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Metabolic responses in discrete, Mice brain regions like CC, CG, H and CQ during PTZ induced epileptic seizures.

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Abstract

Epilepsy, a neurological disorder with recurrent seizures, involves disruption of different metabolic enzymes and its related metabolites altering the normal processes of metabolism, either during the onset or post epilepsy in the brain. In the present work, it is convincingly, observed that the Mice brain regions such as Corpus callosum (CC), Cingulate gyrus (CG), Hippocampus (H) and Corpora quadrigemina (CQ) shows significant changes in the activities of metabolic enzymes such as AST, ALT, LDH; ATPases like Na^+, K^+-ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase along with their metabolites such as Glucose, Pyruvate, Lactate and Glutamate, altering the metabolic integrity during Pentylenetetrazole (PTZ) induced epileptic seizure. We report from the present work that, H and CG are affected completely during epileptic seizure as compared to its control but CC and CQ shows partly altered as far as metabolism in brain is concerned.

Keywords: Corpus callosum, Hippocampus, Cingulate gyrus, Corpora Quadrigemina, Metabolic enzymes and metabolites.

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Introduction

Epilepsy, involves a disruption of brain energy homeostasis and is potentially manageable through principles of metabolic control theory. Metabolism is an essential process underlying all phenotypes and an alteration in metabolic process can modify phenotype. The theory is based on the idea that compensatory genetic and biochemical networks, operating through flexible biological systems, are capable of modulating the bioenergetics of glycolysis, the TCA cycle, electron transport and oxidative phosphorylation [1,2].

Although, the work in this aspect has been done in Cerebral cortex, Cerebellum, Medulla Oblongata, Hippocampus (H) etc, yet the regions such as Corpus Callosum (CC), Cingulate gyrus (CG), Corpora Quadrigemina (CQ) are not explored in this regards to a large extent.

The corpus callosum is the principal anatomical and neurophysiological track linking the cerebral cortices [3]. Corpus callosotomy was first introduced as a surgical technique for the treatment for epilepsy in 1939 [4]. It is often used to treat atonic, clonic, myoclonic and generalized tonic-clonic seizures [5]. The cingulate gyrus is an arched convolution that lies next to the corpus callosum and is separated from it by the sulcus of the corpus callosum. Cingulate gyrus epilepsy is controversial because it may overlap with other frontal lobe epilepsy syndromes. But, an aberrant behaviors observed in epileptic patients completely resolved after lesionectomy of Cingulate gyrus [6]. Hippocampus and Cingulate gyrus forms the part of limbic system. Corpora quadrigemina are reflex centers involving vision and hearing. In the brain, the corpora quadrigemina are the four colliculi, two inferior, two superior located on the tectum of the dorsal aspect of the midbrain [7].

Metabolic enzyme such as aminotransferases like AST and ALT serve as a strategic link between carbohydrate and protein metabolism under pathophysiologic stress [8]. Similarly, LDH is an enzyme that transforms lactate into pyruvate in brain tissue and thus, prevents acidosis and provides a substrate for TCA cycle. ATPases, also play an important role in the maintenance of ionic gradient by coupling ATP hydrolysis with energy processes [9]. ATP itself, as a neurotransmitter and neuromodulator, may influence the release of other neurotransmitters by acting through its own receptors or by altering the neurotransmitter receptors [10,11]. Na^+, K^+ -ATPase is a membrane bound enzyme and inactivation of this enzyme is an important factor in epileptization of neurons [12]. Similarly it is demonstrated that inhibition of microsomal Mg^{2+}-ATPase, Ca^{2+}-ATPase may be associated with long-term plasticity changes associated with epileptogenesis [13]. Idiopathic epilepsies involve the Na^+, K^+, or Ca^{2+} chan-

nels and the activities of Na^+, K^+ - or Ca^{2+} ATPase are responsible for maintaining ionic balance in the cell [14].

