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The effect of pentylenetetrazole-induced seizures on blood-brain barrier integrity in a rat model of preeclampsia

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Abstract: The pathophysiological mechanisms underlying blood-brain barrier (BBB) disruption in preeclampsia/eclampsia have not been elucidated. This study investigated the effect of pentylenetetrazole (PTZ)-induced seizures on the functional and structural properties of the BBB during N (omega)-nitro-L-arginine methyl ester (L-NAME)-induced hypertension and proteinuria in pregnant rats. Animals were treated with L-NAME for 10 days, beginning on day 10 of pregnancy. The BBB permeability was determined by measurements of sodium fluorescein (NaFlu) extravasation into the brain. Occludin and aquaporin (AQP)-4 immunoreactivities were evaluated in brain sections. Severe proteinuria and significantly increased arterial blood pressure were observed following L-NAME treatment. PTZ-induced seizures in pregnant rats treated with L-NAME increased NaFlu levels in all of the brain regions analyzed ($P < 0.01$). A significant increase in the extravasation of NaFlu was also observed in the diencephalon of intact pregnant rats treated with PTZ. PTZ-induced seizures in both L-NAME-treated and untreated pregnant rats significantly decreased occludin immunoreactivity in the hippocampal capillaries. L-NAME administration significantly increased AQP-4 immunoreactivity in the astrocytic endfeet surrounding the parietal cortex microvessels of PTZ-treated and untreated pregnant rats. These findings suggest that PTZ-induced seizures lead to a severe disruption of the BBB in preeclampsia and that the paracellular pathway may play an important role in increased BBB permeability in this context.

Key words: Pregnancy, hypertension, sodium fluorescein, aquaporin-4, occludin

Sıçan preeklampsi modelinde pentilentetrazol ile uyarılan epileptik nöbetlerin kan-beyin bariyeri bütünlüğüne etkisi

Özet: Preeklampsi/eklampside kan-beyin bariyeri (KBB) hasarının altında yatan patofizyolojik mekanizmalar açığa kavuşturulamamıştır. Bu çalışmada N (omega)-nitro-L-arjinin metil ester (L-NAME) ile gebe sıçanlarda oluşturulan hipertansiyon ve proteinüri koşullarında pentilentetrazol (PTZ) ile uyarılan nöbetlerin KBB'nin fonksiyonel ve yapısal

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özellikleri üzerinde oluşturduğu etkiler araştırılmıştır. Gebeliğin 10. gününden itibaren 10 gün boyunca L-NAME uygulanan hayvanlarda beyin dokusuna geçen sodyum fluoresein (NaFlu) boya miktarı ölçülerek KBB geçirgenliği belirlenmiş ve beyin kesitlerinde okludin ve akuaporin (AQP)-4 immunoreaktivitesi değerlendirilmiştir. L-NAME uygulamasını takiben ileri derecede proteinüri ve anlamlı derecede arteryel kan basıncı artışları saptanmıştır. L-NAME uygulanan gebe sıçanlarda, PTZ ile uyarılan nöbetler incelenen tüm beyin bölgelerinde NaFlu boya miktarını arttırmıştır ($P < 0,01$). Sağlıklı gebe sıçanlarda PTZ uygulaması diensefalon bölgesinde NaFlu miktarını anlamlı derecede arttırmıştır. L-NAME uygulanan ve uygulanmayan sıçanlarda PTZ ile uyarılan nöbetler hipokampal kapilerlerdeki okludin immünoreaktivitesini anlamlı ölçüde azaltmıştır. PTZ uygulanan ve uygulanmayan gebe sıçanlarda L-NAME uygulaması parietal korteksteki kapilerleri saran astrosit son ayakçıklarında AQP-4 immünoreaktivitesini anlamlı ölçüde arttırmıştır. Bu bulgular preklampside PTZ ile uyarılan nöbetlerin şiddetli KBB hasarına yol açtığını ve bu koşullarda artan KBB geçirgenliğinde paraselüler yolağın önemli rol oynayabileceğini düşündürmektedir.

