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Immunolocalization of Atrial Natriuretic Peptide and C-Type Natriuretic Peptide In Healthy and Pre-Eclamptic Human Placenta

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ABSTRACT The aim of this study was to determine the immunohistochemical distribution of atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP) in normal and pre-eclamptic human placentas. Tissue samples from maternal and fetal parts of pre-eclamptic (n:6) and healthy human placentas (n:6) were subjected to routine tissue processing. Avidin-biotin-peroxidase complex (ABC) immunohistochemistry was applied to formalin-fixed, paraffin-embedded placenta sections collected onto poly-l-lysine slides to demonstrate ANP and CNP expression. Immunohistochemistry revealed immunoreactivity for ANP and CNP in fetal and maternal tissues. On the fetal side of the preeclampsia group, strong ANP and CNP immunoreactivity were detected in the amniotic epithelium, fetal vessels, villous syncytiotrophoblast plate, villous vascular endothelial cells and smooth muscle cells than the healthy group. On the maternal side of the pre-eclampsia group, decidual cells exhibited lower ANP and CNP immunoreactivity than healthy group. These data indicate that increased ANP and CNP expression relating to preeclampsia might serve as a compensatory function to properly maintain uteroplacental perfusion and fetal development during pregnancy.

Keywords: *Preeclampsia, Atrial natriuretic peptide, C-Type natriuretic peptide, Placenta, Pregnancy*

ÖZ

Sağlıklı ve Pre-Eklampsili İnsan Plasentasında Atrial Natriüretik Peptid ve C-Tip Natriüretik Peptidin İmmunolokalizasyonu

Bu çalışmada sağlıklı ve pre-eklampsili insan plasentalarında atrial natriüretik peptid (ANP) ve C-tip natriüretik peptidin (CNP) immunohistakimyasal dağılımlarının belirlenmesi amaçlandı. Pre-eklampsili (n:6) ve sağlıklı insan plasentalarının (n:6) maternal ve fötal kısımlarından alınan doku örnekleri rutin doku işleme sürecinden geçirildi. ANP ve CNP ekspresyonunu göstermek için poli-lizinli lamlar üzerine alınan kesitlere avidin-biyotin-peroksidaz kompleks (ABC) yöntemi uygulandı. İmmunohistokimya, fetal ve maternal dokulardaki ANP ve CNP immunoreaktivitesini ortaya çıkarıldı. Pre-eklampsili grubun fötal tarafındaki amniyon epitelinde, fötal kan damarlarında, villöz sinsitiyotrofoblast hücrelerinde, villöz damar endotel hücrelerinde ve düz kas hücrelerinde sağlıklı gruba göre güçlü ANP ve CNP immunoreaktivitesi saptandı. Maternal tarafındaki desidual hücrelerde ise sağlıklı grubuna nazaran daha zayıf ANP ve CNP immunoreaktivitesi gözlendi. Bu sonuçlar, pre-eklampsili ile ilgili olarak artan ANP ve CNP ekspresyonunun gebelik sürecinde utero-plasental perfüzyon ve fötal gelişimin uygun bir şekilde devam etmesi için telafi edici bir fonksiyon görebileceğini akla getirmektedir.

Anahtar Kelimeler: *Pre-eklampsi, Atrial natriüretik peptid, C-Tip natriüretik peptid, Plasenta, Gebelik*

INTRODUCTION

Preeclampsia is a multifactorial pregnancy-specific vascular disorder which severely affects an estimated 3% to 8% of all pregnancies worldwide (Khan et al. 2006). It is a prevalent and severe complication arisen in human pregnancy and characterized by maternal hypertension, proteinuria and edema, placental dysfunction and intrauterine growth restriction (Ghuman et al. 2009). The leading cause of the disease is that multiple factors of genetical, dietary, hematological or autoimmunological origin upset the delicate balance between the production of endogenous vasodilator and vasoconstrictor substances (Brown 1995). This endocrine impairment results in vasospasm, increased sensitivity to vasoconstrictors and hypertension (Lim and Gude 1995; Graham et al. 1996). Restriction of fetal growth may be due to insufficient blood supply from placenta which develops secondary to reduced uteroplacental blood flow and placental-bed atherosis (Gude et al. 2000).

