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Title: Serum prolidase enzyme activity level: Not a predictive biomarker for epilepsy

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ABSTRACT:

Oxidative stress and inflammation are held responsible for the pathogenesis of epilepsy. Prolidase is known to have an extremely important role for proline recycling in collagen synthesis. Higher proline levels have been shown to increase oxidative stress. Furthermore, prolidase activity is known to be associated with inflammation during fibrotic process. No study has yet investigated the relationship between epilepsy and prolidase enzyme activity (PEA). In this study, we aimed to contribute to the existing literature by assessing postictal PEA levels, which are known to be correlated to inflammation and oxidative stress, in order to determine whether PEA levels may be used as a biomarker for the disorder.

This study included patients with epilepsy who presented to emergency department within first six hours after a seizure. The epileptic group included 27 patients (16M, 11F) and the control group 31 healthy individuals (11M, 20F). The mean age of the epilepsy group ($n=27$) was 43.1 ± 20.2 , and the healthy control

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group ($n=31$) 51.9 ± 21 years. Serum PEA levels were 1171.9 ± 343.3 in the epileptic group and 1137.1 ± 295.6 in the control group. There were no significant differences between two groups. ($p > 0.05$)

Our study results suggest that, although PEA is an enzyme associated with oxidative stress and inflammation, it is still not an ideal biomarker for epileptic patients. This study is an important one as it investigated PEA in patients with idiopathic epilepsy for the first time.

INTRODUCTION:

Epilepsy, a disorder with an overall population prevalence of 1%, is characterized by recurrent seizures resulting from the hyperexcitability of neurons throughout the cortex or in localized brain regions [1]. A variety of pathophysiological mechanisms have been identified for epilepsy. Markers in physiological pathways have recently gained even more importance in the diagnosis and prognosis of the disease. Oxidative stress and inflammation caused by increased serum prolidase enzyme activity (PEA) are known to play an important role in the pathogenesis of epilepsy. Studies have shown that PEA increases in conditions such as chronic liver disease, lung cancer, hypertension, and acute hemorrhagic stroke [2-5]. This increase has been reported to be secondary to oxidative stress.

In an experimental study, increased proline in rat brain tissue was reported to reduce the total radical antioxidant potential and to cause oxidative stress. This result to be related to an aspect of the neurological dysfunction seen in hyperprolinemia [6]. A clinical study found that prolidase and total oxidant status levels were higher and total antioxidant status levels were lower in Alzheimer patients than in the control group. It has been reported that oxidative stress and collagen degradation play a role in cognitive destruction [7].

Prolidase activity is also related to inflammation in the fibrotic process. Prolidase inhibitors have been reported to cause a reduction in immune mediator and receptor expression [8]. Patients with chronic hepatitis infection have an increased prolidase activity with collagen turnover [9].

No study has yet investigated the relationship between epilepsy and PEA. In this study, we aimed to contribute to the existing literature by assessing postictal PEA levels, which are known to be correlated to inflammation and oxidative stress, in order to determine whether PEA levels may be used as a biomarker for the disorder.

MATERIAL AND METHODS:

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This study included patients with epilepsy who presented to the emergency department of the hospital within 6 hours of an idiopathic generalized tonic-clonic seizure. There were 27 patients with an age range of 18-88 years. The healthy control group consisted of 31 healthy individuals of the same age range. Exclusion criteria included ischemic cerebrovascular accident, aneurysm, arteriovenous malformation, intracranial mass, alcohol use, antidepressant-antipsychotic use, metabolic disorders, prolonged postictal confusion, syndromic seizures, and hypersomnia or insomnia. Laboratory studies were studied from the serum samples remaining from those taken for routine laboratory testing. The local ethics committee approved the study. Written informed consent was obtained from patients who participated in this study.

DATA COLLECTION:

All patients underwent neurological examination, electrocardiography, a complete blood count, and biochemical testing. After the completion of all procedures, the prolidase levels of the serum samples were measured using the ELISA technique.

