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Immunohistochemical Examination of Placental NRG-1 Expression in Pregnant Women Diagnosed with Preeclampsia

Preeklampsi Tanısı Alan Gebelerde Plasental NRG-1 Ekspresyonunun İmmünohistokimyasal Olarak İncelenmesi

Simge KİLİS[1], Mustafa TOSUN[1], Burcu GÜNAYDIN[2], Dilan ÇETİNAVCI[3], Burcu KASAP[4], Hülya ELBE[5]

[1]*Muğla Sıtkı Koçman University Faculty of Medicine, Medical Student, Muğla, Turkey*
[2]*Muğla Sıtkı Koçman University, Institute of Health Sciences, Department of Histology and Embryology, Muğla, Turkey*
[3]*Muğla Training and Research Hospital, Histology and Embryology, Muğla, Turkey*
[4]*Muğla Sıtkı Koçman University Faculty of Medicine, Department of Obstetrics and Gynecology, Muğla, Turkey*
[5]*Muğla Sıtkı Koçman University Faculty of Medicine, Department of Histology and Embryology, Muğla, Turkey*

ABSTRACT

Aim: Preeclampsia is a multisystemic disease characterized by hypertension, proteinuria, increased vascular damage and permeability, and superficial placental invasion that occurs during pregnancy. Neuregulin-1 (NRG-1) is an important ligand for embryogenesis, angiogenesis, nervous system development, myogenesis, and gonadogenesis. In our study it was aimed to examine the importance of NRG-1 in preeclampsia using light microscopy.

Materials and Methods: Study groups: Group 1 (control group) (n=10): healthy normal pregnants, Group 2 (preeclampsia group) (n=10): pregnant women diagnosed with preeclampsia. Hematoxylin Eosin and Periodic Acid-Schiff stainings were done. NRG-1 immunostaining was performed and scored.

Results: In the control group, the syncytiotrophoblast layer, villus stroma, fetal vascular structures and intervillous space were histologically normal. It was observed that the lumen of the villi in the placentas of preeclamptic pregnant women was narrowed and the number of villi was less than that of normal pregnant women. A significant increase in the number of nuclear chains was detected around the syncytial node and villi. It was observed that the number of fetal capillaries and fetomaternal barriers decreased. Intervillous and perivillous fibrin deposition was clearly observed. In the preeclampsia group, it was determined that the staining intensity with anti-NRG-1 around the villus was significantly less than the normal pregnant placentas in the control group ($p<0.05$).

Conclusion: Preeclampsia can cause pathological changes in the placenta at the light microscopic level, as well as a decrease in NRG-1 expression. We think that NRG-1 may be an important marker for the preeclampsia process.

Keywords: Preeclampsia, NRG-1, placenta, immunohistochemistry

ÖZ

Amaç: Preeklampsi; gebelikte ortaya çıkan hipertansiyon, proteinüri, artmış damar hasarı ve geçirgenliği ve yüzeyel plasenta istilası ile karakterize multisistemik bir hastalıktır. Neuregulin-1 (NRG-1), embriyogenez, anjiyogenez, sinir sistemi gelişimi, miyogenez, gonadogenez için önemli olan bir liganddır. Çalışmamızda NRG-1'in preeklampsideki öneminin ışık mikroskobik olarak incelenmesi amaçlanmıştır.

Gereç ve Yöntem: Çalışma grupları: Grup 1 (kontrol grubu) (n=10): sağlıklı normal gebelerden, Grup 2 (preeklampsi grubu) (n=10): preeklampsi tanısı alan gebelerden oluşmaktadır. Hematoksilen-Eozin ve periyodik asit-Schiff boyamaları yapıldı. NRG-1 immün boyaması yapılarak skorlandı.

Bulgular: Kontrol grubunda, sinsityotrofoblast tabakası, villus stroması, fetal vasküler yapılar ve intervillöz aralık normal histolojik yapıda izlendi. Preeklamptik gebelere ait plasentalardaki villus lümeninin daraldığı ve villusların normal gebelere göre sayıca daha az olduğu görüldü. Sinsityal nod ve villusların etrafında nükleer zincir sayısında belirgin artış tespit edildi. Fetal kapiller sayısının ve fetomaternal bariyer sayısının azaldığı gözlendi. İntervillöz ve perivillöz fibrin birikimi belirgin olarak izlendi. Preeklampsi grubunda, villus etrafındaki anti-NRG-1 ile boyanma yoğunluğunun kontrol grubundaki normal gebe plasentalarına göre anlamlı olarak daha az boyandığı tespit edildi ($p<0.05$).

