The potential role of anti tumor necrosis factor-alpha antibodies on some renal functions and vasoregulatory factors in preeclamptic pregnant Wistar rats

Hayam I. Gad, *MSc, PhD.*

ABSTRACT

الأهداف: معرفة دور عامل النخر الورمى على وظائف الكلى وإفراز المواد المنظمة للأوعيه الدموية مثل الأندوثيلين–1, الأنجيوتنسين–2 أنترلوكين–6, موادإلتصاق الأوعيه الدمويه (فيكام–1) وذلك في حالات ماقبل تسمم الحمل.

الطريقة: تم إجراء هذا البحث فى بيت الحيوانات بمستشفى الملك خالد الجامعى بجامعة الملك سعود فى الرياض خلال الفترة من ديسمبر 2011م إلى نوفمبر 2012م. أجرى البحث على 40 فأرا تم تقسيمهم إلى 4 مجموعات بالتساوى. شملت المجموعة الأولى فئران غير حوامل وشملت المجموعة الثانية فئران حوامل تم حقنهم بمحلول الملح. أما المجموعة الثالثة فشملت فئران حوامل تم حقنهم بمثبط للإنزيم المخلق لأكسيد النيتريك كنموذج لمرحلة ماقبل تسمم الحمل. كما شملت المجموعة الرابعة فئران حوامل تم حقنهم بمثبط الإنزيم المخلق لأكسيد النيتريك ومضاد عامل النخر الورمى. فى اليوم العشرين من بداية الحمل تم قياس ضغط الدم الشريانى وبعض وظائف الكلى ووزن الفئران حديثى الولادة. كما تم أخذ عينات الدم لقياس مستوى الإندوثيلين–1والأنجيوتنسين–2 في البلازما. وكذلك مستوى أكسيد النيتريك, أنترلوكين–6 وفيكام–1 في المصل.

النتائج: أوضحت الدراسة أن العلاج بمضاد عامل النخر الورمى أدى إلى نقص فى ضغط الدم الشريان وتحسين وظائف الكلى. كما أدى إلى نقص مستوى الأندوثيلين–1, أنجيوتنسين–2, أنترلوكين–6, فيكام –1. أيضاً كان هناك زيادة ملحوظة فى وزن الفئران حديثة الولادة.

خاتمة: تشيرهذه الدراسة إلى إمكانية إستخدام مضاد عامل النخر الورمى لعلاج حالات ماقبل تسمم الحمل. نوصى بإجراء دراسات أخرى لإثبات هذه النتائج.

Objectives: To investigate the potential role of anti-tumor necrosis factor-alpha (TNF-α) antibodies on some renal functions and release of vasoregulatory peptides using nitric oxide synthase deprived pregnant rats.

Materials: This study was carried out at King Khalid University Hospital, King Saud University, Riyadh, Kingdom of Saudi Arabia from December 2011 to November 2012. Forty female Wistar rats were divided into 4 groups (10 rats each); Group I - included virgin non-pregnant rats. Group II - included pregnant rats that received saline, Group III - received N^G-nitro-L-arginine methyl ester (L-NAME), and Group IV - received both L-NAME and anti TNF-α antibodies. Mean arterial blood pressure, urine volume, creatinine clearance and 24 hours urinary albumin excretion were measured on day 20 of gestation. Blood samples were taken on day 20 of gestation for measurement of plasma endothelin-1 (ET-1), angiotensin II (Ag II) and serum levels of total nitric oxide (NO) products, interleukin-6 (IL-6) and soluble vascular cell adhesion molecule (sVCAM-1). Viable pups were also weighed.

Results: Anti TNF-α antibodies reversed hypertension, improved renal function, decreased release of vasoactive substances and increased pup weight.

Conclusion: Preeclampsia is associated with disturbed renal function, overproduction of cytokines and vasoregulatory factors, and fetal growth restriction. Treatment of pregnant rats with anti TNF-α antibodies, restored urine volume, creatinine clearance, plasma ET-1, serum IL-6 and sVCAM-1 to normal levels. Hence, anti TNF-α antibodies may have beneficial effects in preeclampsia. Additional studies are warranted to confirm these results.

