

Selective Vulnerability to Transcellular Pathway of Blood-Brain Barrier in Chronic Stage of the Kainate Model of Temporal Lobe Epilepsy in Rats

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Citation: Yilmaz CU, Orhan N, Arican N, Ahishali B, Gürses C, et al. (2018) Selective Vulnerability to Transcellular Pathway of Blood-Brain Barrier in Chronic Stage of the Kainate Model of Temporal Lobe Epilepsy in Rats. Int J Cerebrovasc Dis Stroke : IJCD-108. DOI: 10.29011/IJCD-108.100008

Received Date: 01 October, 2018; **Accepted Date:** 22 October, 2018; **Published Date:** 26 October, 2018

Abstract

Background: The blood-brain barrier (BBB) integrity is severely affected in many epilepsy syndromes including temporal lobe epilepsy (TLE).

Aim: In the present study, we investigated the effects of acute and chronic seizures induced by kainic acid (KA) on the transport pathways of BBB in rats.

Methods: Electroencephalography (EEG) was recorded to evaluate seizure activity. Immunohistochemistry for claudin-5, a tight junction protein, caveolin-1, and the glial fibrillary acidic protein (GFAP), a marker of astrocyte activation, was performed. Electron microscopy was used to ultrastructurally assess the presence and route of extravasation of horseradish peroxidase (HRP), a permeability marker, in barrier type of brain capillary endothelial cells.

Results: The immunoreactivity of claudin-5 and caveolin-1 in hippocampus increased by both acute and chronic seizures ($p < 0.01$), while an increase in GFAP immunoreactivity was found in the hippocampus by only acute seizures ($p < 0.01$). The endothelial cells of brain capillaries in hippocampus and amygdala regions of animals in acute and chronic sham groups showed occasional HRP reaction products. Acute and chronic seizures led to the observation of significantly greater extent of accumulation of HRP reaction products in both brain regions of rats compared to acute and chronic sham groups ($p < 0.01$), while tight junctions were intact in all experimental groups.

Conclusion: This study provides immunohistochemical and ultrastructural evidence of BBB disruption through a selective vulnerability of the transcellular transport via an increase in caveolar vesicles in the endothelial cells brain capillaries rather than activation of paracellular pathway in the KA-induced rat model of TLE.

Keywords: Blood-Brain Barrier; Caveolin-1; Claudin-5; Electron Microscopy; Kainic Acid; Temporal Lobe Epilepsy

Introduction

Epilepsy, which is characterized by spontaneous recurrent seizures, is one of the most important pathological conditions of the brain. Temporal lobe epilepsy (TLE), the most common type of human focal epilepsy, frequently develops in adults after head trauma, status epilepticus (SE) and stroke [1]. Recent evidence suggests that one of the mechanisms in the development of TLE might be associated with the alterations in blood-brain barrier (BBB) integrity [2] and the loss of BBB integrity might stimulate epileptogenic processes [3]. Also, Tomkins et al. reported a correlation between disrupted BBB and abnormal neuronal excitability in the course of post-traumatic epilepsy [4].

The effects of kainic acid (KA)-induced seizures on BBB integrity have been investigated in a number of studies using various tracers such as [¹⁴C] Alpha-aminoisobutyric acid ([¹⁴C] AIB; MW: 104 Da), sodium fluorescein (376 Da), horseradish peroxidase (HRP; 44 kDa), evans blue (EB)-binding albumin (69 kDa), and IgG (150 kDa). These studies showed increased tracer accumulation in brain tissue following periods ranging from minutes to 21 days after KA injection. In three of these studies, it is reported that the BBB permeability to [¹⁴C] AIB significantly increased at various time points between 30 min and 24 h after injection of KA into the rats [5-7]. In another study, it is shown that HRP extravasation disappeared in coronal brain sections at 12 h, while endogenous IgG was observed in brain sections until three days after KA injection [8]. The intensity of immunoglobulin leakage as assessed by IgG staining in the hippocampus was found to increase on day 7 of KA-induced seizures [9]. FITC-albumin leakage to limbic regions of the brain was also found to increase following KA-induced seizures [10]. Kainate-induced seizures also led to the extravasation of HRP to the brain parenchyma at one hour after injection of KA in rats [11]. Increased EB extravasation and transendothelial caveolar vesicles were observed in brain tissue at four hours after injection of KA, however endothelial Tight Junctions (TJs) remained intact under the same conditions [12].

