Abstract

Epilepsy is a chronic neurological disorder and generally associated with certain psychiatric comorbidities. Among several comorbidities depressive behavior and cognitive impairment has been reported to be most debilitating comorbidity associated with epilepsy. The present study has been undertaken to evaluate the therapeutic role of Vinpocetine in epilepsy induced comorbid depression and cognitive dysfunction in Pentylenetetrazol (PTZ) kindled rats. The experimental protocol comprised of five groups (n = 8), where a sub convulsive dose of PTZ (30 mg/kg, i.p.) had been administered on every alternate day for a period of 23 days and seizure episodes were noted after each PTZ injection over a period of 30 min. Treatment groups received Vinpocetine (5, 10 and 20mg/kg) from day 1st to 31st day. Memory and Cognitive impairment was assessed using Morris water maze and object recognition task and depression was assessed using tail suspension test. While the oxidative stress parameters Malondialdehyde (MDA), glutathione (GSH) and nitrite levels were estimated in the whole brain. The PTZ administration in rats produced significant impairment in learning and memory in Morris water maze (MWM) and object recognition task (ORT) and depression was assessed using tail suspension test. Biochemically significant elevation of MDA, nitrite levels and depletion of GSH levels were observed in PTZ-kindled rats. Chronic administration of Vinpocetine attenuated PTZ-induced learning and memory deficit as evidence in MWM and ORT and produced antidepressant effect as observed in TST. Moreover, Vinpocetine was also significantly attenuated PTZ-induced oxidative stress. Our results suggest important role of Vinpocetine in the therapeutic management of epilepsy and associated comorbid conditions.

Keywords: Vinpocetine, Epilepsy, Pentylenetetrazol kindled rats, Comorbid depression , Cognitive dysfunction

1. Introduction

Epilepsy is a complex neurological disorder often characterized by abnormal synchronous neural activity. Clinical manifestations include recurrent episodes of seizures, loss of consciousness, memory impairment and sensory disturbances [1]. Depression and cognitive deficit appears to be major debilitating neurobehavioral comorbidity of chronic epilepsy [2-4]. Cognitive impairment has been reported in more than half of the epilepsy patients due to seizures [5, 6]. Although there are wide recognition of epidemiological aspects of these comorbidities but nature, timing, and course of these comorbidities, causal relationships. Clinical interventions for the prevention of comorbidities and related problems still and management of epilepsy patients are issues of substantial concern [7]. Alteration in classic neurotransmitters systems such as the glutaminergic [8] and GABAergic [9] systems have been implicated in the genesis of epileptic seizures. Despite the availability of a number of antiepileptic drugs, the current treatment in epilepsy is not satisfactory. The drugs used to treat these conditions are associated deleterious effects on epilepsy associated comorbid conditions [10]. And there is still a great need for the management of epileptic comorbid and other neurological complications. For the development of epilepsy an animal model pentylenetetrazole (PTZ) is used as one of the most widely used agent. PTZ is a selective blocker blocker of chloride channel coupled to the GABAergic receptor complex and PTZ has been...
found to be down regulates GABA<sub>A</sub> receptors, thus enhanced glutamate-aggravated seizures [11]. Vinpocetine is a synthetic ethyl ester of the alkaloid apovincamine, which is isolated from the leaves of Vinca minor and commonly known as the lesser periwinkle [12]. Vinpocetine also reported for its nootropic effects for the improvement of memory [13]. Vinpocetine selectively inhibits Ca<sup>2+</sup>/calmodulin-dependent phosphodiesterase (PDE) type 1, which maintains levels of cAMP and cGMP. Vinpocetine has also been demonstrated to modulate various neurotransmitters such as monoamines, which may contribute to its antidepressant actions. It also protects neurons from glutamate and NMDA toxicity [15]. Vinpocetine affects GABAergic and serotonergic pathways, peripheral BZD receptors and selectively blocks voltage-sensitive Na<sup>+</sup> and Ca<sup>2+</sup> channels [16] and cause decrease in extracellular calcium (Ca<sup>2+</sup>) ions in striatal nerve endings. Vinpocetine is also having antioxidant activity due to the scavenging of hydroxyl radicals [17]. During the last decade, there has been a rapid explosion of publications reporting the neuroprotective activity of Vinpocetine [18]. Thus, unlike available therapy for epilepsy, vinpocetine would have an advantage, over other drugs, not just to halt epileptogenesis but also can preserve other comorbid conditions. The present study was designed to investigate the therapeuic potential of vinpocetine in the management of experimental PTZ induced kindling and associated comorbid conditions such as learning and memory deficit and oxidative stress in rats.