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From the Physiology Department, College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia.

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Address correspondence and reprint request to: Dr. Hayam I. Gad, Physiology Department, College of Medicine, King Saud University, PO Box 2925 (29), Riyadh 11461, Kingdom of Saudi Arabia. Tel. +966 (1) 4692762. Fax. +966 (1) 4786798. E-mail: hayam_gad@hotmail.com / hgad@ksu.edu.sa

Preeclampsia (also indexed as pregnancy-induced hypertension, gestosis, and gestational hypertension) is a pregnancy-specific multisystem disorder of unknown etiology, recognized from antiquity as a leading cause of maternal and perinatal mortality.[1] It affects 4-5% of pregnancies and is diagnosed by the accompanying increased blood pressure, proteinuria and edema, all of which appear in the second half of pregnancy.[2] These signs are accompanied by several metabolic disorders. It has been termed as the "disease of theories", reflecting the confusion that surrounds the causes and pathophysiology of pre-eclampsia. Recent insights, however, may be clarifying this enigmatic condition. Aberration of the interaction between placental and maternal tissue is probably the primary cause, but the exact nature of the differences from normal pregnancy remain elusive.[3] Attempts to understand the sequence of physiological changes have concentrated on vascular endothelium and oxidative stress issues. While certain types of hemodynamic forces are essential for physiological functions of the vascular endothelial cells under normal conditions, other types can induce endothelial dysfunction by adversely modulating vascular endothelial cells signaling and gene expression, thus contributing to the development of vascular pathologies.[4]

Genetic, immunologic, metabolic susceptibilities and other backgrounds have been investigated to understand the pathogenesis of this disease.[5] In normal pregnancy, the luminal diameter of the spiral arteries enlarges and the walls remodel such that they contain very little smooth muscle. These changes extend into the vessels to the inner third of the myometrium to provide a large bore, flaccid, low-resistance circuit for perfusion of the intervillous space. These modifications are associated with endovascular invasion of fetal trophoblast into these maternal vessels.[6] In preeclampsia, physiologic changes in the spiral arteries are confined to the decidual portion of the arteries; approximately 30-50% of the placental bed's spiral arteries escape entirely from endovascular trophoblast invasion. Myometrial segments remain anatomically intact and undilated. Many vessels are occluded by atherosis. Acute atherosis and the associated thrombosis may cause placental infarctions. Lipoprotein-a deposition is often found in association with atherosis.[7]

The improper remodeling of the spiral arteries leading to placental ischemia is an important initiating event that causes excessive placental release of circulating factors such as inflammatory cytokines and the pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α), which may serve as an important initiator of maternal endothelial activation and/or dysfunction that has been hypothesized as the leading phenomenon responsible for the clinical signs of preeclampsia and to be a major contributor in the pathogenesis of the disorder.[8] In addition, excessive release of TNF-α may have direct detrimental effects mediated by upregulation of other cytokines as interleukin-6 (IL-6), adhesion molecules, as soluble vascular cell adhesion molecules-1 (sVCAM-1) and inflammatory mediators as reactive oxygen species that increase lipid peroxidation.[9] The collective biological properties and activities of TNF-α coupled with the consistently noted metabolic disturbances documented in preeclamptic pregnancies, have led investigators to postulate that TNF-α may play an important role in the pathogenesis of preeclampsia.

Nitric oxide (NO) is probably an important messenger, but certainly not the only one, in inducing vasodilation in normal pregnancy.[10] A previous study[11] demonstrated that NO synthase (NOS) is important in the regulation of blood pressure during pregnancy. Moreover, immunohistochemical studies to localize endothelial NOS in term placentae found strong staining patterns in syncytiotrophoblast.[12] A deficiency of NO could thus result in vasoconstriction, eventually leading to elevated blood pressure and local or disseminated intravascular coagulation, and thereby demonstrating the feature of preeclampsia. In pregnant animals, chronic inhibition of NOS eventually produces a preeclampsia-like syndrome.[13] Several investigators reported the inhibition of NO synthesis with analogues of L-arginine, such as N^G-nitro-L-arginine methyl ester (L-NAME) caused hypertension, proteinuria, fetal growth retardation, and increased fetal mortality without affecting gestational length.[14] These phenomena are remarkably similar to preeclampsia. Therefore, L-NAME-treated rats can be used as an animal model of preeclampsia. This study is carried on NOS deprived pregnant rats as an animal model of preeclampsia. The main objectives of this study are to clarify the effects of anti TNF-α antibodies on renal function and the release of vasoregulatory peptides as endothelin-1 (ET-I), angiotensin II (Ag II), IL-6, and sVCAM-1.