Accumulated data indicate that the ultrastructural basis of BBB disruption that occurs during the acute stage of seizures is more likely due to an increased transendothelial transport by the increase of caveolar vesicles in the barrier type of capillary endothelial cells rather than the opening of the TJ structures [13-15]. As mentioned above, a number of studies have addressed seizure-induced BBB disruption during both acute and chronic stages of TLE induced by KA; however, there are no well-defined studies which delineate whether transcellular (via caveolar vesicles) and/or paracellular (via opening of TJs) pathway(s) are responsible for the increased BBB permeability especially during the chronic stage of TLE.

To learn which BBB transport pathway(s) is/are responsible for the transport of circulatory substances into the brain parenchyma during epileptogenesis may enable us to explore the mechanisms that prevent or reduce the activation of the pathway(s) in question as a next step. Therefore, in this study, our purpose was to investigate the alterations in the transport pathway(s) in barrier type of brain capillary endothelial cells during the acute and chronic stages of KA-induced TLE in rats.

Materials and Methods

Animal protocols

All experiments were performed on adult female 8-12 weeks old Wistar albino rats weighing 180-240 g obtained from Aziz Sancar Institute of Experimental Medicine, Istanbul University. The study design and all surgical and animal handling procedures described in this paper were approved by the Local Ethics Committee of Animal Experiments of Istanbul University (25/2012) according to the rules and regulations of Turkish Ministry of Food, Agriculture, and Livestock (2011/28141). Animals were housed in groups under controlled humidity and temperature, with free access to food and water in the animal facility. The rats were randomized into four experimental groups; acute sham (one day), chronic sham (one month), acute kainic acid (KA; 1 day), and chronic KA (one month). Separate subsets of experimental groups consisting of 8 rats were used for each experimental procedure.

One week before KA injection, electroencephalography (EEG) electrodes were implanted for EEG recording which was used to confirm continuous seizure activity following KA administration. In brief, animals were intraperitoneally (i.p.) anesthetized using chloral hydrate (360 mg/kg), and the scalp was incised and opened from back to front by following the center of the middle line. All stereotaxic coordinates were established by the atlas of Paxinos and Watson under a stereotaxic frame (Stereotact, World Precision Instruments, Inc., USA). Two stainless steel bipolar electrodes (**inner and outer diameter:** 200 and 260 μ m; A-M Systems, Inc. #791900, Carlsborg, WA, USA) were implanted stereotactically into the left and right hippocampus through 0.6 mm-wide burr holes, and they were secured onto the skull via dental acrylic (AP: -3.14 mm, ML: 2.0 mm and DV: 2.8). Two stainless steel support screws and one stainless steel screw for reference electrode (AP: 3.0 mm) were placed on the skull and secured using dental acrylic. A mini-USB connector was used to connect electrodes to an EEG recording system (Micromed S.p.A., Italy). EEG video recording was continuously monitored while the rats were allowed to move freely in their cages for one hour, and one month.

A low-dose systemic KA protocol which was adapted from Hellier and Dedeurwaerdere was used for the study [16,17]. KA (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in isotonic saline (pH:7.4), and administered (i.p.) at a dose of 2.5

mg/kg followed by one or two administration doses at the same concentration once per hour to rats until continual epileptiform activity was observed on visual observation and EEG activity. KA-injected rats that exhibited stage 5 convulsions according to Racine's scale [18], were regarded as having SE. Four hours later, SE was terminated by diazepam (4 mg/kg, i.p.). Three rats died during SE and excluded from the analysis. Animals exhibited behavioral changes such as grooming, rearing, hind limb scratching, wet dog shakes, jaw movements, salivation, urination, defecation, and head nodding within one h after injection of KA. Following the KA injections, rats were placed in clear polyethylene-carbon cages, and motor seizures were evaluated and classified according to Racine's scale. EEG recordings were obtained from KA-treated rats at one month, and behavioral seizure activities were recorded. All animals in the one month-KA group exhibited recurrent seizures, which were also verified by EEG.