Anahtar sözcükler: Gebelik, hipertansiyon, sodyum floresein, akuaporin-4, okludin

Introduction

Preeclampsia/eclampsia is a disorder that adversely affects the mother and the fetus and is the leading cause of maternal death in developed countries. Preeclampsia is characterized by hypertension, proteinuria, and edema. The last 2 symptoms are common indicators of the microvascular leakage associated with this disorder. If left untreated, preeclampsia can progress to eclampsia, which has life-threatening neurovascular complications including severe hypertension and seizures (1,2). It has been reported that the neurologic manifestations of severe preeclampsia/eclampsia are identical to those observed in hypertensive encephalopathy (3). Experimental inhibition of nitric oxide synthesis by N (omega)-nitro-L-arginine methyl ester (L-NAME) has been shown to result in gestational hypertension and proteinuria in rats.

Accumulated data have shown that aquaporin (AQP)-4, which is expressed predominantly in astrocytes, is involved in the regulation of potassium and water homeostasis in the brain and plays an important role in the pathogenesis of both types of brain edema (4-6). AQP-4 is also implicated in the pathophysiological mechanisms related to epileptogenesis (7,8). In addition, AQP-4 expression in the brain has been shown to increase during pregnancy (9,10).

The blood-brain barrier (BBB) maintains neuronal homeostasis and is impaired under certain pathological conditions, such as hypertension and epilepsy (11,12). It is well established that proper functioning of the BBB is especially critical during pregnancy. The decreased expression of tight junction (TJ) proteins, which is associated with increased permeability through the endothelial cells of the umbilical veins, has also been observed in preeclampsia (13). The effect of preeclampsia/eclampsia on the properties of the BBB is far from resolved owing to the limited number of experimental studies using animal models. In the current study, we therefore investigated the effect of seizures evoked by pentylenetetrazole (PTZ) on the integrity of the BBB in a rat model of L-NAME-induced preeclampsia.

Materials and methods

Animal protocol

Sprague-Dawley rats were obtained from the Institute of Experimental Medicine at İstanbul University. The study and all associated procedures were approved by the Local Ethics Committee for Animal Experimentation of İstanbul University. Rats were mated by housing them overnight with fertile males and the first day of pregnancy was verified by the presence of spermatozoa in a vaginal smear. Beginning on day 10 of pregnancy, pregnant rats were treated with L-NAME (0.5 mg/kg; in drinking water) for 10 days to induce hypertension and proteinuria.

Measurements of arterial blood pressure and urinary protein

On the day of the experiment, rats were anesthetized with diethyl ether and polyethylene catheters (PE-50) were inserted into the right femoral artery and vein. One of these catheters was connected to a pressure transducer that interfaced with a data acquisition system and a personal computer in order to continuously monitor the mean arterial blood

pressure (MABP). The second catheter infused sodium fluorescein (NaFlu; MW: 376) tracer and PTZ. The degree of proteinuria was determined using urine reagent strips (Uristik, The Hague, the Netherlands) and scored as 1+, 2+, 3+, or 4+. Severe proteinuria was defined as ≥ 3+ on a urine dipstick and mild proteinuria was defined as < 3+ (14).

Seizure induction

Seizures were evoked by intravenous injection of PTZ (80 mg/kg). The behavioral severity of seizures was scored according to Racine's scale (15).

Measurement of BBB permeability

BBB permeability was assessed by detection of extravasated NaFlu in the analyzed brain regions. NaFlu (2%, 5 mL/kg) was administered 5 min prior to PTZ administration and allowed to circulate for 30 min. Rats were then transcardially perfused with 200 mL of saline to remove intravascular NaFlu. Brains were removed and dissected into the following 4 regions: left cerebral cortex, right cerebral cortex, diencephalon, and cerebellum. Each brain region was weighed, homogenized in 2.5 mL of phosphate-buffered saline and vortexed for 2 min following the addition of 2.5 mL of 60% trichloroacetic acid. Samples were later cooled for 30 min and centrifuged at 14,000 × *g* for 10 min. Tracer concentration in the supernatant was measured with a spectrophotofluorometer at an excitation wavelength of 440 nm and an emission wavelength of 525 nm (Microplate Reader; DTX880, Beckman Coulter, CA, USA). NaFlu levels were expressed as ng/mg of brain tissue against a standard curve.