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Methods. The current study was carried out on 40 female Wistar rats supplied by the Medical College animal house at King Khalid University Hospital, from December 2011 to November 2012. Their average weight was 250-300 g. They were 13-18 weeks old. They were housed in a controlled environment and get free access to water ad labium. All experiments were performed in accordance with the guide for the animal care and the use committee guidelines at King Khalid University Hospital Animal House. Two or 3 cycling female rats were paired with male rats for 24 hours and tested daily by vaginal smear preparation for the presence of vaginal plug or sperms in the smear as evidence of copulation. Those dams that coupled between the second and fourth days after pairing with males were further studied. An eye dropper with approximately 500 µl of distilled water was inserted into the vagina and then sucked back onto a glass slide and analyzed through a microscope.[15] Insemination took place mainly on the second or third days after pairing females with males. The presence of sperms in vaginal smears was considered as day one of pregnancy. Rats were divided into 4 groups (10 rats each). Group I included virgin non-pregnant rats. Group II included pregnant rats that received saline solution (0.5 ml/100 g body weight) subcutaneously and daily starting from day 7 to day 20 of gestation. Group III included pregnant rats that were treated with L-NAME (Sigma, St. Louis, MO, USA) dissolved in sterile saline solution in a dose of 10 mg/0.5 ml/100 g body weight subcutaneously and daily starting from day 7 to day 20 of gestation, to make an animal model of preeclampsia.[15] Group IV: included pregnant rats that were treated by both L-NAME (the same dose as mentioned above) and rabbit anti-mouse TNF-α antibodies (supplied from Sigma, St. Louis, MO, USA) at a dose of 100 ng/rat/day intravenously.[16] through tail vein starting from day 7 to day 20 of gestation. On day 20 of gestation, the mean arterial blood pressure (MAP) was measured from rat-tail by non-invasive blood pressure system.[17] Blood pressure measurements were repeated at least 3 times, to obtain a mean blood pressure value. Urine samples were taken for measurement of urine volume, creatinine, and 24 hours urinary albumin excretion.[18] Blood samples were taken from the eye ball using capillary tubes and collected in EDTA tubes containing aprotinine at 0°C then centrifuged at 1600 g for 15 minutes. Plasma was analyzed for the levels of ET-I using the parameter ET-1 immunoassay kit catalog No. BBE 5, manufactured by R&D Systems, Inc. USA, Minneopolis, MN[19] and Ag II using the competitive EIA kits provided from Peninsula Laboratories, Inc. San Carlos, California. Catalog No. S-1133 (EIAH7002).[20] The serum was also used for measurement of the levels of creatinine, total NO catalog No. DE1600 manufactured by R&D Systems, Inc. USA, Minneopolis, MN,[21] IL-6 using Quatinkine, rat IL-6 immunoassay catalog No. R6000B provided by R&D Systems, Inc. USA, Minneopolis, MN[22] and sVSAM-1 using Quatinkine, mouse sVCAM-1 immunoassay catalog No. MVC00 supplied from R&D Systems, Inc. USA, Minneopolis, MN.[23] Viable pups were also weighed. Control non-pregnant rats were kept separately and exposed to the same environment. Blood and urine samples were taken on the same day for the other pregnant rats.

Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS Inc, Chicago, IL, USA) program for Windows version 17. Data were expressed as mean±SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) with 95% confidence intervals. Differences were considered to be statistically significant at $p<0.05$.