Immunohistochemistry

Immunohistochemical staining for claudin-5, caveolin-1 and GFAP was performed in the brain sections as previously described by Orhan, et al. [19]. Briefly, rats were anesthetized with chloral hydrate (350 mg/kg, i.p) and sodium pentothal (50 mg/kg, i.p.) and after transcardial perfusion with saline followed by fixative, brains were embedded in paraffin. 3- μ m thick sections were deparaffinized and incubated in 1 mM EDTA solution (pH 8.0) for claudin-5 in a microwave oven or in 10 mM citrate buffer solution (pH: 6.0) for GFAP and caveolin-1 in a pressure cooker at 1 ATM pressure to achieve antigen retrieval. Monoclonal mouse anti-GFAP (Neomarker, Fremont, CA; 1/100, 1 hr) and polyclonal mouse anti-claudin-5 (Invitrogen, CA, USA; 1/100, 1 hr) and rabbit anti-caveolin-1 (Abcam, Cambridge, United Kingdom; 2910; 1/100, 1 hr) were used as primary antibodies. Secondary antibodies were used as biotinylated goat anti-mouse (Lab Vision, Westinghouse, CA) for GFAP, and goat anti-polyvalent (ScyTek Laboratories, USA) for claudin-5 and caveolin-1. Images were captured from the hippocampus using a digital camera (Nikon, Coolpix 4500) attached to a light microscope.

For the semiquantitative evaluation of the intensity of immunohistochemical staining, approximately 15 capillary images for claudin-5 and caveolin-1 and approximately 15 microscopic areas for GFAP were taken by 100X objective for claudin-5 and caveolin-1 and 40X magnification for GFAP, respectively. The intensity of staining was scored in the form of 0: no staining, +1: mild intensity, +2: moderate intensity, and +3: high intensity [20]. The H score values for each rat were then calculated as the sum of the values obtained by multiplication of percentages of capillaries staining at each intensity by the weighted intensity of the staining [$HSCORE = \sum P_i (i + 1)$], where i is the immunoreactivity

intensity score, and P_i is the percentage of capillary vessels (for claudin-5 and caveolin-1) or microscopic areas (for GFAP) with immunoreactivity at the corresponding intensity].

Electron microscopic assessment of horseradish peroxidase permeability

We carried out an ultrastructural HRP assay as previously described [21,22]. The rats under chloral hydrate (350 mg/kg, i.p) and sodium pentothal (50 mg/kg, i.p.) anesthesia were administered an injection of HRP (type II, Sigma Chemical Co., St. Louis, MO; 200 mg/kg body weight in 0.2 mL saline, i.v.). HRP was allowed to circulate for 30 min, and then the animals were perfused transcardially with saline followed by fixative. Coronal sections of 50- μ m thickness were cut and incubated in a solution of 0.05% 3,3'-diaminobenzidine in 0.05 M Tris-HCl buffer containing 0.01% hydrogen peroxide to provide HRP-reaction products. For the ultrastructural semiquantitative evaluation of the intensity of HRP extravasation, approximately 15 capillary images taken from the brain regions of each animal in the experimental groups were scored as 0: no HRP extravasation, +1: mild HRP extravasation, +2: moderate HRP extravasation, and +3: severe HRP extravasation. The H score values for each rat were then calculated as the sum of the values obtained by multiplication of percentages of capillaries with HRP extravasation at each intensity by the weighted intensity of HRP extravasation [$H SCORE = \sum P_i (i + 1)$], where i is the HRP extravasation intensity score, and P_i is the percentage of capillary vessels with HRP extravasation at the corresponding intensity].

Statistical analysis

Data are presented as mean (X) \pm Standard Error of the mean (SE). Group differences were determined using the one-way ANOVA followed by Tukey's for relative intensity of caveolin-1, claudin-5, GFAP, and accumulation of HRP reaction products using the Statistical Package for Social Sciences (SPSS) version 21.0. In all cases, differences between the means were considered significant if $p < 0.05$. Differences were considered significant if the p -value was less than 0.05.

Results

Repetitive and long-lasting generalized seizures were observed in animals treated with KA in the acute and chronic groups. EEG recordings after KA injection(s) showed a good build-up with high-frequency activity at the beginning of the seizures and continued to become generalized tonic-clonic seizures (Figure 1). At the same time, all animals were observed for physical evaluation of seizure pattern via Racine scale and all animals in both acute, and chronic KA groups exhibited stage 5 seizures.



