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Effects of Acute Hyperglycemia on Blood Brain Barrier During Pentylenetetrazole-induced Epileptic Seizures

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Abstract: Epileptic seizures are one of the most common neurologic disorder and lead to disruption of Blood-brain Barrier (BBB). In animal experiments, seizure susceptibility has been shown to increase with incremental blood glucose. Moreover, clinical information regarding seizures and parameters of glucose control is lacking. The aim of this study is to examine the effect of acute hyperglycemia on permeability of Blood Brain Barrier (BBB) and the immunoreactivity of Zonula occludens-1 (ZO-1) and Glial Fibrillary Acidic Protein (GFAP) during Pentylenetetrazole (PTZ)-induced epileptic seizures. Experimental rats are divided into four groups. Evans blue was used as BBB tracer; ZO-1 and GFAP were determined by an immunohistochemical method. The BBB permeability of three parts of the brain in acute hyperglycemia+PTZ group compared to PTZ group ($p<0.05$). Immunoreactivity of ZO-1 decreased in acute hyperglycemic rats ($p<0.05$). GFAP immunoreactivity also significantly increased in PTZ and acute hyperglycemia+PTZ ($p<0.01$). This study was conducted to determine if acute hyperglycemia aggravates seizure changes GFAP activation and increases BBB damage during seizures.

Key words: Acute hyperglycemia, blood brain barrier, epilepsy, GFAP, ZO-1

INTRODUCTION

Hyperglycemia is the initiating insult and contributing factor in the pathogenesis of many cerebrovascular events (Salameh *et al*., 1997). Acute hyperglycemia attenuates endothelium-dependent vasodilatation in normal rat arterioles *in vivo*, suggesting that high glucose levels may mediate the abnormality (Williams *et al*., 1998). Exposure to high glucose may cause increased production of free radicals and lead to characteristic dysfunction and morphological changes of the endothelium. In this situation, Blood Brain Barrier (BBB) permeability may increase (Tesfamariam and Cohen, 1992; Yamashita *et al*., 1995). It was shown that increased antioxidant level protected against membrane damage depended on lipid peroxidation in the brain of rats during chronic hyperglycemia (Subash-Babu and Ignacimuthu, 2007). Experimental data indicate that acute hyperglycaemia can aggravate the consequences of epileptic seizures on the permeability of BBB (Kaya *et al*., 2002). Glucose plays a major role in metabolism and cerebral functions. It has been revealed that maternal diabetes affects BBB permeability, amount of cerebrospinal fluid and electrolyte concentration in newborn rats (Tehranipour *et al*., 2007, 2009). However, the effects of hyperglycemia on the Central Nervous System (CNS) and neuronal excitability are not fully understood. (Gispen and Biessels, 2000). It has been suggested that glucose enhances synaptic transmission and propagation, leading to more excitable neurons Seizure types described in patients with hyperglycemia are diverse including epilepsia partialis continua, which is the most frequent type, simple or complex partial seizures and less common recognized symptoms are apnea, somatosensory symptoms, aphasia, and visual disturbance (Tutka *et al*., 1998; Gispen and Biessels, 2000). However, no experimental data are available yet on tight junction protein of BBB such as zonula occludens (ZO)-1 and Glial Fibrillary Acidic Protein (GFAP) indicator of astrocyte recognition in acute hyperglycemia during seizures. The present study aims to address such an effect and to assess possible changes of the staining level

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of GFAP as indicator of ZO-1 and astrocyte in order to determine their influence over BBB permeability in these conditions.