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Although its pathophysiology and etiology still remain elusive, the pre-eclampsia results from the failure to complete spiral artery remodeling, a process that transforms from the uterine spiral arteries into low-resistance, high-capacity vessels enabling adequate blood supply to the growing fetus (Boeldt and Bird 2017). In this process, extravillous trophoblasts invades the maternal spiral arteries, resulting in the derangement of smooth muscle cell and loss of endothelial lining from the vessel wall to dilate its lumen (Harris 2011). For a successful spiral artery remodeling, the process initiated with trophoblast invasion is tightly regulated by multiple factors (Redman and Sargent 2005). Among these factors, natriuretic peptides may have a pivotal role in the regulation of these processes.

Of natriuretic peptides, atrial natriuretic peptide (ANP) is a hormone secreted by the atria in response to increased blood volume, as well as synthesized by other organs such as hypothalamus, pituitary gland, adrenal medulla, gastrointestinal system, thymus, corpus luteum, ovarium and testis (Ivanova et al. 2003). ANP plays a role in female pregnancy by promoting trophoblast invasion and spiral artery remodeling in uterus (Wang et al. 2012; Zhou and Wu 2013). In pregnant rats, ANP receptors are localized to the placenta, placental maternal vessels, uterine smooth muscle, yolk sac and decidual area (Scott 1993). In addition, ANP is also synthesized in human placenta (Lim and Gude 1995; Gude et al. 2000).

Another natriuretic peptide crucial for maintaining fetal-maternal homeostasis, CNP (C-type natriuretic peptide), is widely expressed in other tissues, especially vascular endothelium (Sellitti et al. 2011). Endothelium-derived CNP exerts local effects on vascular smooth muscle cells, thereby enabling vasorelaxation and venodilation (Suga et al. 1993). Although vascular actions of CNP in humans remain to be fully elucidated, studies in small ruminants have recently shown an increase in CNP levels during early and late gestational periods when maternal nutrient uptake is restricted. These suggest that CNP is strongly associated with placental and fetal maturation (McNeill et al. 2009; McNeill et al. 2012; Madhavan et al. 2017). Moreover, CNP may have a compensatory or organ-specific function in the human reproductive tissue under pathophysiological conditions (Stepan et al. 2002). Therefore, maternal plasma CNP concentration may be increased due to fetal growth retardation or impaired placental blood flow (Reid et al. 2014).

Pregnancy triggers extensive alterations in maternal vasculature to deliver a constant supply of blood to the growing fetus. Pregnancy-induced hypertensive complications like preeclampsia may arise when these alterations do not occur properly (Giannubilo et al. 2017). For both mother and fetus, natriuretic peptides appear to be key players in the development of a better adaptation to the pregnancy. However, expression patterns of ANP and CNP in the healthy and pre-eclamptic human placentas have not been fully elucidated. Here, we aimed to determine the immunohistochemical distribution of ANP and CNP in the healthy and pre-eclamptic human placentas.

MATERIALS and METHODS

Tissue Preparation

The present study was conducted with the approval of Suleyman Demirel University, Medicine Faculty, Clinical Research Ethical Committee (dated 27/07/2016 and numbered 128/72867572-050-3022). The study material was obtained from Department of Gynecology and Obstetrics, Research and Practice Hospital, Süleyman Demirel University. Tissue samples harvested from maternal and fetal parts of 6 pre-eclamptic (mean age: 25.17 years, mean weight: 95.67 kg, mean gestational age: 252±15 days) and 6 healthy (mean age: 25.67 years, mean weight: 85 kg, mean gestational age: 268±4.6 days) human placentas were fixed in 10% phosphate-buffered (pH=7.2) formalin solution for 10 days at room temperature. Formalin was removed with running water overnight, followed by dehydration in ascending alcohol series. After dehydration, samples were cleared in xylene and embedded in paraffin wax. Formalin-fixed paraffin-embedded (FFPE) samples were cut into 5–7 µm sections using rotary microtome (RM2125RT, Leica, Nussloch, Germany) and collected on poly-L-lysine coated slides.