Figure 1: Representative EEG traces recorded from right and left hippocampus CA1 areas of the rats before and during KA-induced acute generalized seizures. EEG traces with the Red color show activity before the seizure, and EEG traces with yellow color show seizure activity. High-frequency seizure patterns with an average of 100 milliseconds are shown with arrows.

Caveolin-1 immunostaining was detected in animals in experimental groups along the wall of the blood microvessels (Figure 2). The immunostaining intensity of caveolin-1 significantly increased on the endothelial cells of the capillary wall in the hippocampus region of animals during acute and chronic seizures compared with sham groups (Figure 2); ($p < 0.01$). In these animals, the immunostaining intensity of claudin-5 was also increased on TJ's in the capillaries of the hippocampus region (Figure 3); ($p < 0.01$). An increase in the immunostaining intensity of GFAP was observed in the hippocampus region of rats in only acute seizures (Figure 4); ($p < 0.01$).

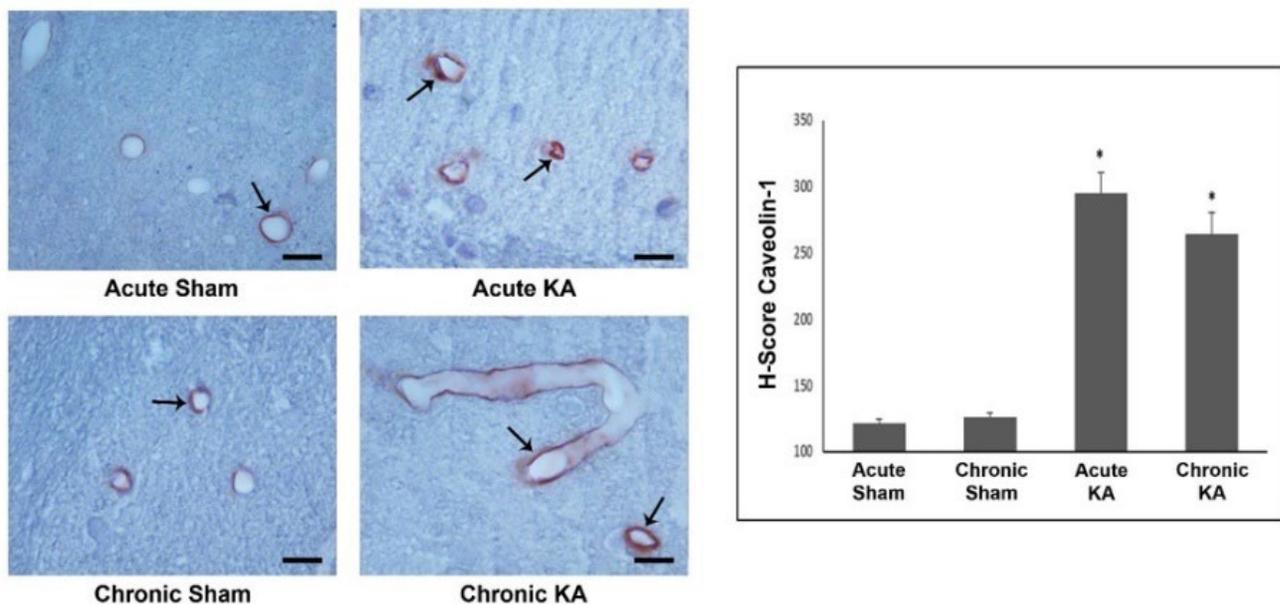


Figure 2: The intensity of caveolin-1 immunostaining (black arrows) along the microvessels in the hippocampus region of animals in acute sham, acute KA, chronic sham, and chronic KA groups. Note that a pronounced increase in caveolin-1 immunostaining intensity was observed in microvessels in the hippocampus region of animals in both acute KA and chronic KA groups as assessed by semiquantitative H scoring. Scale bars = 10 µm.

