Bevacizumab-treated pregnant rats may constitute an experimental model for studying preeclampsia

Gebe sıçanlara bevacizumab uygulayarak preeklampsi çalışmalarında kullanılabilecek deneysel hayvan modeli oluşturulabilir

Abdullah Boztosun, Şafak Olgan, Hatice Ozer, Remzi Atilgan, Sehmus Pala

Abstract

Purpose: To develop a rat model of preeclampsia by administering bevacizumab, an angiogenesis inhibitor.
Materials and methods: Sixteen pregnant rats were randomly allocated to intraperitoneal injection of 10 mg/kg bevacizumab or 0.1 cc intraperitoneal serum physiologic on the 4th and 8th days of gestation. Blood pressure, body weight, and proteinuria were measured on both day 0 (D0) and day 20 (D20). Blood samples were collected on D20 for analysis, including for determining vascular endothelial growth factor (VEGF) and soluble Fms-like tyrosine kinase 1 (sFlt-1) levels. On the same day, the mice were euthanized, the placentas and pups were weighted, and the angiogenesis markers and microvessel density were evaluated using immunohistochemical methods.
Results: Lower serum VEGF (p=0.038) and higher SFlt-1 (p=0.015) levels were observed in bevacizumab-treated pregnant rats. Bevacizumab-treated pregnant rats had significantly higher systolic (p=0.050) and diastolic (p = 0.046) blood pressures compared to the controls. Additionally, the bevacizumab group showed a significant increase in proteinuria on D20 compared to that on D0 (p=0.026). Although higher serum AST, ALT, BUN, and creatinine levels and renal glomerular endotheliosis scores as well as lower placental VEGF and microvessel density were noted in bevacizumab-treated rats, these differences were not statistically significant (p > 0.05 for each).
Conclusion: The promising results of this trial show that bevacizumab treatment in pregnant rats might provide a model to study human preeclampsia.

Key words: Bevacizumab, preeclampsia, rat model

Boztosun A, Olgan Ş, Ozer H, Atilgan R, Pala S. Bevacizumab-treated pregnant rats may constitute an experimental model for studying preeclampsia. Pam Med J 2018;11(3):301-308.

Özet

Amaç: Bir anjiogenez inhibitörü olan bevacizumab uygulayarak sıçanlarda preeklampsi modeli oluşturmak.
Gereç ve Yöntem: Toplam 16 gebe sıçana intraperitoneal 10 mg/kg bevacizumab ya da 0.1 cc serum fizyolojik 4 ve 8. günlerde uygulandı. Sıçanların kan basınçları, ağırlıkları ve proteinüri miktarları 0 ve 20. günlerde değerlendirildi. 20. günde sıçanlardan kan örnekleri alınarak vasküler endotelyal büyüme faktörü (VEGF) ve çözülebilir Fms benzeri protein kinaz (SFlt-1) seviyeleri ölçüldü. Aynı gün ötenazi uygulanılarak plasenta ve yavruların ağırlıkları değerlendirildi. Immünohistokimyasal olarak anjiogenez belirteçleri ve mikro damar yoğunluğu incelendi.
Bulgular: Bevacizumab uygulanan grupta daha düşük VEGF (p=0.038) ve daha yüksek SFlt-1 (p=0.015) seviyeleri saptandı. Benzer şekilde bevacizumab uygulanan sıçanların sistolik (p=0.050) ve diastolik (p=0.046) kan basınçları daha yüksek bulundu. Bevacizumab uygulanan sıçanlarda 20. günde proteinüri miktarları 0. güne göre belirgin olarak daha yüksekti (p=0.026). Her ne kadar daha yüksek AST, ALT, BUN, kreatinin, böbrek glomerüler endotelyosis skorları ile daha düşük plasental VEGF ve mikro damar yoğunluğu gözlenmiş olsa da, istatistiksel olarak anlamlı fark saptanmadı (p>0.05).
Sonuç: Çalışmamızda gebe sıçanlara bevacizumab uygulayarak preeklampsi modeli oluşturulabileceğine dair olumlu sonuçlar elde edilmiştir**.**

Anahtar sözcükler: Bevacizumab, preeklampsi, sıçan modeli

Boztosun A, Olgan Ş, Ozer H, Atilgan R, Pala S. Gebe sıçanlara bevacizumab uygulayarak preeklampsi çalışmalarında kullanılabilecek deneysel hayvan modeli oluşturulabilir. *Pam Tıp Derg* 2018;11(3);301-308.