MATERIALS AND METHODS

The experimental protocols were conducted following guidelines of the Animal Studies Ethics Committee of Istanbul University (Resolution No. 09/12.12.2006). Thirty two adult female Wistar albino rats for each parameters weighing between 180-230 g were selected for the study. The rats were divided into four groups of 8 animals each as follows:

- **Group 1:** Controls
- **Group 2:** Rats treated with PTZ to induce epileptic seizures
- **Group 3:** Acute Hyperglycemic rats
- **Group 4:** Acute Hyperglycemic rats treated with PTZ

The following parameters were evaluated in all cases:

- Assessment of blood-brain barrier permeability
- Assessment of ZO-1 and GFAP

Assessment of BBB permeability: Evans Blue Dye (EBD, Sigma, E-2129) 2% solution was administered at a dose of 4 mg kg^{-1} to trace the blood-brain barrier permeability. Acute Hyperglycemia was induced by intraperitoneal injection of 2 mL 50% glucose solution. Thirty minutes after induction of acute hyperglycemia, rats with a fasting blood sugar level above 250 mg dL^{-1} were included in the study. Ether was used for general anesthesia and Jetokain for local anesthesia. A cannula was inserted into the femoral vein for injections and obtaining blood samples while the femoral artery was used for blood pressure monitoring. Following administration of EBD, 100 mg kg^{-1} of PTZ were injected to induce convulsion. Seizures were monitored for 20 min. All the animals exhibited generalized convulsions characterized with tonic-clonic contractions. Twenty-five min after EBD injection all the rats were killed by perfusion through the heart with saline to avoid artificial staining of the brain during removal. Following this procedure, the brains were removed and the left and right hemispheres and the cerebellum and brainstem were dissected so that each section was analyzed separately. The age and weight of each brain section was recorded. Subsequently, each brain section was homogenized in 2.5 mL phosphate buffer. Following homogenization, 60% trichloroacetic acid was added into each tube and the EBD was separated from albumin by mixing the tube in a vortex for 2 min, cooling at 4°C for 30 min and then centrifuged at 1000 G for 30 min. After the centrifugation, the supernatants were transferred into spectrophotometer tubes and their absorbance values were read at 620 nm, comparing them with an EBD calibration curve. The EBD values were calculated as percentages (mg 100 g^{-1} brain tissue).

Assessment of ZO-1 and GFAP: Immunohistochemical staining was used to visualize ZO-1 and GFAP expressions. The anesthetized rats were first perfused with saline followed by a second perfusion with 4% formaldehyde in pH 7.4 phosphate buffer for 10 min. After perfusion, the brains were kept in the formaldehyde solution at 4°C for 24 h, embedded in paraffin and then cut into 4-5 μm sections that were then heated in microwave oven within citrate buffer (0,1 M, pH 6) for 10 min and then incubated in hydrogen peroxide for an additional 10 min to ensure inactivation of endogenous peroxidase activity. Polyclonal rabbit anti-ZO-1 (Zymed Lab Inc., CA, 1:50, overnight) and monoclonal mouse anti-GFAP (Neomarker; Fremont, CA; 1:100, 60 min) were used as primary antibodies for ZO-1 and GFAP, respectively. Biotinized goat anti-polyvalent was used as secondary antibody for ZO-1 and biotinized goat anti-mouse for GFAP (Lab Vision Co., Westinghouse, CA). After flushing the sections were treated with peroxidase-conjugated streptavidin (Lab Vision Co., Westinghouse, CA) and aminoethyl carbazole chromogen, stained with Mayer hematoxylin and examined under a light microscope. Both the intensity and the distribution of specific ZO-1 and GFAP staining were scored. For each sample, a histological score (HSCORE) value was derived by summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining [HSCORE = S Pi (i+1], where i is the intensity, S is score (0-4) and Pi is the corresponding percentage of the cells]. 0: no staining 1: weak staining 2: middle staining 3: dark staining 4: very dark staining.

Statistical analysis: The Wilcoxon signed rank test was used for the statistical analysis of blood glucose and blood pressure. The EBD results were treated by ANOVA and the Mann-Whitney U test to compare cytokine responses. In all cases $p<0.05$ was set as limit of significance.

RESULTS

Blood pressure and blood glucose: Table 1 shows the blood pressure and blood glucose levels in all of the groups under study. The blood pressure is significantly higher after administration of PTZ, $p<0.01$. The blood glucose values was significantly increased in the acute hyperglycemic animals relative to the non-acute hyperglycemic rats, $p<0.01$.