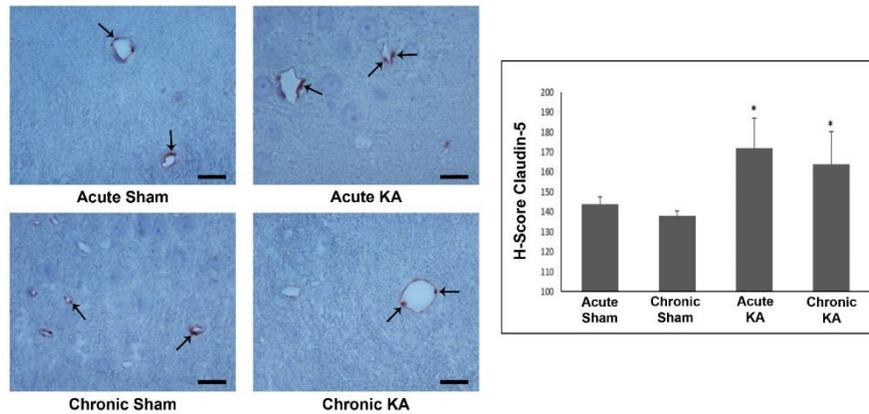


Figure 3: The intensity of claudin-5 immunostaining (black arrows) along the microvessels in the hippocampus region of animals in acute sham, chronic sham, acute KA and chronic KA groups. Note that claudin-5 immunostaining intensity increased in the microvessels of the hippocampus region of animals in both acute KA and chronic KA groups as assessed by semiquantitative H scoring. Scale bars = 10 µm.

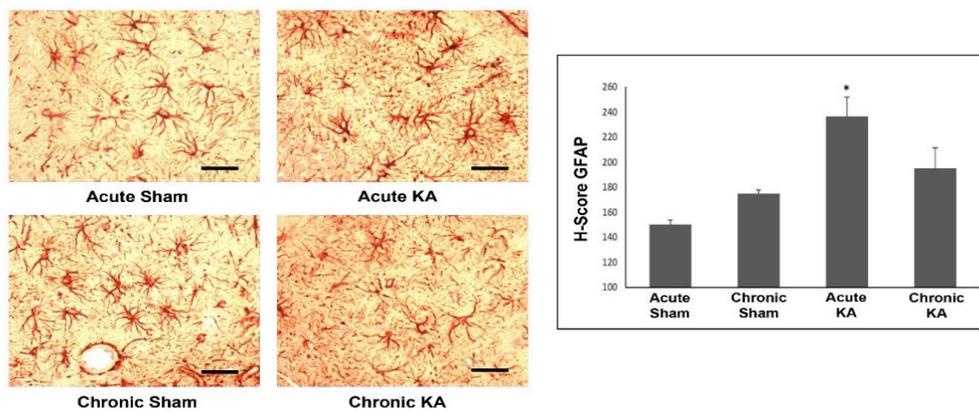


Figure 4: The intensity of GFAP immunostaining of astroglial cells in the hippocampus region of animals in acute sham, chronic sham, acute KA and chronic KA groups. Note the pronounced GFAP immunoreactivity of astrocytic processes surrounding the blood vessels in the hippocampus of animals in acute KA group GFAP activity was increased in only acute KA group compared to other groups. Scale bars = 25 µm.

Macroscopic observation of HRP extravasation in coronal Vibratome sections of brains in experimental groups is shown in Figure 5. There were no evident HRP reaction products in the brain parenchyma in acute or chronic sham groups. A widespread pattern of HRP extravasation was noted in cortical and subcortical regions of the brain in both acute and chronic seizures induced by KA in rats.

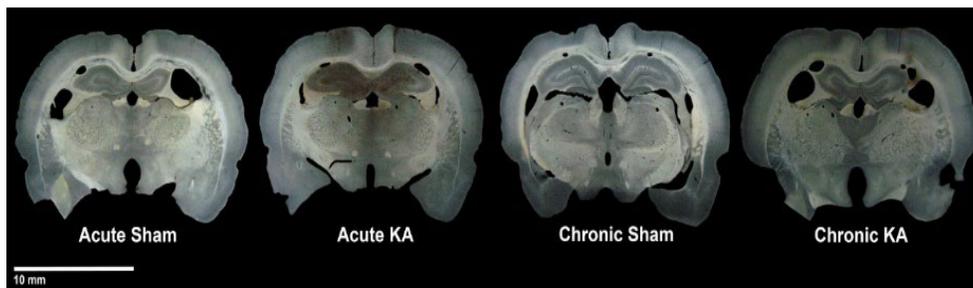


Figure 5: Photomicrographs showing the pattern of HRP extravasation in Vibratome sections of brains of rats in acute sham, chronic sham, acute KA, and chronic KA groups. Note that the animals subjected to KA in both acute and chronic stages showed macroscopic evidence of HRP extravasation (brown colour).