Abdullah Boztosun, Doç.Dr., Department of Obstetrics and Gynecology, Faculty of Medicine, Akdeniz University, ANTALYA, e-posta:abdullahboztosun@akdeniz.edu.tr (orcid.org/0000-0002-1934-7106)

Safak Olgan, Dr.Öğr.Üyesi, Department of Obstetrics and Gynecology, Faculty of Medicine, Akdeniz University, ANTALYA, e-posta: safakolgan@gmail.com (orcid.org/0000-0001-6479-3626) (Sorumlu yazar)

Hatice Ozer, Doç.Dr., Department of Pathology, Faculty of Medicine, Cumhuriyet University, SIVAS, e-posta: haticeozer@gmail.com (orcid.org/0000-0003-2635-7158)

Remzi Atilgan, Dr.Öğr.Üyesi, Department of Obstetrics and Gynecology, Faculty of Medicine, Firat University, ELAZIG, e-posta: remzi_atilgan@hotmail.com (orcid.org/0000-0003-2635-7158)

Sehmus Pala, Dr.Öğr.Üyesi, Department of Obstetrics and Gynecology, Faculty of Medicine, Firat University, ELAZIG, e-posta: sehmuspala@hotmail.com (orcid.or/ 0000-0002-2376-4244)

Introduction

Preeclampsia complicates 3%--5% of pregnancies and remains one of the major causes of maternal and neonatal morbidities [1]. The precise pathophysiologic mechanisms of preeclampsia remain unknown; therefore, effective preventive strategies remain elusive, and delivery remains the main approach for preventing maternal morbidity and mortality [2-5]. A disruption in the balance between angiogenic factors such as vascular endothelial growth factor (VEGF) or placental growth factor (PlGF) and antiangiogenic factors such as soluble Fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) is believed to precede preeclampsia [6, 7].

It is known that VEGF plays a major role in angiogenesis [8, 9]. VEGF-A, the major type of VEGF, is known to potentiate the vascular permeability and induce vasodilatation [10]. Additionally, it regulates the vascular tonus; suppresses endothelial apoptosis; and inhibits vascular adhesion, platelet aggregation, and thrombosis [11]. Several studies have evaluated the effect of anti-VEGF treatment in nonpregnant rats. It was observed that anti-VEGF treatment in adult animals causes proteinuria with glomerular endothelial damage [12]. Moreover, studies of anti-VEGF treatment in the context of antiangiogenic cancer treatment established proteinuria, hypertension, and loss of glomerular endothelial pores [12, 13]. Impaired placental vasculogenesis and early embryo mortality might also occur owing to the absence of the VEGF gene that plays the key role in angiogenesis [14]. Additionally, it was hypothesized that the inhibition of angiogenesis during early placentation would create hypertension concomitant with placental and fetal dysfunction. Suramin, a potent VEGF inhibitor, was studied to mimic preeclampsia in early gestational weeks in pregnant rats [15, 16]. These studies have shown that the inhibition of uterine angiogenesis with suramin increases maternal blood pressure and compromises the development of the fetus and placenta. Similarly, Ahmed [17] proposed that sFlt-1, an alternative mRNA splice variant of the sFlt-1 gene, might be a key factor responsible for the clinical manifestation of preeclampsia because of the loss of circulating free VEGF.

The development of an animal model that replicates this complex pregnancy-related disorder may help to expand our understanding and may hold great potential for the design and implementation of an effective treatment. Although bevacizumab is the first antiangiogenic agent that has been used systemically, its effects on pregnant rats have not yet been studied. Therefore, in this study, we aimed to investigate the effect of bevacizumab on pregnant rats and to generate a maternal and fetal condition resembling human preeclampsia with the goal of developing a useful animal model of this disorder.

