NAME + Etanercept)
MDA (nmol/mg protein)	0.69 ± 0.04	0.70 ± 0.05	1.39 ± 0.09 *	0.72 ± 0.03 #
CAT (µmol/mg protein)	36.69 ± 2.43	38.11 ± 2.97	14.92 ± 1.41 *	35.39 ± 2.27 #
SOD (U/mg protein)	15.95 ± 1.13	16.14 ± 1.42	6.21 ± 0.79 *	15.74 ± 1.48 #
GPx (µmol/mg protein)	11.36 ± 1.24	11.65 ± 1.18	3.63 ± 0.33 *	10.22 ± 1.12 #

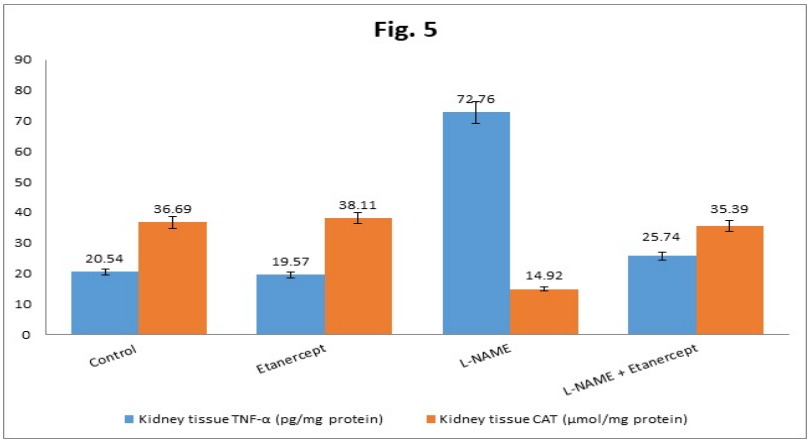
Data is expressed as mean ± standard deviation, *P.* value = probability of chance, $P < 0.05$ is significant tested by using One-way analysis of variance (ANOVA) test and Bonferroni's Multiple Comparison Test.
* Significant difference *vs* the control group
# Significant difference *vs* the L-NAME induced hypertensive group.

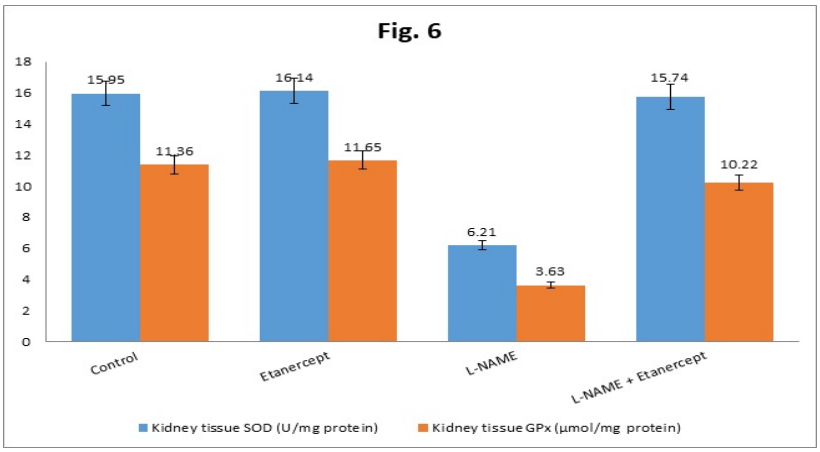
3.6. Effect of etanercept on renal tissue TNF-α and NADPH oxidase (Table 6).

Groups / Parameter	Group I (Control)	Group II (Etanercept)	Group III (L-NAME induced hypertension)	Group IV (L-NAME + Etanercept)
TNF-α (pg/mg protein)	20.54 ± 1.68	19.57 ± 1.47	72.76 ± 3.32*	25.74 ± 1.22 #
NADPH oxidase (nmol/mg protein)	0.01 ± 0.01	0.01 ± 0.03	0.07 ± 0.02*	0.02 ± 0.02 #

Data is expressed as mean ± standard deviation, *P.* value = probability of chance, $P < 0.05$ is significant tested by using One-way analysis of variance (ANOVA) test and Bonferroni's Multiple Comparison Test.
* Significant difference *vs* the control group
# Significant difference *vs* the L-NAME induced hypertensive group.







Discussion

N$^{\omega}$-Nitro-L-arginine-methyl ester (L-NAME) induced hypertension is a well-established model of experimental nitric oxide (NO) deficient hypertension. Its physiological and pathological characteristics resemble essential hypertension [25]. The obtained data in the present study revealed that administration of L-NAME (40 mg/kg/day) for 3 weeks resulted in a significant elevation in SBP and DBP which remained high throughout the experimental period. These results were in line with *Berkban et al.*, *El-Nezhawy et al. and Sung et al.* who found that

administration of L-NAME resulted in a rapid progressive increase in SBP and DBP [14, 26, 27].