2. Materials and methods

2.1. Experimental animals

The experiments were carried out on female Wistar rats (200-250g) obtained from central animal house of ISF College of Pharmacy, Moga, Punjab (India). The animals were kept in polycrylic cages and maintained under standard housing conditions (room temperature 22±0°C and relative humidity of 60-65%) with 12h light/dark reverse cycle. The food in the form of dry pallets and water were made available ad libitum. All behavioral experiments were carried out between 9.00 h to 17.00 hrs. The experimental protocol was reviewed and approved by the ISFCP/IAEC/CPCSEA/Meeting No.13/2014/ Protocol No.206 and the animal experiments were carried out in accordance with the Indian National Science Academy (INSA) Guidelines for use and care of animals.

2.2. Drugs and chemicals

The drugs used in the present study Pentylenetetrazol (PTZ) and Vinpocetine were purchased from Sigma (St Louis, MO, USA). PTZ and Vinpocetine were dissolved in normal saline solution and DMSO respectively. The time gap and the route of administration were selected from the available literature. All other chemicals used in the study were of analytical grade. Solutions of the drug and chemicals were freshly prepared before use [19].

2.3. Kindling procedure by PTZ

For PTZ kindling, a subconvulsant dose of PTZ (30 mg/kg) was injected intraperitoneally on every alternate day for 23 days. The PTZ injections were stopped when the animals showed adequate kindling, i.e. seizure score of 5 on three consecutive injections. Convulsive behavior was observed after each PTZ injection for 30 min. Behavioral changes were scored according to the criteria described by as:

Stage 0 (no response)
Stage 1 (hyperactivity, restlessness and vibrissae twitching)
Stage 2 (head nodding, head clonus and myoclonic jerks)
Stage 3 (unilateral or bilateral limb clonus)
Stage 4 (forelimb clonic seizures)
Stage 5 (generalized clonic seizures with falling).

The number of myoclonic jerks and the latencies to myoclonic jerks were recorded. The latencies were transformed into seizure score [20]. Animals were considered kindled if they exhibited stage 5 of seizures on two consecutive trials.

2.4. Experimental protocol

Animals were randomly divided into five groups (n=8). The first group received vehicle (normal saline and DMSO) other treatment and diseased group received PTZ (30 mg/kg, i.p.) for every alternate days for 23 days and groups 3, 4 and 5 received Vinpocetine (5mg, 10mg and 20mg i.p.) respectively on every alternate day for 31 days. Cognitive deficit was assessed by Morris Water Maze, Object Recognition Task and depression symptoms were assessed by Tail Suspension Test. Following the behavioral test, the animals were sacrificed and the whole brain homogenate was used for oxidative stress parameters. (Fig 1)
2.5. Assessment of Behavioral parameters:

Behavioral parameters were evaluated upon completion of kindling procedure.

2.5.1 Object recognition task (ORT)

The rats were acclimated to the arena (60×60×40 cm for rats) without objects for 30 to 45 min before testing. The ORT consisted of two trial periods (T1 and T2) separated by a 24-h intertrial period. The time required for each animal to complete 15 s of total exploration of the two identical objects, as shown by placing its nose within 2 cm of the object, was determined, with a cut off of 240 s. For the retention trial (T2) conducted 24 h later, one of the objects presented in T1 was replaced with a novel object. Rats were returned to the arena for 3 min, and the duration of exploration of each object was scored. A criterion of minimal level of object exploration was used to exclude animals with low levels of spontaneous exploration; thus, only animals having a minimal level of object exploration of ≥ 5 s during the retention trial T2 (novel+ familiar ≥ 5 s) were included [21].

2.5.2 Morris water maze test

Spatial learning and memory of animals were tested in a Morris water maze (Morris, 1984). It consisted of a circular water tank (180 cm diameter, 60 cm height) filled with water (25±1 °C) to a depth of 40 cm. A non-toxic water dispersible emulsion was used to render the water opaque. Four equally spaced locations around the edge of the pool (North, South, East, and West) were used as start points, which divided the pool into 4 quadrants. An escape platform (10 cm in diameter) was placed in the pool 2 cm below the surface of water. The escape platform was placed in the middle of one of the randomly selected quadrants of the pool and kept in the same position throughout the entire experiment (northeast for this study). Before the training started, the rats were allowed to swim freely into the pool for 120s without platform.

Animals received a training session consisting of 4 trials per session (once from each starting point) for 4 days (days 25, 26, 27 and 28), each trial having a ceiling time of 120 s and a trial interval of approximately 30 s. After climbing onto the hidden platform, the animals remained there for 30 s before commencement of the next trial. If the rat failed to locate the hidden platform within the maximum time of 120 s, it was gently placed on the platform and allowed to remain there for 20 s. The time taken to locate the hidden platform (latency in seconds) was measured. Twenty four hours after the acquisition phase, a probe test (day 29) was conducted by removing the platform. Rats were allowed to swim freely in the pool for 120 s and the time spent in target quadrant, which had previously contained the hidden platform was recorded. The time spent in the target quadrant indicated the degree of memory consolidation which had taken place after the acquisition trial [22].