Materials and Methods

Obtaining Pregnant Rats

Sixteen Wistar albino rats weighing 200-250 g (12-16 weeks old) were obtained from the Animal Laboratory of Cumhuriyet University, Sivas, Turkey. Consent for using and studying the animals was obtained from the Institutional Test Animal Ethic Committee. Care was taken to select animals of similar age and weight. The animals were maintained with 8-mm standard rat pellets in special cages under standard conditions (12-h light-dark cycle, air conditioned, stable temperature). Each female rat was mated with male rats between 5:00 PM and 9:00 AM at 22°C. The next day, vaginal smear tests were performed to seek sperm. Day 0 (D0) of gestation was defined as the day when spermatozoa were found in the vaginal smear. Then, the rats were randomized into two groups (control and bevacizumab groups), with an equal number of rats in each group.

Study Groups

(1) Control group: Eight pregnant rats received intraperitoneal 0.1 cc serum physiologic on the 4th and 8th days.

(2) Bevacizumab group: Eight pregnant rats received intraperitoneal 10 mg/kg (0.1 cc) bevacizumab on the 4th and 8th days.

Experimental Procedure

The rats were killed using 40 mg/kg intraperitoneal ketamine HCl and 1 mg/kg xylazine hydrochloride on day 20 (D20) of gestation after performing a cesarean section under combined general anesthesia (ketamine

HCl 75 mg/kg and xylazine hydrochloride 1 mg/kg). The placentas and pups were removed by Cesarean section. The number and weight of the pups and placentas were recorded, and the placentas were placed in formalin for histopathologic examination after being washed with saline.

Measurement of Weight and Blood Pressure and Collection of Urine Sample

The weight and blood pressure of the rats were measured and urine samples were collected on D0 and D20 of gestation. Blood pressure was measured by using the tail-cuff blood pressure monitoring system (BPHR 9610 Blood Pressure System; Commat, Ankara, Turkey) [18]. The tails of anaesthetized rats were heated to dilate the tail vessels in a closed environment with a constant temperature of 39-40°C.

Protein Measurement in Urine Samples of Rats

The protein quantity in the urine samples obtained on D0 and D20 of gestation was measured by the dipstick method (006T250; Ulti Med Products GmbH, Ahrensburg, Germany). Proteinuria was identified by dropping the urine samples onto colorimetric strips. Proteinuria was graded as "j," "1+," "2+," "3+," or "4+" by comparing the color reaction to the color scale: "j" to "2+" indicates mild proteinuria (30–100 mg/dL) and "3+" and "4+" indicate severe proteinuria (300-2000 mg/dL)

Measurement of VEGF, sFlt-1, and Other Biochemical Markers

On D20, intracardiac blood samples were taken and all rats were sacrificed. The serum in the rat blood samples was separated and stored at -80°C until it was studied. According to the manufacturer's advice (Ray Biotech, Inc., Norcross GA, USA, and Cusabio Biotech Co., Ltd., Hubei, PRC), VEGF and sFlt-1 serum concentrations were determined two times with Elisa kept on serum. Moreover, ALT, AST, BUN, and creatinine levels in the serum samples were measured using an otoanalyser (Advia 1800 Autoanalyser; Bayer Diagnostic, Leverkusen, Germany).

Light Microscopy and Immunohistochemical Evaluation

The placental sections were paraffinized in xylene and dehydrated by migration through 80°, 90°, 96° +ethanol. Then, the endogen peroxidase activity was blocked by incubation in hydrogen peroxide for 15 min, and the sections were heated in ethylenediaminetetraacetic acid (EDTA) solution of pH 8.1 in a microwave pressure cooker for 20 min. Next, the slides were left in the same solutions for 20 min at room temperature, following which they were incubated by UV blockage for 20 min. The sections were incubated for another 2 h with CD31 antibodies (rabbit polyclonal, Cat. #RB-10333-R7, Thermo Scientific, USA), CD105 antibodies (mouse monoclonal clone HZ52, Neomarkers, USA), VEGF (mouse monoclonal clone 2D2, Neomarkers, USA), and sFlt-1 (mouse monoclonal clone 8C8, Neomarkers, USA) at room temperature under damp conditions. Then, the sections were incubated in order for 20 min using a binding solution (link), for 20 min in streptavidin peroxidase, and for 20 min in AEC chromogen. Finally, Mayer hematoxylin was used for counterstaining for 1 min. All sections were rinsed with phosphate buffered saline (PBS) solution after each step.