Hypertension, a sustained elevation of blood pressure, is known to cause structural and functional abnormalities in both the cardiovascular and renal systems. Urea is a major nitrogen containing metabolic compound necessary for protein metabolism and creatinine is endogenously produced and released into body fluids. Creatinine clearance (CrCl) is a measure of the glomerular filtration rate [28]. The outcomes from the existing research revealed that L-NAME induced hypertensive rats showed significantly increased levels of plasma urea and creatinine and significant decrease in creatinine clearance rate when compared to the control group which are in accordance with other earlier findings [29, 30].

Compelling evidence supports the hypothesis that immune system and inflammatory cytokines play a critical role in the development of hypertension and target end organ damage [31]. TNF-α is an important pro-inflammatory cytokine mainly produced by activated immune cells and is involved with pleiotropic vascular effects [32]. TNF-α acts on the vasculature, kidney, and sympathetic nervous system to affect blood pressure [33]. The results of the current study showed that inhibition of TNF-α by etanercept could attenuate the hypertensive response and renal damage in L-NAME model of hypertension which is in accordance with previous reports in various animal models of hypertension, including preeclampsia, Ang II-hypertensive rats, deoxycorticosterone acetate (DOCA)- salt-hypertensive mice and a mouse model of systemic lupus erythematosus [33, 34, 35]. Meanwhile, there were also opposite reports that the inhibition of TNF-α with etanercept failed to reduce blood pressure in Ang II-hypertensive mice and DOCA-salt-hypertensive mice. Although etanercept significantly reduced renal injury. This discrepancy in the antihypertensive effect of TNF-α inhibition may be related to different animal models of hypertension or the treatment duration [13, 36].

L-NAME is responsible to cause imbalance in RAS as it increased the expression of angiotensin II and caused renal dysfunction [37]. Ang. II, the major bioactive peptide of the RAS that plays a major role in the regulation of vascular function and structure as excessive production of Ang. II leads to increase vascular superoxide ($O2^-$) formation through increased expression of NADPH-dependent oxidase [38]. The current study revealed an increase in plasma Ang. II and plasma renin activity in L-NAME hypertensive rats compared to their level in the corresponding control rats which is consistent with previous reports [39, 40, 41]. The present study also revealed that blockade of TNF-α by etanercept led to a significant decrease in plasma renin activity and plasma

Ang. II as compared with L-NAME hypertensive rats. Both in vitro and in vivo studies have demonstrated a cross-talk between Ang II and TNF-α as intracerebroventricular (ICV) treatment with etanercept resulted in reduction of angiotensin converting enzyme (ACE) and angiotensin receptors' expression in the paraventricular nucleus (PVN). [42].

Oxidative stress, characterized by increased bioavailability of reactive oxygen species (ROS), initiates and facilitates damage to membrane lipids, resulting in a decrease of renal cell membrane fluidity. The increased levels of ROS such as superoxide anion, hydrogen peroxide and lipid peroxides were reported in hypertensive patients [43]. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) act together to eliminate ROS, and even a small deviation in their physiological concentrations may have a dramatic effect on the resistance of cellular lipids, proteins, and deoxyribonucleic acid (DNA) to oxidative damage leading to deleterious effects [44, 45]. In this study, L-NAME induced significant increase in renal tissue MDA with a significant decrease in renal tissue SOD, CAT and GPx activity compared with control rats, indicating that oxidative stress plays an important role in the pathophysiology of hypertension which were in consistent with the results of *Veerappan and Senthilkumar and Toba et al.* [41, 46]. The present study also showed a significant increase in renal NADPH oxidase and TNF-α in rats treated with L-NAME and this is in congruent with the previous results [13, 47]. Chronic inflammation can also trigger oxidative stress, which has been associated with hypertension and TNF-α can induce oxidative stress via the stimulation of NADPH oxidase. The kidney is an important organ involved in regulating blood pressure, and chronic kidney disease is one of the most common causes of secondary hypertension. Elevated renal oxidative stress can be seen in the early stages of chronic kidney disease. Inflammation and oxidative stress increase as renal dysfunction progresses [48]. In this study, TNF-α inhibitor, etanercept, significantly decreased MDA, TNF-α and NADPH oxidase in renal tissue homogenate and significantly increased anti-oxidant enzymes (SOD. CAT and GPx) when compared with L-NAME induced hypertensive rats. These findings were in agreement with the previous studies [42, 49]. However, a study of *Goldenberg* indicated that treatment with etanercept did not significantly affect TNF-α expression but decreases its biological activity [50].

Conclusion

TNF-α blockade by etanercept reduces blood pressure and renal impairment in L-NAME-induced hypertensive rats and the antihypertensive effect may be partly mediated by improving renin angiotensin system and attenuating renal oxidative stress and NADPH

oxidase. The findings of the present study suggest that etanercept has the potential to be used as a therapeutic agent for hypertension together with other antihypertensive drugs for preventing renal inflammation and the development of end-organ damage.

Conflict of interest

The authors declare no conflict of interest.

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