Ultrastructurally, occasional HRP reaction products were observed in the cytoplasm of endothelial cells of brain capillaries in the hippocampus and amygdala regions of rats in acute and chronic sham groups (Figure 6). Numerous pinocytotic vesicles of various sizes containing electron-dense HRP reaction products were seen in the cytoplasm of barrier type of capillary endothelial cells in the hippocampus and amygdala of animals in acute and chronic stages of seizures (Figure 6). Semiquantitative H scoring showed significantly higher frequency of HRP reaction products in both amygdala and hippocampus regions of animals with acute and chronic seizures compared to acute and chronic sham groups (Figure 6); ($P < 0.01$). TJs between adjacent endothelial cells were ultrastructurally intact, and no sign of the paracellular passage of HRP reaction products was noted in acute and chronic stages of seizures induced by KA (Figure 6). Astrocytic end-feet around the wall of brain capillaries exhibited normal ultrastructure and revealed no signs of swelling in KA-treated rats.

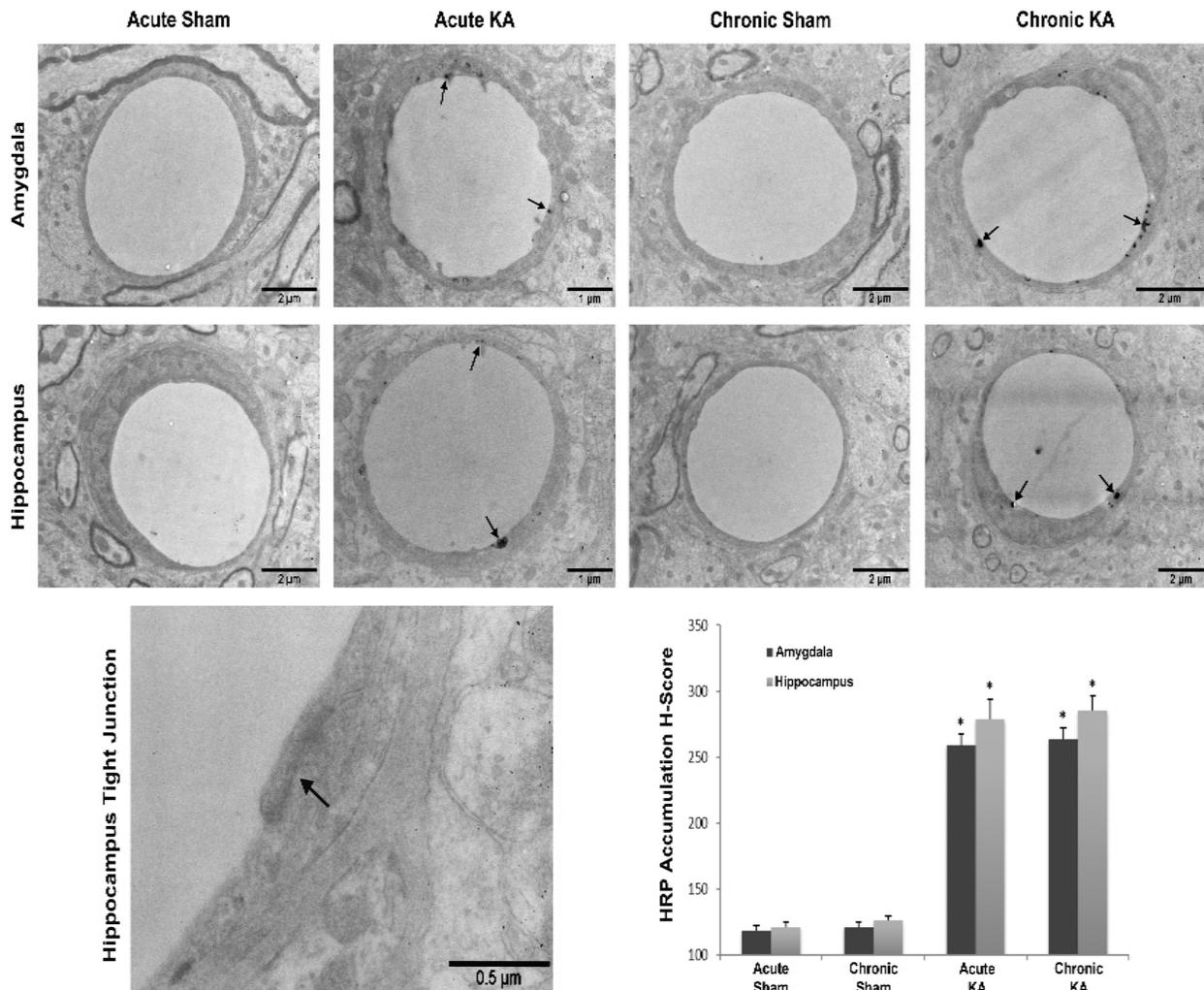


Figure 6: Electron micrographs showing capillaries from hippocampus and amygdala regions of brains in acute sham, chronic sham, acute KA and chronic KA groups following HRP injection. Note that no HRP reaction product was observed in the cytoplasm of endothelial cells of brain capillaries in the hippocampus and amygdala regions of rats in acute and chronic sham groups, while HRP reaction products were frequently observed in the cytoplasm of endothelial cells of brain capillaries in the hippocampus and amygdala of rats in acute KA and chronic KA groups. Assessment of the accumulation of HRP-containing vesicles in the brain capillary endothelial cells in hippocampus and amygdala regions of animals in experimental groups was performed by semiquantitative H scoring. One figure presenting an ultrastructurally intact TJ between adjacent endothelial cells in KA-treated group with no sign of the paracellular passage of HRP reaction products. The data represent mean ± SE. * $p < 0.01$ versus the rest of the groups.

Discussion

In the present study, we investigated the BBB response to the acute and chronic seizures in a kainate model of TLE in rats. Our results showed that BBB permeability increased to HRP, a tracer which cannot normally penetrate into the brain parenchyma. BBB opening not only occurred in the early hours after KA injection but also persisted for up to one month in the chronic stage in our experimental setting.