Immunohistochemistry

To analyze the immunoreactivity of occludin and AQP-4, rats were perfusion fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were then removed, kept in the same fixative at 4 °C for 24 h, and embedded in paraffin. Three-micron thick sections were deparaffinized and incubated with protease (1 mg/mL; Sigma, St. Louis, MO, USA) for 10 min or pressure cooked in citrate buffer for 1 min to achieve antigen retrieval for occludin and AQP-4, respectively. Polyclonal rabbit anti-occludin (Invitrogen, CA; 1/50, 2 h) and anti-AQP-4 (AB-3594, Millipore CA, USA; 1/100, 2 h) primary antibodies were used. The streptavidin-peroxidase detection system (ScyTek, UT, USA) was used and 3-amino-9-ethylcarbazole chromogen was applied for color development. Sections were counterstained with Mayer's hematoxylin to enhance nuclear staining. For negative controls, adjacent sections were processed with the same steps with the exception of the primary antibodies. Images were obtained on a digital camera attached to a light microscope. At least 100 capillaries from each animal were analyzed from the hippocampus and parietal cortex for occludin and AQP-4 immunoreactivity, respectively. The relative density of immunoreactivity for occludin and AQP-4 was quantified using Image Pro Plus 6.0 software (Media Cybernetics Inc.).

Statistical analyses

Group differences were determined using a one-way analysis of variance (ANOVA) followed by Tukey's test and the Wilcoxon test using computer software (SPSS version 11.0). In all cases, differences between the means were considered significant if $P < 0.05$.

Results

MABP values for each experimental group are shown in Table 1. L-NAME administration in pregnant animals resulted in significantly increased blood pressure levels ($P < 0.01$). PTZ administration increased MABP values significantly in pregnant rats treated with or without L-NAME ($P < 0.01$). Table 2 shows the degree of proteinuria in the experimental groups. Intact pregnant rats exhibited mild proteinuria while L-NAME administration led to severe proteinuria in the majority of animals. PTZ administration evoked generalized seizures with Racine scores of 5 in both intact and L-NAME-treated pregnant rats.

When BBB permeability was compared between L-NAME-treated and untreated pregnant rats, permeability to NaFlu was found to be significantly increased in all brain regions in the L-NAME-treated pregnant animals upon the induction of PTZ-induced seizures (Figure 1; $P < 0.01$). NaFlu content in the right and left cerebral cortical regions of pregnant animals in the L-NAME and PTZ co-treatment group was significantly higher than those of pregnant animals in the PTZ single treatment group (Figure 1; $P < 0.01$). PTZ-treated pregnant rats

Table 1. Mean arterial blood pressure values of rats in experimental groups.

Groups	n	Mean arterial blood pressure (mmHg)	
		Baseline	After PTZ administration
Pregnancy	16	105 ± 2	-
Pregnancy + L-NAME	16	141 ± 4†	-
Pregnancy + PTZ	16	102 ± 2	160 ± 6*
Pregnancy + L-NAME + PTZ	16	135 ± 6†	174 ± 6*

Data were presented as mean ± S.E.M.
*P < 0.01 versus baseline values.
†P < 0.01 versus baseline values of pregnancy and pregnancy plus PTZ.
n: number of animals.

Table 2. The degree of proteinuria in experimental groups.

Groups	n	Urine dipstick values			
		1+	2+	3+	4+
Pregnancy	16	14 (87%)	2 (13%)	-	-
Pregnancy + L-NAME	16	-	3 (19%)	6 (37%)	7 (44%)
Pregnancy + PTZ*	16	13 (81%)	3 (19%)	-	-
Pregnancy + L-NAME + PTZ*	16	-	2 (12%)	7 (44%)	7 (44%)

The values are expressed as the number of animals (and percentage of total).
*The degree of proteinuria was determined before PTZ injection.
n: number of animals.