Address for Correspondence: Dilan ÇETİNAVCI MD, Muğla Training and Research Hospital, Histology and Embryology, Muğla, Turkey
Phone: +90 531 352 45 66 **E-mail:** drdilancetinavci@hotmail.com **ORCID ID:** orcid.org/0000-0002-4148-7711
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Sonuç: Preeklampsi, plasentada ışık mikroskobik düzeyde patolojik değişikliklere neden olabildiği gibi, NRG-1 ekspresyonunda da azalmaya neden olmaktadır. NRG-1'in preeklampsi süreci için önemli bir belirteç olabileceğini düşünmekteyiz.

Anahtar Kelimeler: Preeklampsi, NRG-1, plasenta, immünohistokimya

INTRODUCTION

Preeclampsia is a multisystem disease that occurs in 5-10% of pregnant women and is characterized by maternal hypertension, proteinuria, increased vascular damage and vascular permeability, and is one of the main causes of perinatal deaths and disabilities[1,2]. Preeclampsia is defined as *de novo* hypertension accompanied by new-onset proteinuria after the 20th week of gestation. Hypertension is blood pressure measured above 140/90 mmHg at least two times. Proteinuria is defined as the leakage of 300 mg of protein in the urine in 24 hours[3]. Preeclampsia is a disease specific to the human species, and preeclampsia or similar pregnancy pathologies are not observed in other species[4]. Although the cause of preeclampsia is still not fully determined, it is accepted that more than one factor plays a role in it.

The placenta is an organ that continues to exist during pregnancy, with functions such as nutrient delivery to the fetus through the mother, thermoregulation, ensuring fetal immunity, hormone secretion, waste removal and gas exchange[5]. Considering that preeclampsia is a disease specific to pregnancy and that the findings completely regress with the evacuation of the placenta, it has been accepted that the most important role in the development of preeclampsia belongs to the placenta[6]. It is thought that endothelial damage, decreased perfusion and vasospasm in placental vessels play an important role in the pathogenesis of this disease[3,7]. It has been determined that in preeclampsia, there is an increase in placenta-derived oxidative stress and an increase in the levels of oxidative stress products in the maternal circulation, as well as a decrease in antioxidant activity[8]. Recently, many studies have been conducted on the effects of the placenta on the fetus. For this purpose, various placental cells, proteins, hormones and factors have been examined. These factors affecting the development of the fetus and placenta reveal a lot of important information about the fetus and placenta[9].

The roles of angiogenic proteins, cell adhesion molecules and the inflammatory system in microvascular dysfunction are of great importance in the pathophysiology of preeclampsia[3]. Neuregulin-1 (NRG-1), a current signaling pathway molecule investigated in the pathogenesis of various diseases, is a ligand that binds to ErbB receptors, provides activation and mediates intercellular interactions[10,11]. NRG-1 produces fifteen different proteins and is important for angiogenesis, embryogenesis, myogenesis, gonadogenesis and nervous system development[11,12]. NRG-1 sends signals to target cells by interacting with transmembrane tyrosine kinase receptors of the ErbB family. In this way, it stimulates proliferation, migration and differentiation in cells[13]. When scientific publications made to date are examined, it is understood that preeclampsia is an important disease among pregnant women. Although there are studies in the literature examining the connections of NRG-1 with various diseases, there is no study investigating its role in preeclampsia. The immunohistochemical distribution and expression level of NRG-1 in normal pregnant women and pregnant women diagnosed with preeclampsia are unknown. Therefore; in our study, we aimed to examine the importance of NRG-1 in preeclampsia by comparing NRG-1 expression in the placenta of normal pregnant women with the placenta of pregnant women diagnosed with preeclampsia.