As far as energy metabolites in brain are concerned, glucose forms the obligatory energy substrate for brain and it is almost entirely oxidized to CO_2 and H_2O. Glucose can be incorporated into lipids, proteins, and glycogen, and it is also the precursor of certain neurotransmitters such as γ-aminobutyric acid (GABA), glutamate, and acetylcholine [15,16]. Numerous studies have been performed to identify molecules that could substitute for glucose as an alternative substrate for brain energy metabolism. Among the vast array of molecules tested, mannose is the only one that can sustain normal brain function in the absence of glucose [17]. However, mannose is not normally present in the blood and cannot therefore be considered a physiological substrate for brain energy metabolism. Whereas, lactate and pyruvate can sustain synaptic activity in vitro [18,19], hence, act as an alternative energy substrate in the brain. Glutamate is an amino acid neurotransmitter that can behave as an endogenous convulsant as well as energy metabolite. Microdialysis measurements from humans with spontaneous seizures from the hippocampus show transient release of glutamate [20].

An attempt is made in the present work to study the effect of Pentylenetetrazole (PTZ) induced epileptic seizures on the mice Corpus callosum (CC), Cingulate gyrus (CG), hippocampus (H), and Corpora quadrigemina (CQ) since they could be associated with epileptic events. Though metabolic activities of other parts of brain during or after onset of epilepsy have been studied, the metabolic aspect of Corpus callosum, Cingulate gyrus and Corpora quadrigemina has been ignored thus far. Therefore, we decided to throw some light on a few basic aspects such as metabolites and associated key enzymes, which are playing role in epilepsy.

Materials and Methods

Animals

Experiments were carried out on Mice, *Mus musculus*, weighing 25-30 g. The animals were kept on a 12 – h light/ dark cycle and allowed free access to food and water. All experiments were performed between 10.00 am to 12.00 noon in a silent room at 22-24^{0}C. All animal use procedures were approved by the Goa University ethical committee.

The animals were randomly divided into two groups: (a) controls (n=5) were with no previous history of seizure and were given saline injections s.c., (b) Experimentals (n=5) mice that received PTZ s.c.

PTZ treatment

A subcutaneous dose of PTZ sufficient to induce a seizure in 97% of the test animals was referred to as the convulsive dose 97 (CD97). At the time of the test, PTZ (70 mg/kg) was injected s.c. into the loose fold of skin in the midline of the neck. This dose represents the average CD97 in Mice, *Mus musculus*. The site of injection was massaged in order to distribute the PTZ solution prepared in saline throughout the subcutaneous tissue. Mice were then placed in individual cages and observed over the course of next 30 min for the presence or absence of a tonic- clonic seizure.

Sample preparation

Mice were ether anesthetized and sacrificed by decapitation. The brains were quickly removed and placed on ice. The Corpus callosal, Cingulate gyral, Hippocampal and Corpora quadrigeminal regions, were dissected from the brain and immediately stored at -20^{0}C.

Tissue samples were thawed and homogenized in 0.32 M sucrose for AST, ALT, LDH, Na^+/K^+-ATPase, Mg^{+2}-ATPase, and Ca^{2+}-ATPase assay; in distilled water for glucose estimation, and in 0.25 M sucrose for Glutamate and Pyruvate estimation, and in 10% TCA for lactate estimation.

Enzymatic assays

Aminotransferases ie ALT (EC 2.6.1.2) and AST (EC 2.6.1.1) were measured according to 2,4-dinitrophenyl hydrazine (2,4-DNPH) method as described previously [21]. The incubation mixture for ALT contained 500 µl of alanine-α-ketoglutarate, 100 µl of enzyme extract, 500 µl of 2,4- DNPH and 5 ml of 0.4 N NaOH. The incubation mixture for AST contained 500 µl of aspartate-α-ketoglutarate, 100 µl of enzyme extract, 500 µl of 2,4-DNPH and 5 ml of 0.4 N NaOH. Optical density of corresponding brown colored hydrazone formed in alkaline medium was read at 505 nm.

LDH (EC 1.1.1.27) activity were assayed by 2,4- DNPH method, using Span Diagnostic Reagent Kit (Product Code: 25903)

The activities of Na^+/K^+-ATPase, Mg^{+2}-ATPase, and Ca^{2+}-ATPase were determined from both control and experimental set, by following the method of Venugopal et.al. (2005) [22]. The filtrate obtained after the reaction is over, and after precipitating protein i.e., enzyme by 10% TCA was treated with an ammonium molybdate reagent, which reacted with inorganic phosphate to form phosphomolybdic acid. This was reduced by Metol to give blue colour, which was measured spectrophotometrically. Phosphorous standard was used to calculate the concentration of inorganic phosphorous present in the sample.