The microvessel density was determined by counting the number of microvessels stained with CD31 and CD105 (Figure 1) in an entire 1-mm core. In addition to capillaries and venules stained with CD31 and CD105+, accumulated endothelial cells without lumen and individually spreading endothelial cells were also counted. VEGF (Figure 2) and sFlt-1 (Figure 3) expressions were semiquantitatively evaluated by measuring the histological score (H score) according to the method described by Donnez et al. [19]. The histological scores were calculated using the intensity and percentage of staining. The intensity of staining was scored from 0 (-) to 3 (intensely stained cells). The percentage of staining was scored as 1 (<15%), 2 (15%–50%), 3 (50%–85%), 4 (>85%), and 5 (100%). The H score is obtained as the product of these two scores.

All sections were examined by the same pathologist (HO), and the pathologist was not aware of what group the sections belonged to. Renal sections were stained with H&E and PAS, and glomerular endotheliosis was semiquantitatively analyzed under a microscope. For this evaluation, 100 randomly chosen glomerules were used, and histopathological changes such as endothelial swelling (ES), capillary loop occlusion (CO), and protein

droplets (PD) were graded, as shown in Figure 4 [20]. The following grading was used: grade 0=normal; grade 1=mild CO, no PD; grade 2=mild CO, <25% PD; grade 3=severe CO, <50% PD; and grade 4=tremendous CO, >50% PD.
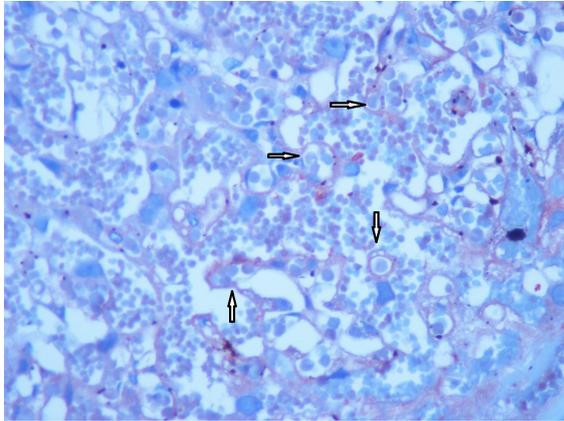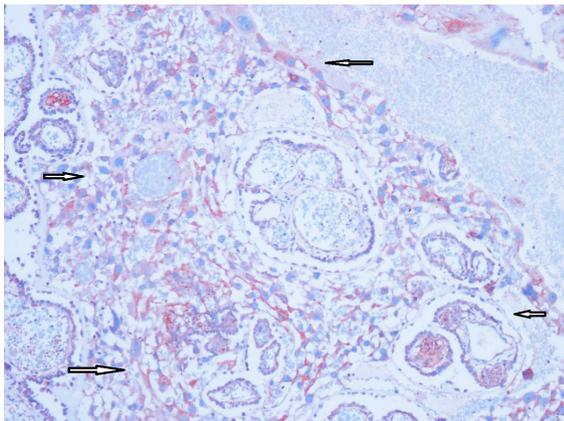

Figure 1. CD105 immunostaining, 400 x magnification
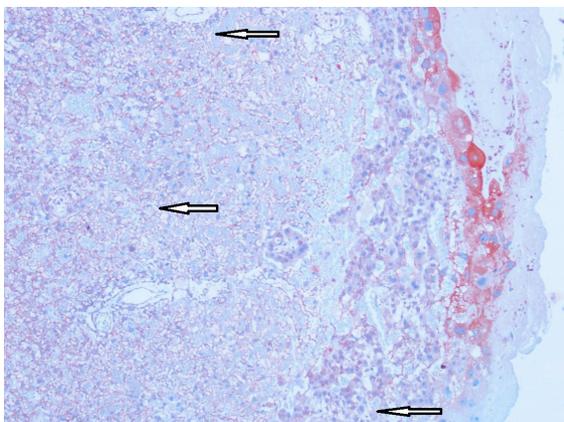