Changes in BBB permeability: Significant increases of permeability of the BBB in the three parts of the brain under study were observed in the PTZ-, acute hyperglycemia+PTZ groups compared to controls ($p<0.01$) (Fig. 1).

Table 1: Before and after PTZ, values of arterial blood pressure and serum glucose for groups

Experiment groups	Arterial blood pressure (mmHg)		Blood glucose levels (mg dL^{-1})
	Before PTZ	After PTZ	
Control	98.0±7.7	-	89±13.0
PTZ	98.0±12.6	171±9.5**	91±15.0
AH	100±7.2	-	376±29.8
AH+PTZ	92.0±8.6	174±9.4**	379±37.5

**p<0.01. AH: Acute Hyperglycemia

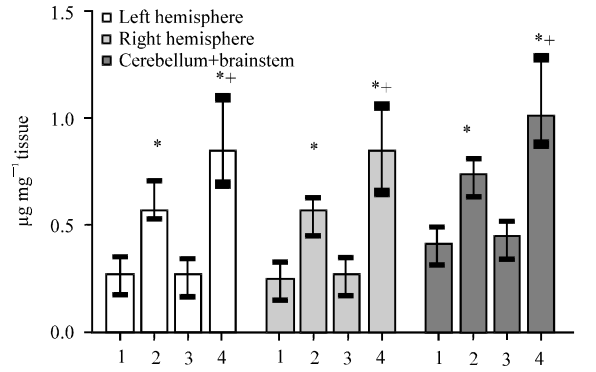

Fig. 1: Contents of the tracer (Evans Blue, µg mg^{-1} tissue) in samples of the left brain hemisphere, right hemisphere and cerebellum+brainstem regions in groups 1-4 of experimental rats. Intragroup Means±SD are shown. *Significant differences at $p<0.01$, respectively from the control group 1 and acute hyperglycemia group 2; +Significant difference at $p<0.05$ from the value in group 2 (PTZ)

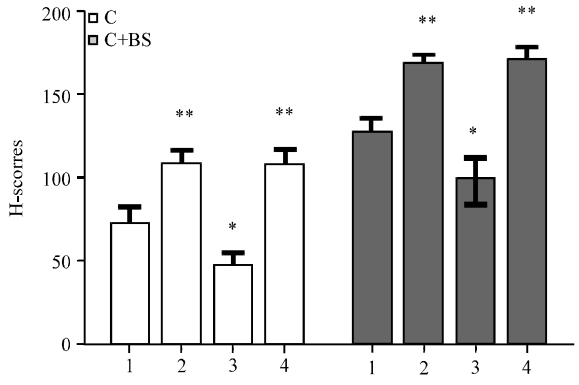

Fig. 2: H-scores values for ZO-1 in samples of the cortex (C) and cerebellum+brainstem (C+BS) regions in groups 1-4 of experimental rats. Intragroup Means±SD. are shown. *, **Significant differences at $p<0.05$ and $p<0.01$, respectively from the control group 1

ZO-1 and glial fibrillar acidic protein changes: ZO-1 immunoreactivity of cerebral endothelial cells in cortex and cerebellum+brainstem sections from acute hyperglycemic groups were lower than in the control group ($p<0.05$). For the PTZ, acute hyperglycemia+PTZ groups It was higher than the controls ($p<0.01$), (Fig. 2, Images 1-2).