SAMPLES:

The blood samples were taken into non-EDTA tubes, centrifuged at 4 ° C and at 3000 rpm for 10 minutes to obtain serum. Serum samples and other biochemical parameters were stored at -80°C for prolidase activity measurement. (Siemens) All measurements were done in the same series after thawing the samples.

PROLIDASE ASSAY:

Serum prolidase activity was quantified using the method described by Myara et al and optimized by Özcan et al. [9,10]. Spectrophotometric method was used to measure proline level. 100 µL serum and 500 µL preincubation solution (50 mmol/L Tris-hydrochloride solution at pH 7.8, with 1 mmol/L endogenous antioxidant glutathione (GSH)), 5 mmol / L manganese (II) chloride (MnCl₂) and 0.1 Triton X-100) were mixed; this mixture was then incubated at 37°C for 3 hours. A 100 µL volume of the preincubation serum was added to 100 µL 144 mmol / L Gly-Pro solution and incubated at 37°C for 30 minutes. Following incubation 1 ml 0.45 mol / L trichloroacetic acid solution was added to incubation tube and the reaction was stopped. This mixture was centrifuged at 1500 rpm for 5 minutes, and 500 µL upper phase was

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removed. The supernatant was used for proline quantification with the method described by Myara et al. and modified by Chinard [10,11,12].

STATISTICAL ANALYSES:

The Kolmogorov-Smirnov test was used to test the normality of the distribution of variables. Continuous parametric variables were compared using the independent-samples t-test. Continuous non-parametric variables were compared using the Mann-Whitney U test. The continuous variables were reported as mean \pm standard deviation. Pearson's chi-square test is used to discover for detect relationship between two variables. Statistical significance was set at $p < 0.05$. All analyses were performed using IBM SPSS Statistics, Version 22.0 (Armonk, NY: IBM Corp.).

RESULTS:

The epileptic group consisted of 27 patients (16 males, 11 females); the control group consisted of 31 healthy individuals (11 males, 20 females) (Table 1). The mean age of the epileptic patients was 43.1 ± 20.2 , and the control group ($n = 31$) 51.9 ± 21 years (Table 2). The demographic variables of both groups were similar. Serum PEA levels were 1171.9 ± 343.3 in the epileptic group and 1137.1 ± 295.6 in the control group. (Table 3). There were no significant differences between patients with epilepsy and the members of the healthy control group. ($p > 0.05$)

DISCUSSION:

In our study, the serum level of PEA, an enzyme related to oxidative stress and inflammation, was evaluated as a biomarker of the pathogenesis of epilepsy among patients with primary generalized epilepsy. There were no statistically significant differences in serum PEA levels between patients with epilepsy and members of the healthy control group.

Prolidase, a protease that degrades imidopeptides into proline or hydroxyproline residues, plays a role in the metabolism of collagen [13]. Proline and hydroxyproline form approximately 25% of collagen connective tissue and play an important role in maintaining its rigidity. The prolidase enzyme has been detected in erythrocytes, leukocytes, dermal fibroblasts, the kidney, the brain, the heart, the thymus, and

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the uterus. A wide tissue distribution suggests that changes in PEA may play an important role in the pathogenesis and result of many disorders.

Epilepsy is a disorder of the central nervous system which is characterized by recurrent seizures as a result of neuronal hyperexcitability [1]. Various physiological mechanisms have been defined for epilepsy. The cellular mechanisms of epileptogenesis include cellular damage, gliosis, increased expression of intermediate-early genes (c-fos, c-jun), increased growth factors, neurogenesis, synaptogenesis, alterations of glutamate and GABA signaling, inflammatory mediators, alterations of voltage-gated ion channels, and excitotoxic antibodies [14]. Prolidase activity has been related to oxidative stress during the process of fibrosis and in various diseases [15]. Inflammation and oxidative stress play an important role in the pathogenesis of epilepsy.

Oxidative stress resulting from excessive free radical release probably plays a role in the initiation and progression of epilepsy [16]. Maintaining low levels of reactive oxygen species (ROS) is critical to normal cellular functions, and thus prolonged increments of ROS constitute a risk for an increase of the neurodegeneration seen in epilepsy. Brain is particularly sensitive to oxidative stress. Increased serum PEA increases proline and hydroxyproline levels. In a study on rat brains, both acutely and chronically increased proline affected some oxidative stress parameters and augmented in vivo and in vitro oxidative stress, suggesting this as the mechanism of brain dysfunction among patients with increased proline levels [17].