2.5.3 Tail suspension test

Tail suspension test was performed on day 31th. The total duration of immobility induced by tail suspension was measured according to the method described by [23]. Rats both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Rats were considered immobile only when they hung passively and completely motionless. Immobility time was manually recorded during a 6-min period [24].

2.6. Assessment of Biochemical parameters:

Upon completion of the behavioral tests, the animals were decapitated under ether anesthesia and the brains were quickly removed, cleaned with ice-cold saline and stored at−80 °C till further analysis within the next 7 days.

2.6.1. Brain tissue homogenate preparation

Animals were sacrificed by decapitation and brains were removed and rinsed with ice-cold isotonic saline. Brain samples were then homogenized with ice-cold 0.1 mol/l phosphate buffer (pH 7.4) 10 times (w/v). Then homogenate was centrifuged for 15 min at 10,000 g for 15 min and aliquots of supernatant were separated and used for biochemical estimation.

2.6.2. Protein estimation

Protein was measured in all brain samples by the method using Lowery method [25].

2.6.3. Estimation of malondialdehyde (MDA)

The quantitative measurement of MDA—end product of lipid peroxidation - in brain homogenate was performed according to the method of Will [26]. The amount of MDA was measured after its reaction with thiobarbituric acid at 532nm using spectrophotometer (Shimadzu, UV-1700). The concentration of MDA was determined from a standard curve and expressed as nMol/mg protein [27].

2.6.4. Estimation of reduced glutathione (GSH)

Reduced glutathione in brain was estimated according to the method described by Ellman method [28]. 1ml supernatant was precipitated with 1 ml of 4% sulfosalicylic acid and cold digested at 4°C for 1h. The samples were centrifuged at 1200g for 15 min. To 1 ml of the supernatant, 2.7ml of phosphate buffer (0.1M, pH 8) and 0.2 ml of 5,5′dithiobis (2-nitrobenzoic acid) (DTNB) were added. The yellow color that developed was measured immediately at 412nm using a spectrophotometer. The concentration of glutathione in the supernatant was determined from a standard curve and expressed as µMol/mg protein [27].
2.6.5. Estimation of nitrite

Nitrite level was determined with a colorimetric assay with Greiss reagent (0.1% N-(1-naphthyl) ethylene diaminedihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid) as described by Green and his coworkers. Equal volumes of the supernatant and the Greiss reagent were mixed; the mixture was incubated for 10 min at room temperature. The absorbance was determined at 540 nm with the double beam UV–VIS spectrophotometer [UVPharmaspec 1700, Shimadzu (Japan)]. The concentration of nitrite in the supernatant was determined from a sodium nitrite standard curve and was expressed as micromole per liter [7].

2.7. Statistical analysis

The results were expressed as mean ± SEM. Behavioural parameters were analyzed by repeated measure two way ANOVA followed by Bonferroni’s post hoc test for multiple comparisons and biochemical parameters were analyzed by one way ANOVA followed by Bonferroni’s post hoc test. Values with P<0.05 was considered to be statistically significant.

3. Result

3.1. Results of behavioral parameters

3.1.1. Effect of Vinpocetine on memory performance in object recognition task in PTZ kindled rats:

On day 30 (T1) when both the objects were similar (familial), all the rats took similar time to achieve 15s of object exploration. PTZ did not show significant difference when compared with that of vehicle control. Similarly Vinpocetine did not showed significant difference when compared with that of vehicle control (p>0.05) (Fig.2A).

Note: PTZ= Pentylenetetrazole, Vin= Vinpocetine.

On day 31, when animals exposed with novel objects replaced with one of the familial object. Only the rats treated with vehicle or Vinpocetine showed significant discrimination between novel and familial objects and spent more time in exploring novel object. Whereas, rats treated with PTZ did not show any significant discrimination between the familial and novel objects (Fig.2B).

Fig 2B: Effect of Vinpocetine on total exploration time of FO1 and NO during T2 in PTZ kindled rats.

Values are expressed as mean ± SEM; *P<0.05 v/s Fam of respective group.