Results. **Table 1** summarizes the estimated measures in non-treated pregnant rats on day 20 gestations resembled that of virgin non-pregnant rats (control) denoting that normal pregnancy has no effect on these measures. The mean arterial blood pressure, 24 hour urinary albumin excretion, plasma ET-1, plasma Ag II, serum IL-6, and serum sVCAM-1 increased in L-NAME treated rats while urine volume, creatinine clearance, serum NO, and pup weight decreased compared to controls, non treated pregnant rats and anti TNF-α antibodies treated rats ($p<0.001$). Treatment of pregnant rats with anti TNF-α antibodies restored the urine volume, creatinine clearance, plasma ET-1, serum IL-6, and serum sVCAM-1 to normal levels on day 20 of gestation. However, it showed no effect on serum NO. Also, pup weight was insignificantly changed in anti TNF-α antibodies treated rats when compared to non-treated pregnant rats (**Table 1**).

In fact, the administration of anti TNF-α antibodies to rats from day 7 up to day 20 of gestation decreased significantly the 24 hour urinary albumin excretion and plasma Ag II when compared to L-NAME treated rats, but still showed significant increase as compared to controls ($p<0.001$).

Discussion. Preeclampsia is a leading cause of maternal and perinatal morbidity and mortality. Although the precise cause is unknown, endothelial cell dysfunction and inflammation are important aspects of the overall puzzle.[9] In this study, we used an animal model of preeclampsia using L-NAME, an NOS

Table 1 - Mean values ± SD of the measured parameters in control rats (Group I), non treated pregnant rats (Group II), N^G-nitro-L-arginine methyl ester (L-NAME)-treated rats (Group III), and anti-tumor necrosis factor-alpha (TNF-α) antibodies-treated rats (Group IV).

Variables	Control non pregnant rats (Group I)	Non treated pregnant rats (Group II))	L-NAME-treated preganant rats (Group III)	Anti TNF-α antibodies-treated pregnant rats (Group IV)	F-value	*P*-value
Mean arterial blood pressure (mm Hg)	101.10 ± 4.93	98.50 ±7.04	145.40 ± 9 .31*	103.40 ± 2.91	117.50	<0.0001*
Urine volume (ml/24 hour)	7.50 ± 0.36	7.60 ± 0.35	6.42 ± 0.28*	7.59 ± 0.30	31.44	<0.0001*
Creatinine clearance (ml/min)	2.99 ± 0.37	3.09 ± 0.46	0.83 ± 0.35*	2.64 ± 0.40	71.41	<0.0001*
24 hours urinary albumin excretion (mg/dl)	31.1 ± 1.85	32.1 ± 2.33	143.7 ± 7.48*	61.7 ± 4.45†	1325.60	<0.0001*
Plasma endothelin-1 (pg/ml)	0.42 ± 0.04	0.43 ± 0.06	0.55 ± 0.05*	0.44 ± 0.03	18.49	<0.0001*
Plasma angiotensin II (ng/ml))	1.00 ± 0.06	0 .97 ± 0.07	1.94 ± 0.08*	1.45 ± 0.06†	447.20	<0.0001*
Serum nitric oxide (μmol/l)	1.04 ± 0.09	1.03 ± 0.12	0.40 ± 0.04*	0.41 ± 0.04‡	199.04	<0.0001*
Serum interleukin-6 (pg/ml)	193.61 ± 4.81	191.73 ± 7.70	244.66 ± 4.09*	195.8 ± 2.87	242.67	<0.0001*
Serum vascular cell adhesion molecule-1 (ng/ml)	91.46 ± 4.21	90.90 ± 3.73	113.33 ± 3.38*	92.87 ± 3.43	85.492	<0.0001*
Pup weight (g)		5.78 ± 0.27	4.31 ± 0.28*	5.68 ± 0.43	59.51	<0.0001*

Significance was considered at $p<0.05$, 95% confidence interval. *Significant changes between Group III and each of Group I, Group II and Group IV.
†Significant increase in Group IV as compared with Group I and Group II, significant decrease in Group IV as compared with Group III.
‡Significant decrease in Group IV as compared with Groups I and II.

inhibitor. Several investigators found that L-NAME treated pregnant rats show preeclampsia-like symptoms consisting of hypertension, intrauterine growth restriction, proteinuria, and renal glomerular injury. These findings are remarkably similar to those seen in human preeclampsia and support the hypothesis that a decrease in the bio-availability of NO might thus play a role in the development of the disease.[13] In the current study, L-NAME treated pregnant rats showed significant rise of both mean arterial blood pressure and 24 hour urinary albumin excretion together with significant reduction of each of creatinine clearance, urine volume and pup weight as compared to pregnant rats received saline solution consistent with the previous reports.[24] As a result, these animals seem to be appropriate model for preeclampsia.