Recently, many clinical studies have been published supporting this relationship. Gönüllü et al. [5] reported increased oxidative stress levels, reduced antioxidant levels, and increased PEA among patients with acute hemorrhagic stroke compared to the controls. Similarly, a study showed that serum PEA was increased and antioxidants were reduced among obese patients, suggesting oxidative stress as the cause [18]. Patients with diffuse anxiety disorder were shown to have significantly increased total antioxidant levels, oxidative stress indexes, and serum PEA compared to those in the control group [19]. On the other hand, our study failed to demonstrate any significant difference between the serum PEA levels of the epileptic patients and healthy controls. A study with results similar to our own demonstrated no significant difference between the serum PEA levels of patients with major depressive disorder and those of the controls [20].

Recent preclinical studies on acute seizures and chronic epilepsy models have revealed that neuroinflammation developing in the brain contributes to underlying neuronal hyperexcitability in seizure onset and generalization. [21] As for the relationship of epileptogenesis with inflammation, it has been found in animal studies that the synthesis of cytokines and adhesion molecules were increased by

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inflammation. [22,23] The levels of circulating inflammatory proteins have been considered to be potential biomarkers for epileptogenesis. [24]. Experimental studies have shown that immune mediators (IL- β , TNF- α , toll-like receptors, TGF- β) have a prominent role in seizure onset. [25]. IL- β is related to febrile seizure and status epilepticus while TNF- α acts through glutamate receptors. Toll-like receptors are released by injured or dead neurons and play a role in seizure spread and recurrence. TGF- β is related to the opening of the brain-blood barrier. One study reported that prolidase inhibitors caused a reduction in TGF- β 1 and receptor expressions [8]. The prolidase enzyme probably exerts an inflammatory effect through TGF- β 1. Patients with chronic hepatitis exhibit increased collagen turnover and increased prolidase activity [9]. Similarly, it was reported that serum PEA may be used for the diagnosis and management of idiopathic pulmonary fibrosis, a disease state created by a reaction to an injury [26]. However, a review reported that prolidase activity per se may not reflect disease activity, and is clinically important only in combination with other biochemical markers [27].

Our study aimed to investigate whether PEA, a marker related to inflammation, could be a novel biomarker for both diagnosis and treatment (for the management of future treatment models involving anti-inflammatory agents) in epileptic patients. This study is significant because of its pioneering role in the investigation of PEA among patients with idiopathic epilepsy. It is important to develop effective biological markers in order to diagnose epilepsy early in its course and to determine its prognosis. In this study, however, no significant difference was found between the epilepsy patients and the control group with respect to the serum levels of PEA. According to our results, although PEA has been reported to have a relationship with oxidative stress and inflammation, it is not an ideal biomarker for epileptic patients.

The limitations of our study are the small sample size, the single measurement of the PEA level, and the lack of measurement of the levels of inflammatory mediators or oxidative stress levels. There is a need for larger and more detailed studies to elucidate the relationship between inflammation and oxidative stress, and the role of PEA in the epileptogenesis of the latter.

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	Female N (%)	Male N (%)	Total N (%)
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Epileptic group	16 (28)	11 (19)	27 (47)
Healthy group	11 (19)	20 (34)	31 (53)

Mann-Whitney U test

Table 1. Gender evaluations of epileptic and healthy control groups

	Mean age±SD
Epileptic group	43.1±20.2
Healthy group	51.9±21

Mann-Whitney U test

SD: standard deviation, ^[1]_[SEP]

Table 2. The mean age of epileptic and healthy control groups.

	PEA level Mean±SD	p
Epileptic group	1171.9 ± 343.3	> 0.05
Healthy group	1137.1± 295.6	> 0.05

Pearson's chi-squared test

SD: standard deviation, ^[1]_[SEP] p<0.05: statistically significant

Table 3. Serum PEA levels of epileptic and healthy control groups

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