Estimations of Energy metabolites

Glucose was estimated by using kits from Span Diagnostics (Product code: SKU # 93MB100-64).

Pyruvate: 0.1 ml supernatant, with1 ml DNPH reagent incubated for 15 minutes at 37^0C, then 4N NaOH was added and readings were taken at 440nm after 5 minutes. 1mM pyruvate was used as a standard [23].

Lactate: The filtrate obtained after centrifugation of homogenate, 20% $CuSO_4$ solution with 8 ml H_2O was added. To this 1 gm of powdered Calcium hydroxide was added and kept at room temperature for 30 min. After centrifugation, 0.05 ml of 0.4% $CuSO_4$ added to the supernatant, followed by 6 ml of Conc. H_2SO_4, kept in boiling water bath for 5 min. After cooling, 0.1 ml of p-hydroxydiphenyl reagent added and allowed to stand at room temperature for 30 min and then in boiling water bath for 90 seconds. After cooling OD was obtained at 560 nm. For Standard, lactate solution (0.01 mg/ml) was used [24].

Glutamate: The glutamate was assayed by running a parallel procedure with another 0.1 ml supernatant. The protein-free homogenate was incubated with 0.2 ml ninhydrin solution at 60^0C for 30 min, cooled, and then mixed with sodium tartarate reagent. The summated absorbance of both glutamate and GABA was obtained spectrofluorophotometrically at excitation and emission wavelengths of 377 and 451 nm, respectively. The absorbance of glutamate was determined by subtracting the absorbance of GABA from the respective summated absorbance [25].

Determination of Enzyme Protein

Determination of the enzyme protein was made according to the method of Lowry et.al. [26].

Statistics

All experimental data is presented as the mean ± SD. Student's t-test was used to analyze the results, using Graphpad Quickcalc software. $P < 0.05$ was considered significant.

Results

Behavioral changes

Subcutaneous dose (CD97) of PTZ induced generalized tonic-clonic seizures exhibiting repeated myoclonic jerks, clonic forelimb and hindlimb seizures with loss of righting reflex along with tonic extension of forelimbs and hindlimbs, Seizure lasted for 3 to 5 minutes.

Changes in Metabolic enzymes and metabolites

AST activity elevated significantly in CC, H, CG, by 175.15 %, 120.099 %, 61.21% respectively. While it decreased by 30.71 % in CQ (Fig: 1). Similarly, ALT activity (Fig: 2) increased in CC and CG by 181.57% and 31.08 % respectively but in Hippocampus it had no significant change, ALT activity decreased in CQ by 48.04 %. LDH activity remained unaltered in CC and CQ as compared to controls. However, it decreased in H and CG by 79.209% and 39.83 % respectively (Fig: 3). Na^+/K^+-ATPase activity lowered in CG, H and CQ by 19.26%, 84.23% and 87.62% respectively, while it increased by 33.36 % in CC (Fig: 4). Besides, significant decrements in the activities of Mg^{+2}-ATPases (Fig: 5) and Ca^{+2}-ATPases (Fig: 6) were observed in CC, H, and CG with no change in CQ. Mg^{+2}-ATPase activity decreased by 54.248%, 35.74% and 48.04% in CC, CG and H, respectively. Similarly, Ca^{2+}- ATPase activity decreased by 34.27, 29.688 and 36.148% in CC,CG and H respectively during PTZ induced epileptic seizure.