There is a variety of experimental models to induce TLE-like syndrome in rodents to enhance our understanding of the development of epileptogenesis [23,24]. To induce TLE model in rats, we chose to use KA, which is known to cause pathological changes similar to those of human TLE. Since high mortality rates along with inconsistent rates of development of spontaneous and recurrent seizures have been encountered by systemic treatment with a single high dose KA, we used a low-dose systemic KA protocol adapted from Hellier et al. and Dedeurwaerdere et al. with individual dosing schedules for each rat [16,17].

In several animal models, prolonged seizures disrupt BBB integrity while impairment in BBB integrity induces the development of epileptogenesis in normal brain [3,25]. Following intra-amygdala microinjection of KA in mice, the immunostaining intensity zonula occludens (ZO)-1, a tight junction protein, was shown to decrease and the expression of the protein remained unaltered in endothelial cells of microvessels in hippocampus, while protein levels of IgG in the hippocampus increased at day 21 [26]. Moreover, the loss of TJ proteins and BBB impairment were reported mainly in early stages after SE in animal models of TLE [27,28]. Although the data from the above-mentioned studies point out the involvement of the paracellular pathway in the early course of TLE, our study suggests the enhancement of transcellular pathway as evidenced by electron microscopic observations of increased HRP reaction products in the cytoplasm of barrier type of capillary endothelial cells along with intact TJs during acute stages of KA-induced seizures. We believe that, in our setting, the superior resolving power of the electron microscope and the use of HRP tracer enables a more decisive assessment of the functional transport pathway(s) and the mechanism(s) that is/are responsible for the increased BBB permeability.

In parallel with our findings, vesicular transendothelial transport was shown to increase at four h after KA injection, and TJs in the barrier type of endothelial cells were reported to be intact in earlier studies [12]. On the other hand, the BBB response to the chronic stage of KA injection has not been elucidated yet. A recent review discussed the role of BBB in TLE and pharmacoresistance [29], and Van Vliet et al. reviewed the literature data to highlight the changes in the BBB characteristics in epileptogenic brain tissue observed during the acute and chronic stages after KA-, pilocarpine-, pentylentetrazol-, bicuculline- and electrically

induced seizures [30]. Despite the mentioned current knowledge, ultrastructural data on the functional status of transcellular and paracellular transport pathways across the BBB were still lacking in especially during the chronic stage of TLE development. In this regard, our data showed for the first time that the BBB damage via increased transcellular transport of substances occurred not only in the acute stages but also in chronic stages of KA-induced seizures.