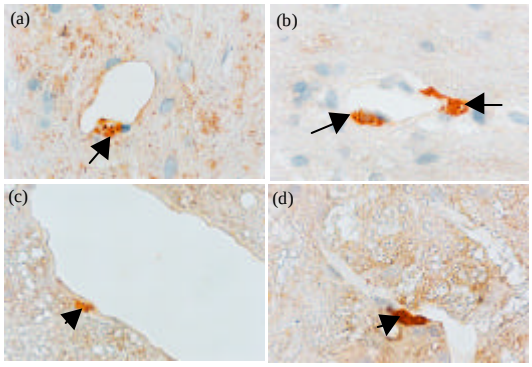

Image 1(a-d): The photos showing the staining grade of zonula occludens–1 in brain capillary endothelial cells of cortex region in experimental groups of rats, (a) Control, (b) PTZ, (c) Acute hyperglycemia and (d) Acute hyperglycemia+PTZ

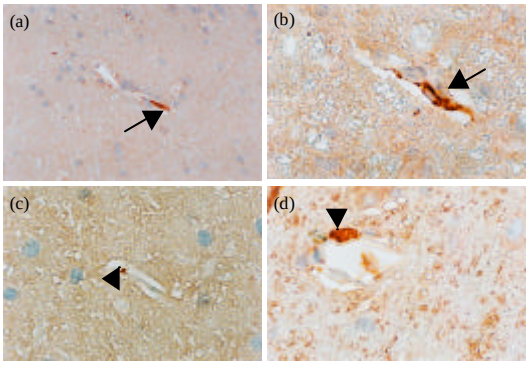

Image 2(a-d): The photos showing the staining differences of zonula occludens-1 in the brain capillary endothelial cells of the brain regions cerebellum+brain stem in the experimental groups of rats,(a) Control, (b) PTZ, (c) Acute hyperglycemia and (d) Acute hyperglycemia+PTZ

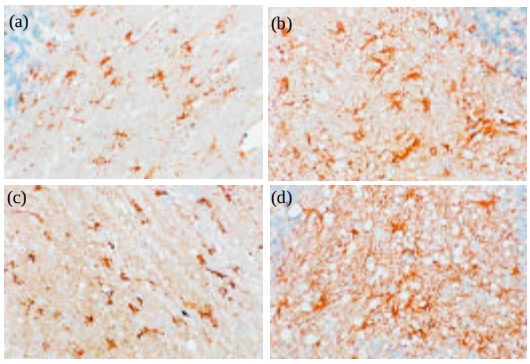

Image 3(a-d): The photos showing the staining degree of Glial Fibrillar Acidic Protein in the brain sections from the cortex region of the experimental groups of rats, (a) Control, (b) PTZ, (c) Acute hyperglycemia and (d) Acute hyperglycemia+PTZ

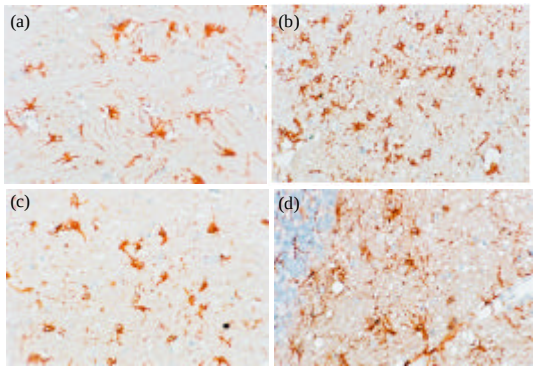

Image 4(a-d): The photos showing the staining differences of Glial Fibrillar Acidic Protein in the brain sections from the cerebellum+ brain stem of the experimental groups of rats, (a) Control, (b) PTZ, (c) Acute hyperglycemia and (d) Acute hyperglycemia+PTZ

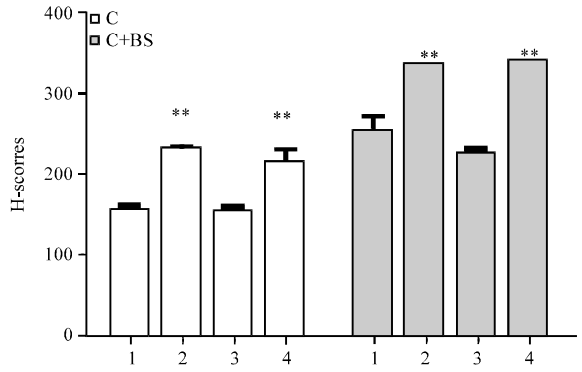

Fig. 3: H-scores values for GFAP in samples of the cortex (C) and cerebellum+brainstem (C+BS) regions in groups 1-4 of experimental rats. Intragroup Means±SD are shown. **Significant differences at $p<0.01$ from the control group 1

In the acute hyperglycemia group the GFAP immunoreactivity was near that of the controls ($p>0.05$). It was increased in the PTZ, acute hyperglycemia+PTZ ($p<0.01$), (Fig. 3, Images 3-4).