Immunohistochemistry

Sections were dewaxed with xylene and rehydrated through a descending series of ethanol. In order to unmask antigenic sites, tissue sections were boiled in sodium-citrate buffer (pH= 6.0) for 15 min in a microwave oven at full-power. After cooling for 20 min, slides were incubated for 10 min in 0.3% H_2O_2 in ethanol to quench endogenous peroxidase activity, and then rinsed in phosphate-buffered saline (PBS, pH=7.2) for three times, each for 5 minutes. After this point, all incubations were performed in a humidified chamber at room temperature. All dilutions were made according to the manufacturer's instructions. Non-specific binding was blocked with normal goat serum (diluted 1:10, Sigma, USA) for 30 min. Following removal of excess serum, slides were incubated with primary anti-ANP (1:100, Elabscience, EAP0498) and anti-CNP (1:100, Elabscience, E-AB-16009) for one hour. After three PBS washes, the slides were incubated with biotinylated goat anti-rabbit IgG antibody (1:400, Sigma, USA). After three PBS washes again, the slides were treated with ExtrAvidin–Peroxidase (1:100, Sigma, USA). The resulting signal was developed with 0.05% 3,3 diaminobenzidine (DAB-Plus substrate kit, Invitrogen, Camarillo, CA) for 10 min. The slides were then counterstained with Gill (III) Hematoxylin, dehydrated and mounted in entellan (107960, Merck, Darmstadt, Germany). ANP and CNP immunostaining was evaluated under a light microscope (Olympus CX41, Tokyo, Japan) by the presence of a brown color chromogen (DAB). Photos were taken using a DP26 digital camera (Olympus, Tokyo, Japan). All dilutions were as specified by the respective manufacturer's instructions. Non-specific immune serum was substituted for primary antibody as the negative control. Rat heart was used as the positive control. All protocols were performed in a humidity chamber at room temperature unless stated otherwise

RESULTS

Immunohistochemistry revealed ANP reactivity in the endothelium and smooth muscle cells of the stem villi in both healthy (Figure 1A) and pre-eclamptic (Figure 1B) samples, the former displaying higher intensity staining. Within the healthy group, the same regions of arteries were also positive for CNP staining (Figure 1C). In addition, within the pre-eclampsia group, ANP and CNP immunoreactivity was present in the atheromatous plaques of stem villi (Figure 1B and D). ANP (Figure 2A) and CNP (Figure 2C) immunoreactivity was observed in endothelium and smooth muscle cells of fetal blood vessels in the healthy group.

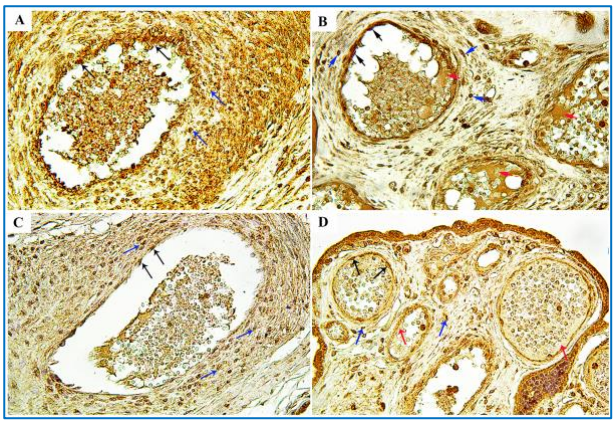

Figure 1. ANP and CNP immunoreactivity in the healthy (A, C) and pre-eclampsia (B, D) group, respectively. Endothelial cells (black arrows), media layer (blue arrows) and atheromatous plaques (red arrows). ABC, 40x.