In a recent study, Morin-Brureau, et al. reported that KA decreased the *in vitro* expression of ZO-1 in hippocampal slices, however, occludin and claudin-5 levels remained essentially unaltered [28]. Caveolar transport of a fluorescent tracer was found to be increased at 6 h of stroke, but paracellular transport increased at two days following a stroke in a transgenic mouse strain [31]. However, in a recent informative study, it is reported that TJ proteins such as claudin-3 and claudin-5 were being dynamic structures, and they could direct the transmigration of leukocytes to the brain while undergoing rapid remodeling during transmigration to maintain the tightness of the BBB [32]. In our experimental setting, we found increased immunostaining intensity of claudin-5 in both acute and chronic seizures, and we did not observe any open TJs probably due to their dynamic properties.

Albumin concentration in the frontal cortex and striatum increased up to day three but declined back to normal levels at nine days after KA (10 mg/kg) injection in rats [33]. On the contrary, no macroscopic evidence of EB dye extravasation was observed in brain parenchyma during early epileptogenesis in a rat model of TLE [17]. However, van Vliet et al. showed increased EB-bound albumin in the piriform cortex at the light microscopic level in chronic epileptic rats at the end of 4 months, although the study lacked evidence of the transport pathway responsible for the albumin transport at the electron microscopic level [34]. It is also reported that the circulatory albumin that gained access to the brain parenchyma following BBB disruption was significantly absorbed by neurons, which might be associated with the neuronal damage and death during epileptic and KA-induced seizures [35,36]. On the other hand, generalized seizures induced by KA administration induce the expression of caveolin-1 in activated microglia in rat brain [37]. Since albumin endocytosis in endothelial cells occurs through caveolae [38], increased transcellular transport of albumin in the brain parenchyma might be related to the enhancement of epileptogenic activity during epileptic seizures induced by KA. Our data regarding caveolin-1 immunohistochemistry showed that the intensity of immunostaining of caveolin-1 increased in both acute and chronic seizures. These findings are also in agreement with our electron microscopic data regarding significantly increased caveolar vesicles containing HRP tracer in both acute and chronic stages of KA-induced TLE. In the light of these data, we suggest that transcellular caveolar transport could be mainly responsible for the transport of HRP into the brain parenchyma

from circulation. Also, we believe that the ultrastructural features of barrier type of endothelial cells are significantly important to elucidate the response of BBB to KA-induced TLE. On the other hand, the HRP tracer that we used in our ultrastructural assay is smaller in size than IgG and EB-bound albumin, which may account for, in part, some of the contradictory results from studies using different tracers.

Astrocytic end feet that surround the endothelial cells of microvessels in brain parenchyma may play a significant role in the induction and maintenance of BBB integrity [39]. In our study, we observed increased GFAP immunostaining intensity in the capillary endothelial cells in the hippocampus during acute seizures induced by KA while the intensity of immunostaining remained essentially unchanged in chronic seizures at day 30 after KA injection. Increased GFAP immune positive areas were also observed in the hippocampus of TLE patients [40]. However, an increase of GFAP immunoreactivity is also reported in the hippocampus in up to 35 days after KA injection in rats [41]. Our data on GFAP immunoreactivity at the acute stage of seizures are in line with studies that suggest that the loss of BBB integrity during seizures stimulates the activation of astrocytes, which in turn might also contribute to the development of epileptogenesis [42].

Conclusion

Our results show that BBB is still leaky one month after KA injection in rats. Given our ultrastructural data regarding the increased caveolar transendothelial transport and intact TJs in barrier type of brain capillaries during both the acute and chronic stages of KA-induced TLE, we suggest that BBB disruption occurs by augmentation of the transcellular pathway.

Acknowledgments

The work was supported by the Research Fund of Istanbul University (22096/2012).

Declarations

Authors' Contributions

CUY, MK, and CG designed the study. CUY, NO, NA, BA, CG, MK, IE, ET, and MK were involved in data collection and analysis. All authors have read and approved the latest version of the manuscript.

Conflict of interest

The authors have declared that there are no conflicts of interest.

Ethical Approval

All procedures performed in the study involving animals were by the ethical standards of the Local Ethics Committee of

Animal Experiments of Istanbul University (25/2012) and Turkish Ministry of Food, Agriculture, and Livestock (2011/28141).

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