Studies in hypertensive pregnant women and experimental animal models suggested that reduction in uteroplacental perfusion pressure due to inadequate cytotrophoblast invasion of uterine spiral arteries and ensuing placental ischemia/hypoxia during late pregnancy may trigger the release of placental factors that initiate a cascade of cellular and molecular events leading to endothelial and vascular smooth muscle cell dysfunction and thereby increased vascular resistance and arterial pressure. Thus, it can be hypothesized that the preeclamptic placenta may become increasing hypoxic with advancing gestation leading to production of a variety of biologically active factors such as TNF-α or yet unidentified factors that act directly or indirectly to induce widespread endothelial dysfunction[25] and affect the neuronal control mechanisms of blood pressure.[26] The increased sympathetic tone combined with systemic decrease in endothelial dependent vascular relaxation may contribute to the increased resistance and blood pressure associated with preeclampsia.[27] Previous studies suggested that TNF-α is involved in limiting trophoblast invasion. This hypothesis is supported by the increased plasma concentration of TNF-α in women with preeclampsia and suggests that increased placental production of TNF-α may contribute to the widespread endothelial dysfunction that characterizes preeclampsia.[28]

According to the above cited data, the overall goal of the present study is to assess the role of anti TNF-α antibodies treatment on renal function and production of ET-1, Ag II, total NO, IL-6, and sVCAM-1 in L-NAME treated pregnant rats as a model of preeclampsia. Gilbert et al[29] explained the pathways by which placental ischemia may lead to endothelial and cardiovascular dysfunction during preeclampsia. They postulated that placental ischemia could result in increased synthesis of many factors as, TNF-α and IL-6. Elevations in these factors are proposed to result in endothelial dysfunction by decreases in bio-available NO and increased reactive oxygen species (ROS) and ET-1, which in turn results in altered renal function, increased total peripheral resistance and ultimately hypertension. This could explain why administration of anti TNF-α antibodies starting from day 7 of gestation during the present study reversed hypertension, decreased 24 hours urinary albumin excretion and increased creatinine clearance. Evidence from several studies indicates that NO generated by renal NOS may

be involved in the long-term control of systemic and renal hemodynamic changes that occur during normal gestation. Therefore, variations in renal NOS expression may also contribute to changes in arterial pressure and renal function seen in preeclampsia.

Cellular hypoxia is a well known inducer of ET-1 release. Plasma ET-1 concentrations are increased in women with preeclampsia and could contribute to the hypertension that characterizes preeclampsia.[30] Our study demonstrated elevated ET-1 levels in L-NAME treated pregnant rats on day 20 of gestation as compared with control rats. These results are consistent with the study of Baksu et al[31] who reported significant increase in plasma ET-1 level in preeclamptic women in comparison with both nonpregnant and normotensive pregnant women. The mechanism of the ET-1 increase in preeclampsia is unknown, but some hypotheses can be made. First, ET-1 is basically local factor acting at the junction between the endothelium and the vascular smooth muscle layer. The disruption and destruction of these anatomical boundaries can lead to a leak of ET from its local environment to the circulation with subsequently higher peripheral blood levels. Second, an abnormal production of ET by the affected endothelium might be a source for its increase, both locally and in the peripheral blood. Third, the increased production of ET-1 from the placental or fetal tissue in preeclamptic pregnancies or increased diffusion into the maternal circulation might explain the increased levels found in preeclampsia.[32]