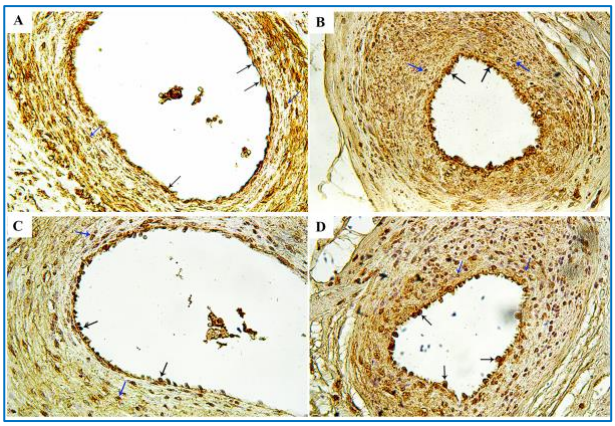

Figure 2 ANP and CNP immunoreactivity in the healthy (A, C) and pre-eclampsia (B, D) group, respectively. Endothelial cells (black arrows) and media layer (blue arrows) of fetal blood vessels. ABC, 40x.

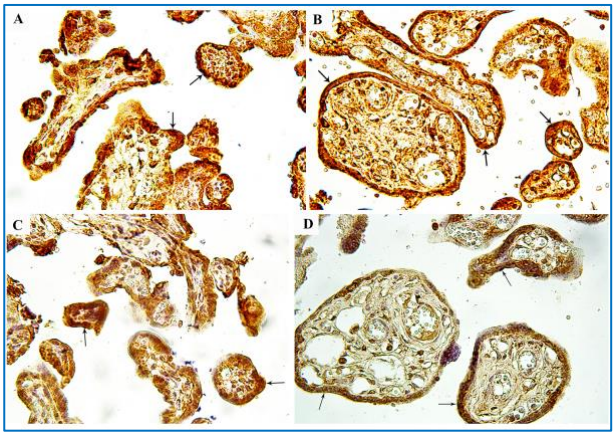

Figure 3. ANP and CNP immunoreactivity in the healthy (A, C) and pre-eclampsia (B, D) group, respectively. Syncytiotrophoblast cells (black arrows). ABC, 40x.

In the pre-eclampsia group, these areas exhibited more intense ANP (Figure 2B) and CNP (Figure 2D) immunoreactivity. Furthermore, ANP and CNP staining in syncytiotrophoblast cells of the stem villi was more prominent in the pre-eclampsia group (Figure 3B and D) than that in the healthy group (Figure 3A and C). While the decidual cells displayed an intense ANP (Figure 4-A) and CNP (Figure 4-C) immunoreactivity in the healthy group, they had a weak ANP (Figure 4-B) and CNP (Figure 4-D) staining in the preeclampsia. ANP (Figure 5B) and CNP (Figure 5D) immunostaining were more intense in the amniotic epithelial cells of the pre-eclampsia group compared to the healthy group (Figure 5A and C). While the epithelial cells of the preeclamptic amnion had similar levels of ANP and CNP expression, the same cell group in the healthy amnion had more pronounced ANP immunolabelling compared to CNP.

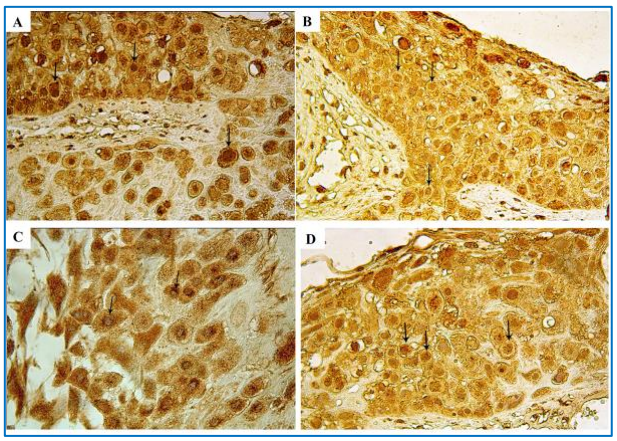

Figure 4. ANP and CNP immunoreactivity in the healthy (A, C) and pre-eclampsia (B, D) group, respectively. Decidual cells (black arrows). ABC, 40x.

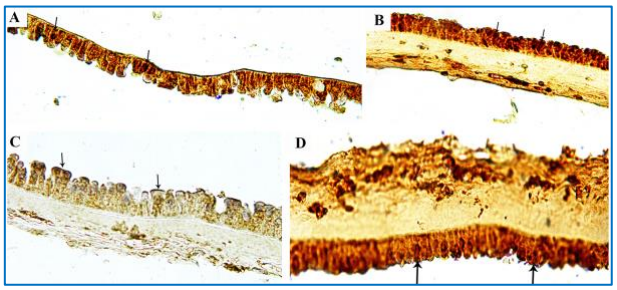

Figure 5. ANP and CNP immunoreactivity in the healthy (A, C) and pre-eclampsia (B, D) group, respectively. Amnion epithelial cells (black arrows). ABC, 40x.