Figure 2. VEGF immunostaining, 100 x magnification

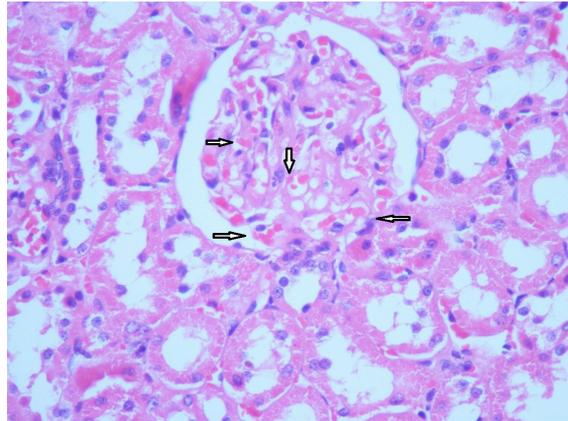**Figure 3.** sFlt-1 immunostaining, 40 x magnification

Figure 4. Protein droplets, image of renal glomerular endotheliosis. H&E staining, 400 X

The data were analyzed using Microsoft Statistical Package for Social Sciences (SPSS) for Windows version 22.0. The Mann–Whitney U test was conducted to analyze continuous and discrete ordinal variables. A Wilcoxon signed rank test was used to compare continuous variables measured on two different occasions. $p<0.05$ was considered statistically significant.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Cumhuriyet University, Sivas, Turkey

Informed Consent: N/A.

Results

VEGF was significantly lower in bevacizumab-treated pregnant rats than in the controls (p = 0.038) (Table 1). By contrast, the sFlt-1 concentration was found to be significantly higher in bevacizumab- treated pregnant rats than in the controls (p=0.015). With regard to the biochemical parameters, there were no differences in AST, ALT, BUN, creatinine, and proteinuria levels between the two groups (p > 0.05). Although the blood pressures on D0 were similar between the bevacizumab- treated and control groups (p>0.05), both systolic (p=0.050) and diastolic (p=0.046) blood pressures were found to be significantly higher in bevacizumab-treated pregnant rats than in the controls. With regard to the systolic and diastolic blood pressures and the proteinuria levels measured on D0 and D20, the Wilcoxon signed rank test did not reveal a statistically significant difference on two occasions in the control group. Similarly, the maternal blood pressures of the bevacizumab-treated pregnant rats on D0 and

D20 were found to be comparable. However, bevacizumab-treated pregnant rats showed a statistically significant increase in proteinuria on D20 compared to that on D0 ($p=0.026$) (Table 1).

The maternal weight and weight gain on D0 and D20 were comparable between the bevacizumab-treated pregnant rats and the controls ($p>0.05$) (Table 2). Similarly, there were no significant differences in the number of pups per litter and the mean fetal weight between the two groups ($p>0.05$). The immunohistochemical evaluation of the placental sections revealed that the microvessel density was comparable between the bevacizumab-treated pregnant rats and the controls ($p>0.05$). Moreover, the glomerular endotheliosis scores obtained from the histopathological evaluation were found to be similar between the two groups ($p>0.05$). Although there was no significant difference in placental VEGF ($p > 0.05$), placental sFlt-1 expression was significantly higher in the bevacizumab-treated pregnant rats than in the controls ($p=0.050$) (Table 2).

Discussion

Because preeclampsia occurs only in humans and primates, no appropriate rodent model is available for this disease.

Therefore, understanding the pathogenesis of preeclampsia remains challenging [21]. The imbalance of angiogenesis (elevated sFlt-1 and decreased PlGF and VEGF) owing to genetic, immunologic, and undefined factors might cause placental hypoxia, thereby starting a vicious cycle that results in preeclampsia [22]. The spontaneous or induced therapeutic effects of VEGF on hypertension and renal dysfunction have been reported previously [20, 23]. Thus, VEGF, which plays a crucial role in angiogenesis, might be the main pathophysiologic mechanism for this disease. It is known that invasive cytotrophoblasts secrete VEGF, PlGF, and sFlt-1. Zhou et al. found that the excretion of these proteins is impaired in preeclampsia [24]. Insufficient invasion of cytotrophoblasts might disrupt this pathway in the early gestational weeks of preeclamptic women [25]. Suramin, an angiogenesis inhibitor, has been used to realize a preeclampsia model for 10 years [15, 16, 26]. In this model, endothelial dysfunction, the major factor in the physiopathology of preeclampsia, was demonstrated [27]. Although bevacizumab is the first antiangiogenic agent that has been used systemically, its effects on pregnant rats have not yet been studied. This model might be advantageous in that there was no direct intervention to VEGF receptors. When compared with the model in which the sFlt-