MATERIALS AND METHODS

Groups

The study was carried out with placenta tissue samples taken from volunteer pregnant women who applied to Muğla Sıtkı Koçman University Faculty of Medicine, Obstetrics and Gynecology outpatient clinic and wanted to participate by reading and signing the informed consent form. Study groups: Group 1 (control group): Healthy normal pregnant women without any disease (n=10). Group 2 (preeclampsia group): Pregnant women diagnosed with preeclampsia (n=10). Control group consists of volunteer healthy pregnant women who applied to the outpatient clinic and did not have coronary artery, liver, kidney disease or diabetes. Pregnant women were given a routine pregnancy examination by a Obstetrics and Gynecology specialist. The diagnosis of preeclampsia was made after the 20th week of gestation with blood pressure of 140/90 mmHg and above and proteinuria of 0.3 g/24 hours and above.

Criteria for inclusion in the study:

- Healthy control group - 38-40. gestational week - Volunteer to participate in the research - Between 18-40 years old,

- Preeclampsia group - 38-40. gestational week - Volunteer to participate in the research - Between 18-40 years of age - Diagnosed with preeclampsia.

Criteria for exclusion from the study:

Those who have a severe physical illness, use of alcohol, cigarettes and drugs, body mass index >30, gestational

diabetes, pregestational diabetes, liver and kidney failure, any endocrine disorder, hematological disease, a history of gastric or intestinal surgery and received medical treatment for any reason in the last three months, cases with chronic inflammation or infection, and with fetal biometry outside the 10-90% percentile on obstetric ultrasound were excluded from the study.

Histological Analysis

Tissue samples taken from the placentas of the pregnant women who participated in the study after giving birth by cesarean section or vaginal delivery in the 38th to 40th week for analysis were washed with physiological saline in the operating room, then transferred to 10% formaldehyde solution and brought to the Histochemistry Laboratory of the Department of Histology and Embryology. After the placenta tissues were fixed in formaldehyde, they were subjected to routine histological follow-up procedures. The samples were first cut into 3-4 mm pieces and fixed in plastic tissue tracking cassettes at room temperature for 48 hours. After the fixation process was completed, the pieces were washed in running tap water for 24 hours, then dehydrated in increasing alcohol levels (70%, 80%, 96%, 100%), made transparent in xylene and embedded in paraffin. 5 µm sections taken from paraffin blocks with the help of a microtome device were placed on slides for histochemical and immunohistochemical examinations. After these prepared slides were kept in an oven at 37 °C for 2 hours to increase the adhesion of the tissue to the slide, Hematoxylin-Eosin (H-E) and Periodic-acid Schiff (PAS) staining methods were applied to observe the general histological structure. The sections were examined and photographed with a Nikon Eclipse 80i light microscope and Nikon image analysis system.

In this study, immunohistochemical staining method was applied using anti-NRG-1 antibody. After the sections were deparaffinized in an oven at 58 °C for 1 hour, they were kept in xylene for 15 minutes, then passed through a decreasing alcohol series (100-70%) and taken into distilled water. The sections that were kept in distilled water for 5-10 minutes and the sections that were placed in boiling citrate buffer to reveal the antigens were treated with citrate for 7 minutes and left to cool without being removed from the buffer. After the cooled sections were washed first with distilled water and then with phosphate buffer solution (PBS) for 1-2 minutes, the borders of the tissue sections were drawn with a hydrophobic pen (pappen) and placed in a humid medium container. Then, hydrogen peroxide was applied to the sections and it was waited for 10 minutes. Boiled water was placed on the platform to keep the medium warm and the lid of the platform was closed. Then, hydrogen peroxide was removed from the tissue and the sections were washed with PBS 3 times for 5 minutes, then the protein block was added and left for 5 minutes. Then, the protein block was removed by shaking the sections very well.

Before washing the sections, anti-NRG-1 (sc-393006) primary antibody, diluted at the rate recommended by the company, was added and kept in a humid environment for 2 hours. Then, after washing with PBS 3 times for 5 minutes, it was treated with secondary antibody for 10 minutes and washed with PBS 3 times for 5 minutes. In the next step, after streptavidin was treated with horse-radish peroxidase complex for 10 minutes, it was washed with PBS 3 times for 5 minutes and incubated with substrate-chromogen (AEC; 3, aminoethyl 9, carbazole) solution for 3 minutes (until a color reaction was observed). After washing with PBS 3 times for 5 minutes and shaking in distilled water, Mayer's hematoxylin stain was applied to the sections (1 minute). The sections were washed first in tap water and then in distilled water, and then sealed with entellan.