DISCUSSION

During fetal development, natriuretic peptides are involved in the regulation of blood pressure and body fluid homeostasis, and in the growth and development of bones and cardiovascular tissues (Cameron et al. 1996). Vascular endothelial and smooth muscle cells produce a large number of vasoactive proteins, notably among which are endothelium-derived relaxant factor, ANP, CNP and endothelin (Rampersad et al. 2011). Furthermore, ANP receptors are found to be localized to the endothelial cells and smooth muscle cells of the arteries and veins, which suggests an association of ANP with the regulation of regional blood flow in the placenta (Scott 1993). In line with the current literature, we have also observed the ANP and CNP immunoreactivity in the vascular endothelial and smooth muscle cells. Of those, ANP is thought to play a role in the uterine relaxation during pregnancy since natriuretic peptide receptors are expressed in human placenta and myometrium (Itoh et al. 1994). Given the vasodilatory nature of ANP, increased ANP release in hypertensive pregnancies may act as a compensatory mechanism for vasospasm (Lumme et al. 1992). Using *in*

vitro autoradiography, Scott (1993) demonstrated that placenta, visceral yolk sac and decidua possess binding sites for ANP during the 16th, 18th, and 20th days of the rat gestation. Researcher also found that in the yolk sac, ANP localized to the villous vessels has a particular binding affinity for the receptors located on the maternal blood vessels supplying the decidual region and placenta. ANP was shown to be localized to rat (Huang et al. 1992) and human term (Gude et al. 2000) placental cytotrophoblasts but not to syncytiotrophoblasts. This contradicts with our observations showing the ANP immunoreactivity in the syncytiotrophoblasts. ANP has been reported to be neither synthesized nor stored within the normal term placenta (Inglis et al. 1993). Contradictions between these studies in terms of the absence (Inglis et al. 1993) or presence of the ANP expression in two trophoblastic cell types (Huang et al. 1992; Gude et al. 2000) may arise from methodological differences. The surprising finding of our study is that ANP might be produced by syncytiotrophoblasts under pre-eclamptic conditions. This needs to be further clarified using current cell isolation techniques and molecular methods.

Our study revealed that both healthy and pre-eclampsia groups had ANP expression in the endothelial and smooth muscle cells of the villous vessels, as well as in the decidual cells. However, ANP staining was more prominent in the healthy group. Lower ANP expression in the preeclampsia group might result from the poor blood flow. The present findings and the current literature (Lumme et al. 1992; Scott 1993; Itoh et al. 1994; Gude et al. 2000; Rampersad et al. 2011) reinforce the notion that placental ANP might play a role in the regulation of maternal-fetal circulation. Our study showed higher immunoreactivity for ANP in the decidua in the healthy group compared to preeclamptic group. CNP expression was detected throughout the media layer, but not the adventitia layer of the all vessels, as well as in the cultured human smooth muscle cells (Kelsall et al. 2006). Those reported by Kelsall et al. (2006) are consistent with the present findings of CNP expression. The literature (Cameron et al. 1996; Kelsall et al. 2006) and the present study suggest that CNP expressed by vascular smooth muscle cells may have the ability to regulate vascular tone independently of the endothelium.

Pregnancies complicated by preeclampsia are associated with decreased plasma volume and central venous pressure. Concentrations of ANP were measured in a study by a specific radioimmunoassay to explore the hypothesis that plasma ANP concentrations are low in the preeclampsia due to deficient secretion (Thomsen et al. 1987). They found that the mean plasma concentration of immunoreactive ANP in healthy pregnant women was higher than that in non-pregnant healthy individuals. The mean concentration in pregnancies with mild and severe pre-eclampsia was also significantly higher than in normal healthy pregnancies. In another study (Minegishi et al. 1999) investigating the possible role of ANP in the physiology of pregnancy and pathophysiology of preeclampsia, ANP plasma concentrations were measured in nonpregnant women, healthy, and preeclamptic pregnancies. They reported that while plasma ANP concentrations in healthy pregnancies remained constant throughout pregnancy, plasma ANP levels in the preeclamptic pregnancies were significantly increased. Our results are in line with the those reported by Thomsen et al. (1987) and Minegishi et al. (1999).