Table 1. Comparison of blood pressures, proteinuria and biochemical parameters between bevacizumab-treated pregnant rats and the control group

		Control Group (n=8)	Bevacizumab Group (n=8)	*p* value
Blood VEGF (pg/ml)		28.84±13.83	15.31±17,62	**0.038**
Blood Sflt-1 (pg/ml)		108.11±26.50	168.47±59,87	**0.015**
AST (U/L)		146.87±29,31	162.25±22.48	0.382
ALT (U/L)		76.75±22.46	83.87±10.92	0.328
BUN (mg/dl)		18.75±4.92	21.00±3,77	0.382
Creatinine (mg/dl)		0.31±0.04	0.36±0.06	0.105
Systolic blood pressure (mm Hg)	D_0	120.87±6.89[a]	126.25±6.94[a]	0.130
	D_{20}	120.62±11.09	132.37±14.16	**0.050**
Diastolic blood pressure (mm Hg)	D_0	78.62±5.04[a]	83.37±6.69[a]	0.195
	D_{20}	78.00±3.81	84.75±7.62	**0.046**
Proteinuria	D_0	0.13±0.23[a]	0.13±0.23[b]	1.000
	D_{20}	0.50±0.46	0.88±0.64	0.084

VEGF, vascular endothelial growth factor; Sflt-1, soluble fms-like tyrosine kinase 1, AST, aspartate aminotransferase, ALT, alanine transaminase, BUN, blood urea nitrogen. Mann-Whitney U test and Wilcoxon Signed Rank Tests were used for analysis. Data are presented as mean ± SD. Boldface data indicates statistical significance ($p<0.05$).

[a] $p >0.05$, D_0 vs. D_{20}, [b] $p =0.026$, D_0 vs. D_{20}

Table 2. Comparison of fetal-maternal weight, histopathology and immunohistochemical results between bevacizumab-treated pregnant rats and the control group.

		Control Group (n=8)	Bevacizumab Group (n=8)	*p* value
Maternal weight (gr)	D_0	213.38 ± 6.93^a	213.50 ± 5.71^a	0.959
	D_{20}	242.50 ± 7.65	240.25 ± 10.36	0.878
Maternal weight gain (gr) (D_{20}-D_0)		29.13 ± 4.73	26.75 ± 7.30	0.505
Number of pups per litter		7.5 ± 0.92	7.37 ± 0.74	0.878
Mean fetal weight (gr)		2.19 ± 0.19	2.00 ± 0.17	0.274
Placental VEGF		6.31 ± 4.54	5.55 ± 3.64	0.328
Placental Sflt-1		5.62 ± 4.14	8.12 ± 4.82	**0.050**
Placental CD31		85.31 ± 5.96	81.93 ± 4.55	0.328
Placental CD105		83.81 ± 6.48	80.56 ± 5.80	0.234
Glomerular endotheliosis		1.75 ± 0.71	1.88 ± 0.84	0.798

VEGF, vascular endothelial growth factor; Sflt-1, soluble fms-like tyrosine kinase 1. Mann-Whitney U test and Wilcoxon Signed Rank Tests were used for analysis. Data are presented as mean ± SD.
Boldface data indicates statistical significance (*p*<0.05).
[a] *p* =0.012, D_0 vs. D_{20}

1 level is elevated by indirect VEGF inhibition owing to adenoviral transfer, it seems much easier and more specific.