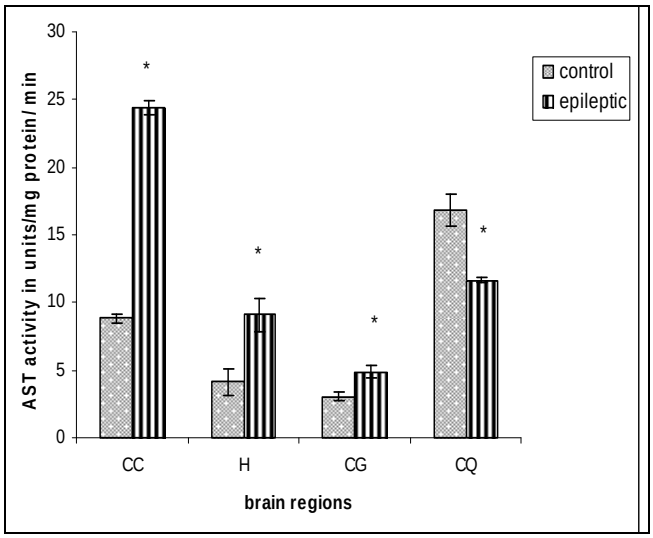

Figure 1. *AST activity in mice brain regions during PTZ induced epileptic seizure.*

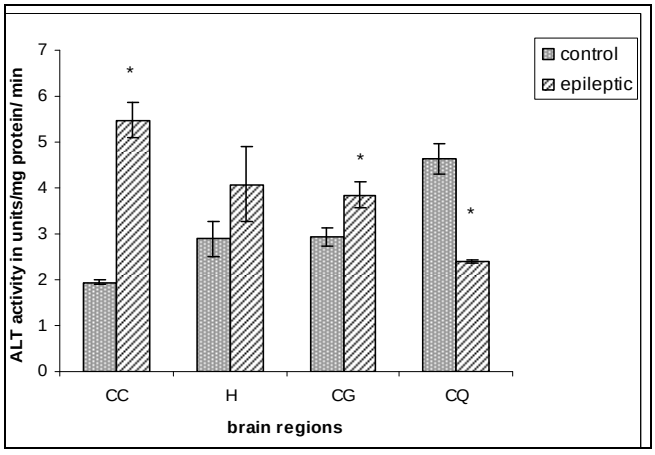

Figure 2. *ALT activity in mice brain regions during PTZ induced epileptic seizure.*

Energy metabolites such as Glucose, Pyruvate, Lactate and Glutamate showed significant changes in the discrete mice brain regions during PTZ induced epileptic seizures. Glucose level increased by 44.30% in CC, and decreased