Evaluation of IHC staining: Immunostaining coverslips were evaluated under a light microscope (Nicon Eclipse 80i, Japan). Antibody-labeled areas on the coverslips were examined at x20 magnification. The staining intensity of the cells was scored as no staining (-), weak (+), moderate (++) and strong (+++) and photographs were taken with the image analysis system.

Statistical Analysis

Statistical analysis of the data was performed with Statistical Package for the Social Sciences 14 statistical program. Whether the data showed normal distribution was evaluated with the Kolmogorov-Smirnov test. Data that did not show normal distribution were analyzed using the Kruskal-Wallis variance test, and pairwise comparisons were performed using the Mann-Whitney U test. $P<0.05$ was considered statistically significant.

RESULTS

Histopathological Findings

Placental chorionic villi were examined histologically in sections stained with H-E and PAS. In the sections of the control group, the syncytiotrophoblast layer, villus stroma, fetal vascular structures and intervillous space were observed with a normal histological structure. It was determined that the number of free villi increased from the maternal side to the fetal side. The syncytiotrophoblast layer surrounding the villi was observed as a regular, thin layer. Syncytial knot and syncytial nuclear chain structures were detected very rarely. Fetal capillaries in the villi were seen to be numerous and scattered throughout the villus stroma. Fetomaternal barriers were observed to be thin and smooth (Figure 1).

When the H-E and PAS stained sections of preeclamptic pregnant women are examined; it was observed that the

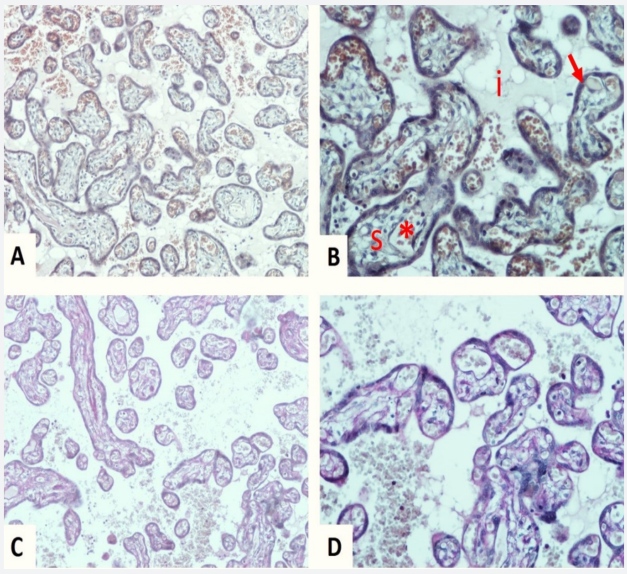

Figure 1. Placenta sections of the control group (taken from normal pregnant women). A) H-E; x10. B) H-E; x20. C) PAS; x10. D) PAS; x20. Star; fetal vascular structure, arrow; syncytiotrophoblast, s; stroma, i; intervillous space