The present study demonstrated the beneficial lowering effects of anti TNF-α antibodies treatment of pregnant rats on plasma ET-1 level. Decreased plasma ET-1 level and the associated reduction in the mean blood pressure in anti TNF-α antibodies treated pregnant rats prove the role of this contracting factor in pregnancy-induced hypertension. It is possible that TNF-α could have a specific role in stimulating secretion of ET-1, particularly into an intrauterine vascular bed that has retained its smooth muscle coat, capable of contraction.[33] The renin-angiotensin system (RAS) has been implicated in the pathogenesis of preeclampsia. In the gravid state, in addition to RAS in the kidney, there is a tissue-based RAS in the uteroplacental unit. Although the etiology of preeclampsia remains unknown, the most compelling studies indicate that the placenta is the culprit. Nevertheless, there is discrepancy of available data whether RAS is upregulated in preeclampsia. In the circulating levels, Ag II appears to be increased in preeclampsia[34] consistent with our results where Ag II levels were significantly increased in preeclamptic non-treated rats on day 20 of gestation. Increased renin expression in human preeclampsia and in transgenic mouse models shows that activation of the uteroplacental RAS, with Ag II entering the systemic circulation, may mediate the pathogenesis of preeclampsia. Vascular maladaptation in manifest preeclampsia may be explained on the basis of Ag II mediated mechanisms through Ag receptor type I (AT1) activation. In previous study, novel Ag II-related bimolecular mechanisms have described in preeclampsia and may explain the primary clinicopathologic features of preeclampsia.[35]

Anti TNF-α antibodies treatment decreased plasma levels of Ag II on day 20 of gestation as compared to L-NAME treated pregnant rats on the same day of gestation, but it was significantly increased as compared to control and non-treated pregnant rats. The placenta is known to be a major source of IL-6 during pregnancy. Bowen et al[36] found that trophoblast cells from preeclamptic placentas produced more IL-6 than those from normal placentas when cells were cultured under normoxic condition. They further found significant increases in IL-6 productions by trophoblast cells when they were cultured under hypoxic condition from both normal and preeclamptic pregnancies. Anti TNF-α antibodies were able to reduce the levels of IL-6 on day 20 of gestation, which indicates its possible beneficial effect in reducing oxidative stress.

Our results agree with those of Takacs et al[37] who reported elevated plasma IL-6 in preeclamptic women compared with normal pregnant and control non-pregnant women. These authors explained the overproduction of IL-6 in preeclampsia not likely to be of placental origin; rather IL-6 may drive from other tissues as the Kupffer cells or the endothelium, possibly in response to TNF–stimulation.

In the present study, we found significantly higher sVCAM-1 concentrations in the serum of L-NAME treated preeclamptic rats on day 20 of gestation. The increase in sVCAM-1may reflects leukocyte adhesion to the endothelium or an endothelial dysfunction. These results are in agreement with Catarino et al[38] who found higher concentrations of sVCAM-1 in addition to IL-6 in preeclamptic pregnant women as compared with normotensive pregnant women. They reported that preeclampsia is associated with an enhanced maternal inflammatory condition. Moreover, they explained enhanced inflammatory state seems to be related to endothelial dysfunction and increased cytokine synthesis, rather than with neutrophil activation. However, controversial results have been published.[39] Some authors proposed that the susceptibility to inflammation might be a common underlying risk

factor for preeclampsia.[40] These authors explained the mechanism for this inflammatory susceptibility to be attributable to a generalized up regulation of a number of inflammatory processes or could be a specific over activity of a particular tissue such as adipose tissue.[40] Treatment of pregnant rats with anti TNF-α antibodies, restored serum sVCAM-1 level to normal levels on day 20 of gestation.

Study limitation. The sample size is small. But because of the limited budget and many parameters measured, we could not increase the sample size.

In conclusion, preeclampsia is associated with disturbed renal function, overproduction of cytokines and vasoregulatory factors, and fetal growth restriction. Treatment of pregnant rats with anti TNF-α antibodies, restored urine volume, creatinine clearance, plasma ET-1, serum IL-6 and sVCAM-1 to normal levels. Hence, anti TNF-α antibodies may have beneficial effects in preeclampsia. Additional studies are warranted to confirm these results.

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