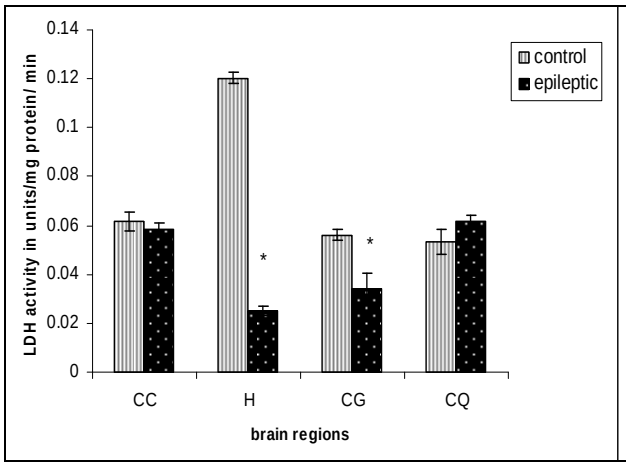

***Figure** 3. LDH activity in mice brain regions during PTZ induced epileptic seizure.*

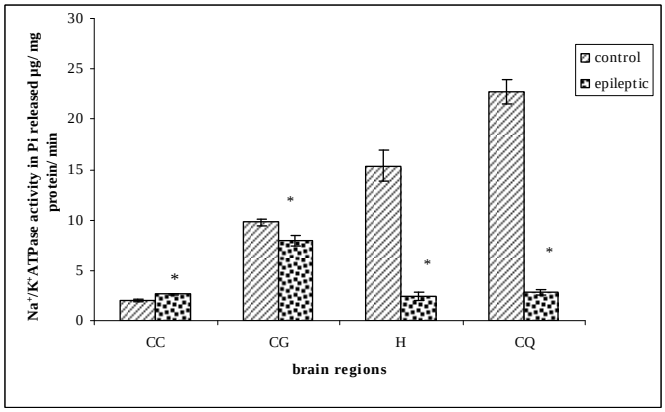

***Figure 4**. Na^{+}/K^{+}-ATPase activity in mice brain regions during PTZ induced epileptic seizure.*

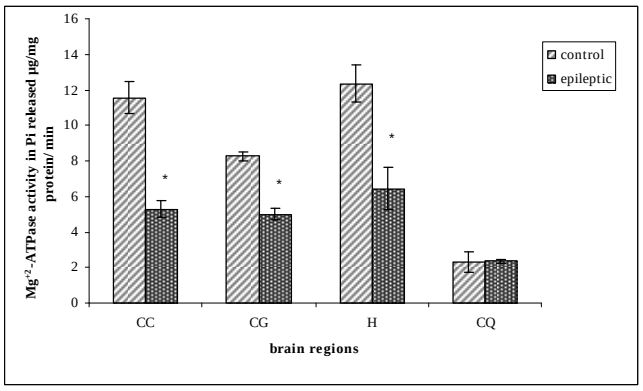

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***Figure 5.** Mg^{2+}-ATPase activity in mice brain regions during PTZ induced epileptic seizure.*