H-E: Hematoxylin-Eosin, PAS: Periodic-acid Schiff

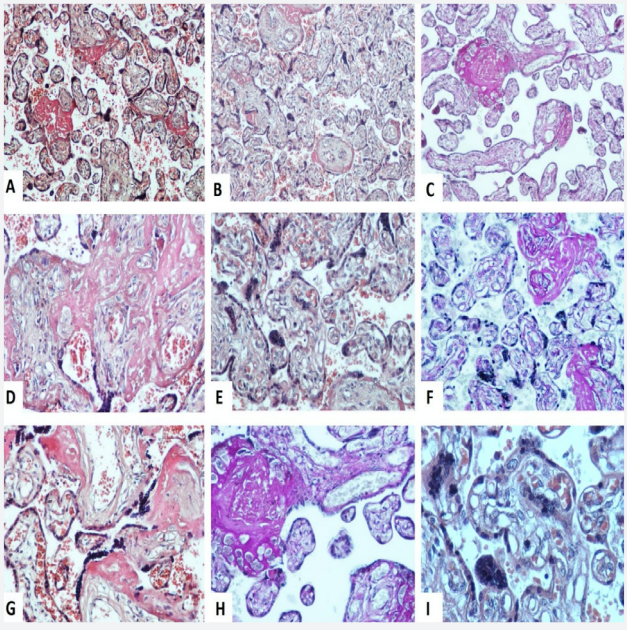

Figure 2. Placenta sections of pregnant women with preeclampsia (Group 2). A) H-E; x10. B) H-E; x10. C) PAS; x10. D) H-E; x20. E) H-E; x20. F) PAS; x20. G) H-E; x20. H) PAS; x20. I) H-E; x40

H-E: Hematoxylin-Eosin, PAS: Periodic-acid Schiff

villus lumen in the placentas narrowed and the villi were less in number than in normal pregnant women. A significant increase in the number of nuclear chains was detected around the syncytial nodes and villi. It was observed that the number of fetomaternal barriers and the number of fetal capillaries decreased. There was dilatation in some of the veins. It was observed that the terminal villous stroma was rich in collagen. Intervillous and perivillous fibrin accumulation was clearly observed (Figure 2).

Immunohistochemical findings were evaluated according to anti-NRG-1 staining intensity. In the placenta, anti-NRG-1 expression was observed in the nucleus and cytoplasm of the cells. Placental NRG-1 expression in the control group was observed to have strong (+++) staining in the trophoblast cells around the villi, and moderately weak (++) staining in the villus mesenchymal connective tissue cells and capillary endothelium. In the preeclampsia group, the intensity of anti-NRG-1 staining around the villi was found to be significantly less compared to the normal pregnant placentas in the control group ($p<0.05$) (Table 1, Figure 3).

DISCUSSION

Preeclampsia is a disease seen during pregnancy being, one of the leading causes of maternal and perinatal mortality and

Table 1. Anti-neuregulin-1 immunoreactivity

Groups	Staining intensity in trophoblasts	Villus mesenchymal staining intensity
Group 1: Normal	+++	++
Group 2: Preeclampsia	++	+

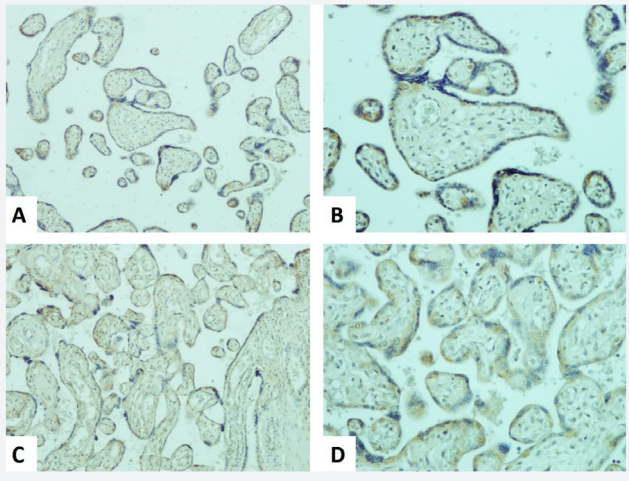

Figure 3. Anti-NRG-1 immunoreactivity. A) Group 1: Control, Normal placenta; x10. B) Group 1: Control; x20. C) Group 2: Preeclampsia; x10. D) Group 2: Preeclampsia; x20

NRG-1: Neuregulin-1

morbidity and affecting many systems in the body, including the kidney, liver, brain and lung, through endothelial damage[14]. In a study conducted to determine vascular endothelial growth factor (VEGF) protein levels in placenta biopsies of pregnant women with preeclampsia, Akercan et al.[15] revealed that in the preeclamptic group compared to normal pregnant placenta; the three layers in each villus, consisting of the outermost syncytial layer, intermediate cytotrophoblast, and inner fetal capillaries supported by mesenchyme, produce very strong VEGF immunoreactivity. In a study conducted on 34 pregnant women to demonstrate the relationship between NF-κB expression and trophoblastic cell apoptosis in pregnancies complicated by preeclampsia or intrauterine growth restriction; immunohistochemical examination of the placenta of pregnant women with preeclampsia showed that NF-κB expression increased, M30 expression increased, caspase-3 expression and bcl-2 expression decreased compared to normal pregnant women[16].