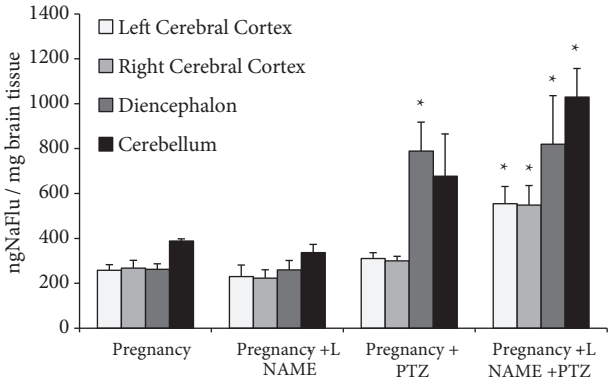

Figure 1. The sodium fluorescein content in different rat brain regions. Data are shown as means ± S.E.M. *P < 0.01 versus other groups.

showed increased BBB permeability to NaFlu in the diencephalic and cerebellar regions when compared with intact pregnant animals. The difference observed in the diencephalon was statistically significant (Figure 1; P < 0.01).

Figure 2 shows immunostaining for occludin in the hippocampal capillaries in brain sections from experimental rats. The relative density of occludin immunoreactivity as assessed by image analysis is depicted in Figure 3. A significant decrease in occludin immunoreactivity was noted following PTZ administration in both intact and L-NAME-treated pregnant rats (Figures 2C, 2D, and 3) compared to PTZ untreated animals (Figures 2A, 2B, and 3; P < 0.01).

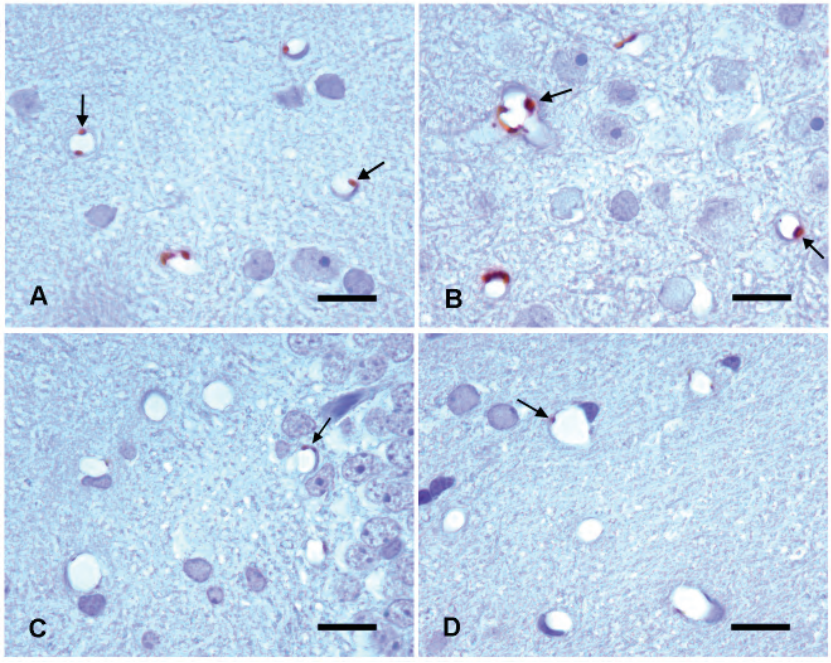

Figure 2. Immunostaining for the tight junction protein occludin (arrows) in the hippocampal microvessels of pregnant animals (A), pregnant plus L-NAME treatment (B), pregnant plus PTZ treatment (C), and pregnant plus L-NAME and PTZ co-treatment (D) groups. Scale bars = 10 μm.