In our study, lower serum VEGF as well as higher sFlt-1 levels were observed in bevacizumab-treated pregnant rats. We also found that bevacizumab-treated pregnant rats had significantly higher systolic and diastolic blood pressures compared to the controls. Additionally, the bevacizumab-treated group showed a significant increase in proteinuria on D20 compared to that on D0. Although higher serum AST, ALT, BUN, and creatinine levels and renal glomerular endotheliosis scores as well as lower placental VEGF and microvessel density were noted in bevacizumab-treated rats, these differences were not statistically significant in our study. Consequently, a post hoc, two-sided sample size calculation was performed. The results show that a sample size of 12-16 rats in each group is needed to realize a power of 80% with alpha error level of 5% for biochemical parameters, glomerular endotheliosis, and microvessel density. Thus, the present sample size might not be enough to demonstrate small differences in these variables. As the implantation of the hatched rat blastocyst was previously shown to occur on the 5th day of pregnancy [28], in our study,

the initial bevacizumab dose was administered on the 4th day just before implantation. With regard to dose assignment, a few recent studies have investigated the intraperitoneal use of bevacizumab [29-32]. In a previous study, healthy rabbits underwent a standardized procedure of debulking surgery and were randomized to receive intraperitoneal bevacizumab (25 mg/kg) or placebo [30]. It was found that the plasma concentration of bevacizumab increased to a peak level at 24 h post-administration. However, interestingly, bevacizumab was not detected in the plasma of animals in which surgery was not performed. Pavlidis et al. evaluated the effect of intraperitoneal 5 mg/kg bevacizumab on an abdominal wound healing model in rats. They did not observe any significant effect when evaluating this low dosage [32]. In contrast, a much lower dose of bevacizumab (2.5mg/kg) was demonstrated to be effective in cecal abrasion and uterine horn models for evaluating postoperative bands [29, 31]. Consequently, the results of previous studies on the effectiveness of intraperitoneal usage of bevacizumab are considered conflicting. Therefore, we considered previous studies' outcomes and decided to use a higher dose than that used in the adhesion [29] and a lower dose than that used in debulking surgery models [30]. Furthermore, bevacizumab was administered

two times, as in previous adhesion models. Despite these measures, the bevacizumab (10 mg/kg) administered two times might still be insufficient to realize a complete model mimicking the maternal and fetal condition of preeclampsia. This might be because our rats had no peritoneal defects that prevented efficient absorption and adequate concentrations of the drug in rats' serum. To solve this problem, higher doses (e.g., 25 mg/kg) or alternative drug administration routes could be used. Measuring the serum bevacizumab concentrations could justify the adequate dose as well.

This study demonstrates that bevacizumab treatment reduces the circulating levels of VEGF and increases those of sFlt-1. This is followed by proteinuria and hypertension, in turn resulting in a maternal condition in pregnant rats that resembles human preeclampsia. Dose response studies and the evaluation of alternative administration routes for bevacizumab are still required to clarify all the key features of the disorder.

Conflict of Interest: No conflict of interest was declared by the authors.

References

1. ACOG practice bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002. Obstet Gynecol 2002;99:159-167.

2. Barton JR, Sibai BM. Prediction and prevention of recurrent preeclampsia. Obstet Gynecol 2008;112:359-372.

3. Makrides M, Duley L, Olsen SF. Marine oil, and other prostaglandin precursor, supplementation for pregnancy uncomplicated by pre-eclampsia or intrauterine growth restriction. Cochrane Database Syst Rev 2006;3:CD003402.

4. Roberts JM, Myatt L, Spong CY, et al. Vitamins C and E to prevent complications of pregnancy-associated hypertension. N Engl J Med 2010;362:1282-1291.

5. Xu H, Perez-Cuevas R, Xiong X, et al. An international trial of antioxidants in the prevention of preeclampsia (INTAPP). Am J Obstet Gynecol 2010;202:1-10.

6. Levine RJ, Maynard SE, Qian C, et al. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 2004;350:672-683.

7. Levine RJ, Lam C, Qian C, et al. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N Engl J Med 2006;355:992-1005.

8. Tischer E, Mitchell R, Hartman T, et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 1991;266:11947-11954.

9. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003;9:669-676.

10. Ku DD, Zaleski JK, Liu S, Brock TA. Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. Am J Physiol 1993;265:586-592.

11. Zachary I. Signaling mechanisms mediating vascular protective actions of vascular endothelial growth factor. Am J Physiol Cell Physiol 2001;280:1375-1386.