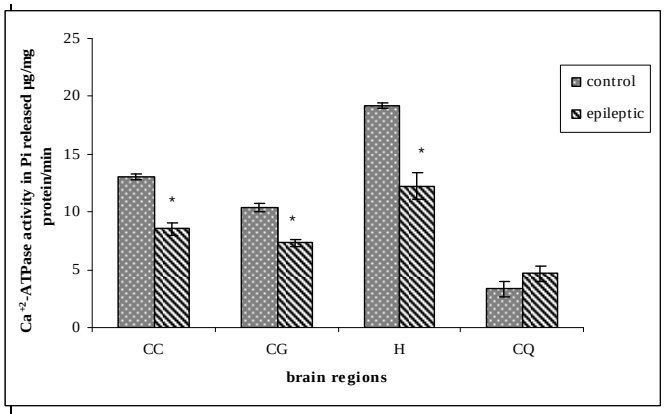

***Figure 6.** Ca^{2+}-ATPase activity in mice brain regions during PTZ induced epileptic seizure.*

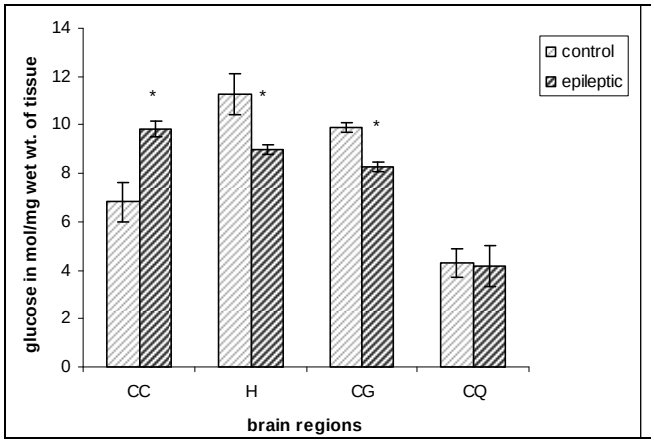

***Figure 7.** Glucose level in mice brain regions during PTZ induced epileptic seizure.*

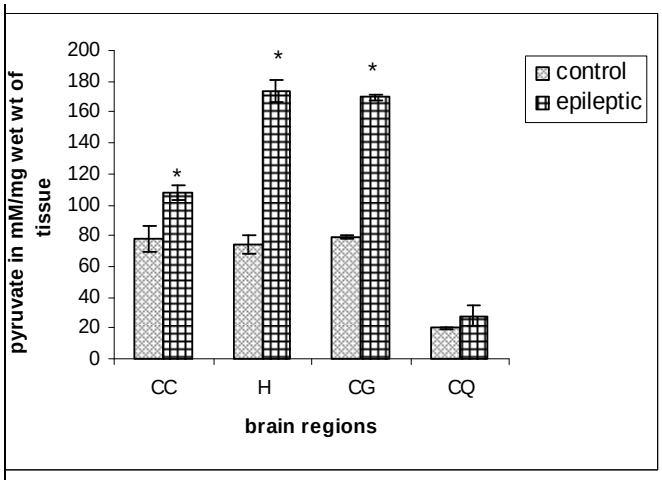

***Figure 8**. Pyruvate level in Mice brain regions during PTZ induced epileptic seizure.*

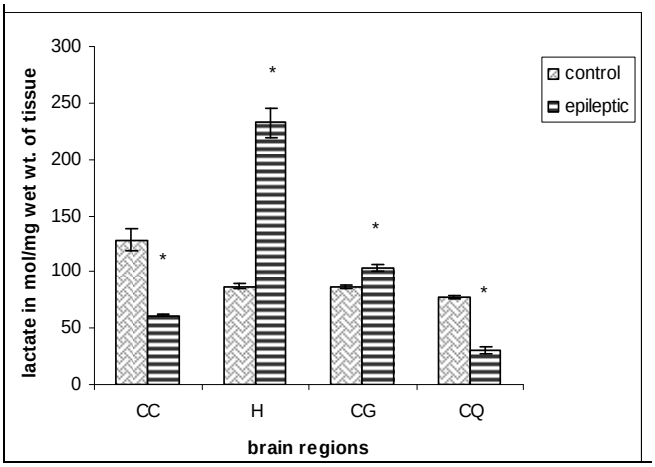

Figure 9. *Lactate level in mice brain regions during PTZ induced epileptic seizure.*

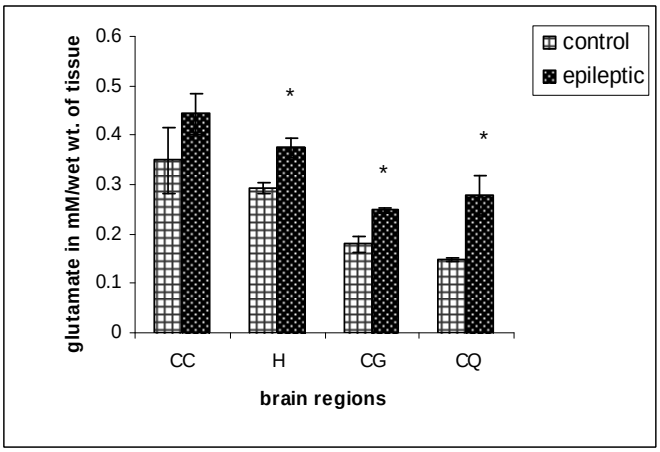

Figure 10. *Glutamate level in mice brain regions during PTZ induced epileptic seizure.*

by 20.21 and 16.65 % in H and CG respectively, with no significant change in CQ (Fig: 7). Whereas, Pyruvate level increased in CC, H and CG by 37.54%, 133.106% and 115.44% respectively, (Fig: 8), with no significant change in CQ. Similarly, Lactate level decreased by 52.311% and 16.28% in CC and CQ, but increased by 166.77% and 19.77% in H and CG respectively, (Fig: 9). Glutamate increased significantly by27.52%, 38% and 89.13% in H, CG and CQ respectively, without any change in CC (Fig: 10).

Figs.1-10: Data represents mean ± SD. * $P < 0.05$ significant different was calculated by Student's t-test.

Discussion

The main objective of our study was to understand the effect of epileptic seizure on, CC, CG and CQ in comparison to H, a region known to be affected by epilepsy.

The hippocampus is often the focus of epileptic seizures: hippocampal sclerosis is the most commonly visible type of tissue damage in temporal lobe epilepsy [27]. It is not yet clear, though, whether the epilepsy is usually caused by hippocampal abnormalities, or the hippocampus is damaged by cumulative effects of seizures [28]. In experimental settings where repetitive seizures are artificially induced in animals, hippocampal damage is a frequent result: this may be a consequence of the hippocampus being one of the most electrically excitable parts of the brain. It may also have something to do with the fact that the hippocampus is one of very few brain regions where new neurons continue to be created throughout life [29]. In the present study, PTZ induced epileptic seizure is observed in Mice, with alteration in the metabolic activities in H, CQ, CC, CG. Injection of convulsive dose of PTZ is thought to modulate patterns of generalised tonic- clonic seizures in humans [30, 31, 32]. Based on the behavioral study in the present work the CD97 dose of PTZ is enough to induce generalized tonic-clonic seizure in mice, which akin to a type of epilepsy seen in humans.