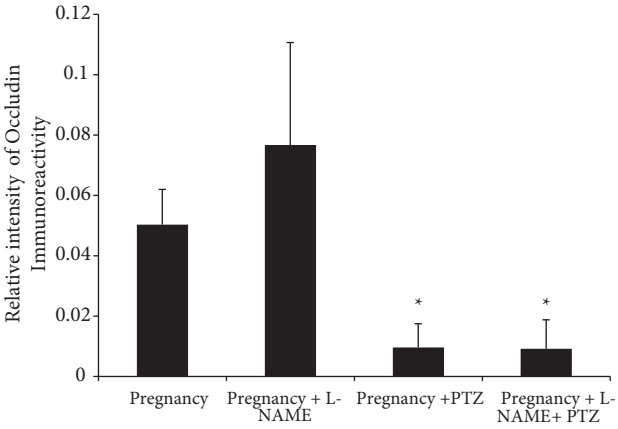

Figure 3. Quantification of occludin staining intensity in the hippocampal microvessels of experimental rats. Note the significantly decreased staining intensity in PTZ-treated animals. *P < 0.01 versus other groups.

Figure 4 shows immunostaining for AQP-4 in the astrocytic endfeet surrounding microvessels of the parietal cortex in sections from experimental rats. The relative immunoreactivity density for AQP-4 as assessed by image analysis is depicted in Figure 5. A significant increase in AQP-4 immunoreactivity was observed following L-NAME administration in pregnant rats treated with or without PTZ (Figures 4B, 4D, and 5) compared to L-NAME untreated animals (Figures 4A, 4C, and 5; $P < 0.05$).

Discussion

The principal finding of the current study was that the permeability of the BBB to NaFlu markedly increased in all of the analyzed brain regions upon the induction of seizures by PTZ in an animal model of preeclampsia. A significant increase in BBB permeability to NaFlu was also observed in the diencephalon of intact pregnant animals following PTZ-induced seizures. In addition, L-NAME treatment increased arterial blood pressure and led to severe proteinuria, indicating that L-NAME treatment is an experimental means of inducing a preeclamptic state in pregnant rats.

Recent studies have indicated that acute hypertensive episodes disrupt BBB integrity in pregnant animals (9,16). Experimentally induced

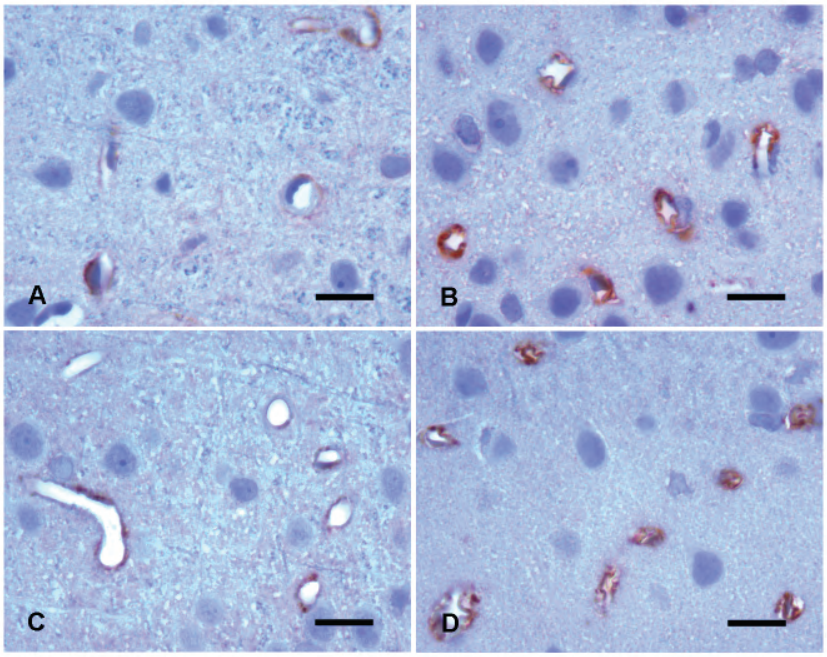

Figure 4. Immunostaining for AQP-4 in the astrocytic endfeet around microvessels in the parietal cortex of pregnant rats (A), pregnant plus L-NAME (B), pregnant plus PTZ treatment (C), and pregnant plus L-NAME and PTZ co-treatment (D) groups. Scale bars = 10 μm.