12. Sugimoto H, Hamano Y, Charytan D, et al. Neutralization of circulating vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1) induces proteinuria. J Biol Chem 2003;278:12605-12608.

13. Zhu X, Wu S, Dahut WL, Parikh CR. Risks of proteinuria and hypertension with bevacizumab, an antibody against vascular endothelial growth factor: systematic review and meta-analysis. Am J Kidney Dis 2007;49:186-193.

14. Claesson-Welsh L. Vascular Endothelial Growth Factors. Springer 1999:123.

15. Waltenberger J, Mayr U, Frank H, Hombach V. Suramin is a potent inhibitor of vascular endothelial growth factor. A contribution to the molecular basis of its antiangiogenic action. J Mol Cell Cardiol 1996;28:1523-1529.

16. Nash P, Wentzel P, Lindeberg S, et al. Placental dysfunction in Suramin-treated rats--a new model for pre-eclampsia. Placenta 2005;26:410-418.

17. Ahmed A. Heparin-binding angiogenic growth factors in pregnancy. Trophoblast Res 1997;18:215-58.

18. Buñag RD, Butterfield J. Tail-cuff blood pressure measurement without external preheating in awake rats. Hypertension 1982;4:898-903.

19. Donnez J, Smoes P, Gillerot S, Casanas-Roux F, Nisolle M. Vascular endothelial growth factor (VEGF) in endometriosis. Hum Reprod 1998;13:1686-1690.

20. Li Z, Zhang Y, Ying Ma J, et al. Recombinant vascular endothelial growth factor 121 attenuates hypertension and improves kidney damage in a rat model of preeclampsia. Hypertension 2007;50:686-692.

21. Podjarny E, Baylis C, Losonczy G. Animal models of preeclampsia. Semin Perinatol 1999;23:2-13.

22. Bdolah Y, Karumanchi SA, Sachs BP. Recent advances in understanding of preeclampsia. Croat Med J 2005;46:728-736.

23. Yang R, Ogasawara AK, Zioncheck TF, et al. Exaggerated hypotensive effect of vascular endothelial growth factor in spontaneously hypertensive rats. Hypertension 2002;39:815-820.

24. Zhou Y, McMaster M, Woo K, et al. Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. Am J Pathol 2002;160:1405-1423.

25. Wang A, Rana S, Karumanchi SA. Preeclampsia: the role of angiogenic factors in its pathogenesis. Physiology (Bethesda) 2009;24:147-158.

26. Carlström M, Wentzel P, Skøtt O, Persson AE, Eriksson UJ. Angiogenesis inhibition causes hypertension and placental dysfunction in a rat model of preeclampsia. J Hypertens 2009;27:829-837.

27. Turgut NH, Temiz TK, Bagcivan I, Turgut B, Gulturk S, Karadas B. The effect of sildenafil on the altered thoracic aorta smooth muscle responses in rat pre-eclampsia model. Eur J Pharmacol 2008;589:180-187.

28. Maeda K, Ohkura S, Tsukamura H. Physiology of reproduction. In: Krinke GJ, editor. The Laboratory Rat, New York: Academic Press, 2000;145-176.

29. Ignjatovic D, Aasland K, Pettersen M, et al. Intra-abdominal administration of bevacizumab diminishes intra-peritoneal adhesions. Am J Surg 2010;200:270-275.

30. Passot G, Dupré A, Rivoire M, Mohamed F, Bakrin N, Glehen O. Intraperitoneal bevacizumab combined with cytoreductive surgery: a pre-clinical study of tolerance and pharmacokinetics in an animal model. Clin Transl Oncol 2012;14:931-936.

31. Moraloglu O, Işik H, Kiliç S, et al. Effect of bevacizumab on postoperative adhesion formation in a rat uterine horn adhesion model and the correlation with vascular endothelial growth factor and Ki-67 immunopositivity. Fertil Steril 2011;95:2638-2641.

32. Pavlidis ET, Ballas KD, Psarras K, et al. Intraperitoneal administration of bevacizumab intraoperatively does not affect abdominal wound healing in rats. Eur Surg Res 2011;47:45-51.