Metabolic enzymes like AST, ALT, LDH and Total ATPases shows drastic changes in their activities as compared to controls. AST and ALT facilitates conversion of aspartate and alanine to glutamate, and serve as a link between carbohydrate and protein metabolism under stress. Increased AST and ALT level in brain regions indicate pronounced metabolism of amino acids associated with seizure [33]. Lactate dehydrogenase catalyses the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. It converts pyruvate, the final product of glycolysis to lactic acid when oxygen is absent or in short supply, and it performs the reverse reaction during the Cori cycle in the liver. The results indicate reduction of LDH in the hippocampus and CG during PTZ induced epileptic seizure. There could be some factors that inhibit its activity in these regions of brain during seizure or it may be just because of increase in lactate as well as pyruvate levels together. However, it needs further investigation.

The Na^+,K^+-ATPase harness energy stored in the phosphate bond to exchange Na^+ ions inside to K^+ ions outside of the axonal membrane [34]. This transmembrane gradient of Na^+ and K^+ ions facilitates the neurons to propagate nerve impulses from one point to another. Other enzymes such as Mg^{2+}- ATPase and Ca^{2+}-ATPase also take part in energy production and transmission of nerve impulses. In the present investigation these three ATPases show a significant decrease in activity as compared to controls which is in agreement with the observations of Visweswari et al. [35] except incase of CC wherein Na^+,K^+-ATPase activity was high and in CQ, Mg^{2+}- ATPase and Ca^{2+}-ATPase was not altered. Inhibition in the activity reveals induction of

energy crisis through disruption in oxidative phosphorylating process during epileptic seizure. While rise of ATPase in CC indicate significantly increased role in impulse transmissions as well as stress on ionic balance in epilepsy. As CC is the principal anatomical and neurophysiological track linking the cerebral cortices, the increased ATPases clearly indicate the increased neural transmission load in it.

The brain is extremely active metabolically, with its local energy demands fluctuating rapidly between high and low activity states. However, the brain's endogenous stores of metabolic energy substrates are small. Consequently, normal brain function requires a continuous supply of exogenous substrates obtained through blood flow. Under normal physiological conditions, glucose is the dominant exogenous energy substrate in the adult brain [36]. However, other exogenous or endogenous energy substrates can serve as alternatives to glucose under certain physiological and pathological conditions. In vitro studies have shown that cultured brain cells or isolated brain tissues can oxidize a variety of substrates. These substrates include glycolytic intermediates such as pyruvate [37] and lactate [38, 39], amino acids such as glutamate and glutamine [40]. Seizures usually lead to uncoupling between glucose uptake and oxygen consumption and increases lactate accumulation while energy homeostasis is maintained [41]. In the present study, glucose level is seen to be decreased in H and CG. This may be due to the high level of lactate and pyruvate which act as an alternative substrate for glycolysis besides glucose. Decreased glucose consumption was reported by Eloqayli et al. (2002) [42] in cerebellar granule cells exposed to PTZ. Elevated lactic acid concentration is observed in the brain of piglets with experimentally induced convulsions and in the extracellular fluid of the hippocampus of humans during spontaneous seizures [43,44]. CQ did not show much change in glucose and pyruvate but a decrease in lactate could be due to the presence of sufficient substrate for activity. Also an increase in glutamate level is observed in this study which is in agreement with the observations of Li et al. 2004; Rowley et al. 1995; Takazawa et al. 1995; Ueda and Tsuru, 1995 [45,46,47,48]. Thus, provide evidence that glutamate, an excitatory neurotransmitter is involved in seizure in almost all parts of brain, except CC.

The present work shows that during epileptic seizure, the metabolism is altered in the brain tissue, which is actually the impact of the seizure that is seen in human with generalised epilepsy. A detailed study in this field is required which will help to treat the post epileptic effect, infact the antiepileptic drug that could be given as a precautionary medicine should have the ability of not altering the basic metabolic processes, which, may in turn lead to control the seizure impact. Besides, H and CG is seen to be affected completely, but CC and CQ are partly altered as far as metabolism is concerned. So, probably Corpus Callosotomy may not be required as a part of treatment of epilepsy, instead the imbalance in metabolic processes should be avoided through an appropriate antiepileptic drugs.

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Conflict of interest

The authors declare that they have no conflict of interest.

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