DISCUSSION

The significant increase of the BBB permeability in the left and right hemispheres and in the cerebellum and brainstem in normal rats with induced epileptic seizures is consistent with earlier literature reports (Lorenzo *et al*., 1975; Bolwig, 1989; Sahin *et al*., 2003). The observed increase in staining levels of ZO-1 and GFAP among PTZ-treated rats is consistent with the results of Arican *et al*. (2006). Passing of EBD-albumin complex into the brain presents us with a contradiction that will require clarifying. There are two important mechanisms that explain elevations of BBB permeability in epileptic seizures. One such mechanism involves the increase of pinocytic activity in capillary endothelial cells (Petito *et al*., 1977; Nitsch *et al*., 1986; Westergaard *et al*., 1978), Others claim that intraluminal pressure elevation leads to capillary dilatation in epileptic seizures, causing tight junctions to open, resulting in a rise of BBB permeability (Chan and Fishmann, 1984; Harik, 1986). The observed increase of ZO-1 may suggest a reduction of paracellular passage, which in turn may indicate preservation of tight junctions during epileptic seizures, supporting the first but not the second of these mechanisms. A sudden elevation of blood pressure was seen in all the epileptic rats. Hypertension during stroke was found to play an important role on the increase of pinocytic activity in brain endothelial cells in support of our proposal (Cipolla *et al*., 2004). In our study, acutely high dose glucose did not effect on BBB permeability of brain regions importantly. Similarly, it was shown that acute hyperglycemia had no effect on BBB permeability (Kaya *et al*., 2002). However, *in vitro* high glucose caused breakdown of the capillary blood-retinal barrier in diabetes (Gillies *et al*., 1997). We observed that acute hyperglycemia did not change in GFAP immunoreactivity but it decreased ZO-1 immuno-reactivity. Reduction of ZO-1 immuno-reactivity occurs increase of paracellular

permeability. This situation may cause the increase of BBB permeability. However, in acute hyperglycemia with Evans Blue Tracer, we did not observe any changes in BBB permeability. In spite of changing in ZO-1 immunoreactivity reason of changing in BBB permeability that we could not observe may be because of the Evans blue tracer binding to albumin which is at 69000 mol weight. In this study, the content of Evans blue dye in the brain regions of animals in the acute hyperglycaemia plus epileptic group was higher than that of normoglycemic plus epileptic animals. Acute hyperglycemia may cause increased production of free radicals and lead to changes of BBB permeability (Tesfamariam and Cohen, 1992; Yamashita *et al*., 1995). Glucose having a fundamental importance for brain oxidative metabolism is increasingly carried to brain as well as consumed there during epileptic seizures. (Cornford *et al*., 1998). However in addition to these changes in epileptic seizures, increased reactive oxygen species may lead to brain endothelial cells (Lagrange *et al*., 1999). In acute hyperglycemia+epileptic animals we observed an increase of immunoreactivity in both ZO-1 and GFAP. Seizures may responsible for these increasing. Also, in this group increase in pinocytotic activity may cause BBB damage as convulsion group. We think that the changes in the BBB permeability in rats undergoing acute hyperglycemic epileptic seizures, may be caused from pinocytic activity elevation or a possible damage in the transcellular pathway.

CONCLUSION

The reduction of zonula occludens-1 suggests that acute hyperglycemia is not an innocent agent for BBB permeability. Controlling hyperglycemia may be decreases BBB damage during epileptic seizures.

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