In the light of the above findings, there might be several reasons for the increased concentration of plasma ANP in the pre-eclampsia. A possible reason is that reduced metabolic clearance of ANP by NPR-C due to renal impairment may lead to an increase in circulating plasma concentrations of ANP in pre-eclamptic pregnancies. High blood pressure could be another reason, as reported by Sagnella (1986). These data suggest, therefore, that pre-eclampsia is associated with increased plasma concentrations of ANP. Furthermore, increased blood volume during pregnancy might be linked with the increased levels of maternal plasma ANP and the fetus could produce its own ANP in response to stimulating factors.

Extravillous trophoblasts invade the uterine wall (interstitial invasion) and the spiral arteries (endovascular invasion), which in turn changes the cells of the vessel wall and forms a high-flow low-resistance vessel (Cartwrighta et al. 2002). Impaired trophoblast invasion prevents the spiral arteries from dilating (Greer et al. 1989). According to the endothelial dysfunction theory, the pathogenesis of preeclampsia is associated with angiogenic and anti-angiogenic factors. Imbalance in both of these factors affects successful trophoblast invasion and spiral artery remodeling (Keman et al. 2009). Pregnant ANP-deficient mice developed high blood pressure and proteinuria, both of which are characteristics of pre-eclampsia. In these mice, trophoblast invasion and uterine spiral artery remodeling were severely impaired (Wang et al. 2012). Data from these studies (Keman et al. 2009; Wang et al. 2012; Zhou and Wu 2013) indicate that ANP is crucial to physiological changes at the maternal-fetal interface, suggesting that defects in ANP function may result in pre-eclampsia. ANP also regulates trophoblast invasion and spiral artery remodeling and increases uteroplacental perfusion (Cui et al. 2012; Zhou and Wu 2013).

CNP, which possesses anti-atherogenic properties, is expressed in different cell types depending on the progression of atherosclerosis (Naruko et al. 1996). CNP has potent anti-proliferative and anti-migratory effects on vascular smooth muscle cells (SMCs). Vascular cell proliferation and migration are important in the pathophysiology of atherosclerosis. Natriuretic peptide system in human coronary arteries is implicated in the pathogenesis of intimal plaque formation and plays a key role in vascular remodeling (Casco et al. 2002). CNP expression was previously detected in the endothelial cells, media smooth muscle cells and macrophages under normal physiological conditions (Ishizaka et al. 1992; Casco et al. 2002). Depending on the progression of atherosclerotic plaques, CNP immunoreactivity was present in macrophages in advanced lesions, but most of the endothelial cells and smooth muscle cells were CNP-negative (Naruko et al. 1996). Such observations intimate that the severity of atherosclerotic lesions is inversely correlated with the CNP expression (Casco et al. 2002). In our study, while weak CNP immunoreactivity was detected in endothelial and smooth muscle cells of vessels resulting in atheromatous plaque, more intense CNP immunoreactivity was observed in these cells of the preeclampsia group that was affected by atherosclerosis. However, there was no significant difference in CNP immunoreactivity in the atheromatous plaques between the healthy group and preeclampsia group.

CONCLUSION

In conclusion, the most noteworthy finding from this study is that, on the fetal side, multiple cells types including the amniotic epithelium, fetal vessels, villous

syncytiotrophoblast plate, villous vascular endothelial cells and smooth muscle cells had more intense ANP and CNP immunoreactivity in pre-eclampsia group contrary to the healthy group. On the maternal side, however, the healthy group demonstrated a more intense ANP and CNP immunoreactivity in decidual cells. It is considered that increased ANP and CNP expression relating to preeclampsia might serve as a compensatory function to properly maintain uteroplacental perfusion and fetal development during pregnancy.

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