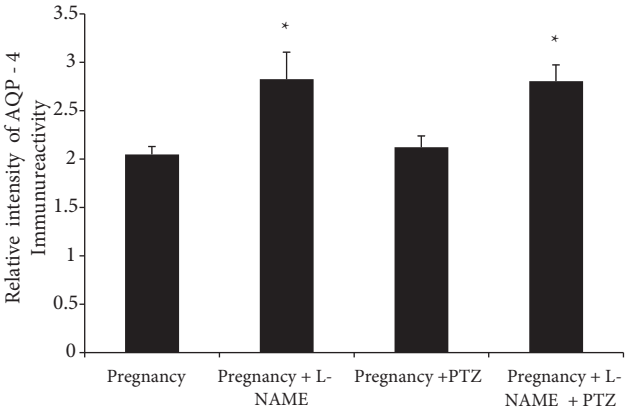

Figure 5. Quantification of AQP-4 staining intensity in the microvessels of the parietal cortex in experimental animals. Note the significantly increased AQP-4 staining intensity in L-NAME-treated animals. *P < 0.05 versus other groups.

seizures were also shown to cause BBB breakdown in intact pregnant rats (17). When the relationship between blood pressure values and quantitative BBB permeability measurements is considered in the context of our study, PTZ-induced seizures seem to play a central role in the association of BBB leakage with increased blood pressure. Our results suggest that hypertension in pregnancy renders the BBB more vulnerable and that severe BBB disruption occurs during PTZ-induced seizures.

Our study is the first to show that preeclampsia predisposes the BBB to integrity disruption following PTZ-induced seizures in a rat model of preeclampsia. Consistent with our findings, an increase in BBB permeability has been shown to be associated with the decreased expression and activity of endothelial nitric oxide synthase in an in vitro study of endothelial cells isolated from preeclamptic pregnancies (18). Recent data indicate that acute intraluminal exposure to preeclamptic plasma significantly increases BBB permeability in cerebral microvessels (19). Moreover, the expression of TJ proteins cadherin and occludin was decreased in an in vitro study of umbilical vein endothelial cells isolated in the setting of preeclampsia (13). These in vitro studies also reported increased monolayer permeability to horseradish peroxidase. The authors, however, did not discuss whether molecules crossed

the monolayers via a transcellular or paracellular route. Here, we show that occludin immunoreactivity is decreased in the hippocampal microvessels in untreated or L-NAME-treated PTZ-administered pregnant animals. Considering our observations that BBB permeability to NaFlu increases during PTZ-induced seizures in preeclamptic animals, it is reasonable to hypothesize that the paracellular pathway, which involves the passage of molecules through TJs, may be primarily responsible for the observed increase in BBB permeability.

AQP-4 has been considered as a potential contributor to the pathogenesis of preeclampsia/eclampsia. An increase in AQP-4 expression, particularly in astrocytes and to a lesser extent in barrier type endothelial cells, has been documented in the brain tissue of pregnant animals. It has been suggested that a number of stress factors, including hypertension and seizures, in preeclampsia/eclampsia may facilitate the increase of AQP-4 expression and contribute to both the impairment of BBB integrity and the development of cerebral edema (8-10). Alternatively, recent reports have suggested that AQP-4 not only facilitates water influx during brain edema formation but also serves as an efflux route for water in brain edema elimination (20,21). In addition, AQP-4 in astrocytes is thought to contribute to BBB function by removing excess water that has entered the brain following BBB disruption (22). Our results show that AQP-4 expression was increased in the astrocytic endfeet that ensheath brain microvessels in L-NAME-induced preeclampsia. Considering the above data demonstrating that BBB permeability is increased predominantly in PTZ-induced seizures, we assume that the increase in AQP-4 expression may alter water and ion distributions in the pericapillary milieu, rendering the BBB more susceptible to disruption by seizure activity. However, whether the alterations in AQP-4 expression represent a causative factor or a defense mechanism in the progression from preeclampsia to eclampsia is difficult to explain and has yet to be determined.

In conclusion, in the current study, we demonstrate marked increases in BBB permeability during PTZ-induced seizures in an experimental model of preeclampsia. On the basis of our observations that BBB impairment is associated with decreased occludin immunoreactivity in brain microvessels, we suggest that the increase in BBB permeability in this setting is primarily mediated by the paracellular pathway.

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