In the study by Shu et al.[17] where they investigated the clinical importance of apoptosis in the placenta with preeclampsia using the annexin V method; they found that annexin V was detected in trophoblasts in the placenta and that the staining intensity of annexin V in the placenta from these patients was reduced compared to normal placenta. Apoptosis is involved in every step of the pathogenesis of preeclampsia. Reduced apoptosis may induce a maternal immune response against the fetus, while increased apoptosis may influence the process of placental ischemia and subsequent systemic endothelial damage[18]. In our study, it was observed that, compared to the placentas of normal pregnancies, the villus lumen in the placentas of preeclamptic pregnancies was narrowed and the villi were fewer in number, and there was a significant increase in the number of nuclear chains around the syncytial nodes and villi. In addition, it was observed that the number of fetal capillaries and the number of fetomaternal barriers decreased in the placentas of preeclamptic pregnant women, and dilatation was observed in some of the veins. Terminal villous stroma was found to be rich in collagen. Intervillous and perivillous fibrin accumulation was clearly observed.

In recent studies; NRG-1 has been shown to be an important molecule for cardiovascular system development and maintenance of adult heart function and is a positive regulator of angiogenesis[19]. Russell et al.[20] reported that NRG-1 and ErbB molecules are expressed in vascular endothelial cells. However, in a study on myocardial cells carried out by Xu et al.[21] it has been shown that NRG-1 protects myocardial cells against oxidative damage by regulating endoplasmic reticulum stress. NRG-1 promotes trophoblast growth and differentiation[22]. Recent studies have shown that NRGs are endocrine regulators of metabolic events. NRG-1 plays an essential role in the growth and development of skeletal muscle, cardiac muscle and nervous tissue. The NRG-1/ErbB pathway is considered a potential target for the treatment of neuromuscular and cardiac disorders[23].

Preterm newborns with low NRG-1 levels have been found to be at higher risk of developing short-term morbidity[24]. It has been reported that NRG-1 may be a potential endogenous protector against perinatal brain white matter damage[25]. Fock et al.[22] showed that NRG-1 is also expressed by human decidual stromal cells and that the NRG-1-dependent ErbB2-ErbB3 signaling pathway is necessary for the survival of differentiated trophoblast cell populations. NRG-1 is also required for blastocyst implantation[26]. NRG-1 has been observed in the human placenta in both the maternal and fetal parts. It is thought that this may also be responsible for placental growth[11]. In our study, it was determined that the intensity of anti-NRG-1 staining around the villi in the preeclampsia group was significantly less than that of normal pregnant placentas in the control group ($p<0.05$).

Study Limitations

Although our study reveals the results we expected, it has some limitations. The most important limitation is that since preeclampsia is a multisystemic disorder, biochemical data should also have been available. In addition, data obtained from electron microscopy examinations can also make the results more reliable.

CONCLUSION

Preeclampsia, which may occur during pregnancy, may cause pathological changes in the placenta at the light microscopic level, as well as a decrease in NRG-1 expression. We think that NRG-1, which is important for angiogenesis, embryogenesis, myogenesis, gonadogenesis and nervous system development, may be an important marker for the preeclampsia process. This study is the first study to address the role of NRG-1, as well as showing the histopathological changes of the placenta in case of preeclampsia in pregnant women. In this respect, it has made a significant contribution to the literature at the basic knowledge level.

Ethics

Ethics Committee Approval: For this study, approval was received from Muğla Sıtkı Koçman University Clinical Research Ethics Committee with the decision dated 26.02.2020 and numbered 04/II, stating that it is ethically appropriate to conduct the study.

Patient Consent: Patient approval was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: B.K., Concept: H.E., S.K., M.T., Design: H.E., S.K., M.T., Data Collection or Processing: B.G., D.Ç., S.K., M.T., Analysis or Interpretation: H.E., D.Ç., S.K., M.T., Literature Search: D.Ç., S.K., M.T., Written by: D.Ç., H.E.

Conflict of Interest: The authors declared no conflict of interest regarding this article.

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