3.1.2. Effect of Vinpocetine on memory performance in Morris water maze (MWM) task in pentylenetetrazole (PTZ) treated rats

All the rats showed gradual decrease in escape latencies to reach submerged platform through the entire observation period and did not showed significant difference in escape latencies on day 27. However PTZ produced significant effects on escape latencies on day 25, 26, and 27 as compare with vehicle control rats. Repeated measure two way ANOVA analysis indicated overall significant effect of treatment (p<0.001), time (p< 0.001) and a time × treatment interaction (p< 0.001). Although Vinpocetine produce significant decrease in escape latencies on day 26, 27 and 28 as compare with PTZ control (Fig.3A).

Note: PTZ= Pentylenetetrazole, Vin= Vinpocetine, Fam= Familiar, Nov= Novel

Fig 3A: Effect of Vinpocetine on escape latencies in Morris water maze (MWM) task in pentylenetetrazole (PTZ) treated rats.
3.1.3. Effect of Vinpocetine on tail suspension task (TST) in PTZ administered rats:

TST was performed on day 31; evaluate the immobility time and mobility time. However, administration of Vinpocetine (5, 10 and 20 mg/kg) significantly decreased the immobility time as compare to PTZ kindled rats (Fig 4).

3.2. Results of Biochemical parameters

3.2.1. Effect of Vinpocetine on lipid peroxidation (LPO level) in PTZ kindled rats:

Kindled rats had significantly increased level of LPO in brain after sub convulsive PTZ (30 mg/kg) administration on alternate days for a total period of 23 days in comparison to normal control group. However, administration of Vinpocetine (5, 10 and 20 mg/kg) significantly attenuated the increased level of the lipid peroxidation in PTZ kindled rats (Fig 5)(Table 1).
Values are expressed as mean ± SEM; *P<0.05 v/s Vehicle control, †P<0.05 v/s PTZ (30), P<0.05 v/s Vin (5).

Note: PTZ= Pentylenetetrazole, Vin= Vinpocetine.

3.2.2. Effect of Vinpocetine on reduced glutathione (GSH level) in PTZ kindled rats:

Chronic, sub-convulsive PTZ (30 mg/kg) administration on alternate days for a total period of 23 days resulted in decreased in antioxidant enzymes in brain. Treatment with Vinpocetine (5, 10 and 20 mg/kg) significantly restored the GSH level in PTZ kindled rats (Fig 6) (Table 1).

Fig 6: Effect of Vinpocetine on reduced glutathione (GSH level) in PTZ kindled rats.

Values are expressed as mean ± SEM; *P<0.05 v/s Vehicle control, †P<0.05 v/s PTZ (30), P<0.05 v/s Vin (5).

Note: PTZ= Pentylenetetrazole, Vin= Vinpocetine.

3.2.3. Effect of Vinpocetine on nitrite levels in PTZ kindled rats:

Kindled rats had significantly increased level of nitrite in brain after sub convulsive PTZ (30 mg/kg) administration on alternate days for a total period of 23 days in comparison to normal control group. However, administration of Vinpocetine (5, 10 and 20 mg/kg) significantly attenuated the increased level of the nitrite in PTZ kindled rats (Fig 7) (Table 1).

Fig 7: Effect of Vinpocetine on nitrite levels in PTZ kindled rats.

Values are expressed as mean ± SEM; *P<0.05 v/s Vehicle control, †P<0.05 v/s PTZ (30), P<0.05 v/s Vin (5).

Note: PTZ= Pentylenetetrazole

4. Discussion

Present study, demonstrates the therapeutic potential of Vinpocetine in comorbid depression and cognitive deficit in pentylenetetrazole (PTZ) kindled rats. Results showed that treatment with Vinpocetine reduced the seizure severity score and attenuates the kindling associated depression like behavior and cognitive deficit. Epilepsy is one of the major neurological disorder still awaiting safer drugs with improved antiepileptic effects and lesser side effects. The most prominent feature of epilepsy is the seizures but memory impairment, learning disabilities, behavioral problems and poor social outcome also involved. Epilepsy has many comorbid conditions but depression and cognitive dysfunction are the most debilitating conditions [29, 30].

In the present study, we have used PTZ kindling model to induce kindling in rats. PTZ kindling is a well established model of epilepsy used for understanding neurobiology and evaluating the effectiveness of antiepileptic drugs. Chemical kindling has also been used to study the several psychiatric comorbidities associated with epilepsy [20, 25, 31].PTZ has been found to induce kindling in various findings and as a common agent to epileptic models [32]. PTZ is a selective blocker which selectively blocks chloride channel coupled to the GABAergic receptor complex. Repeated administration of subconvulsive doses of PTZ was found to be downregulates the sensitivity of GABA_A receptors, thus favoring glutamate-aggravated seizures [11]. In line with these findings our results also confirmed the kindling effect of PTZ and seizure was induced in the PTZ treated groups. Kindled seizures have been shown to cause a neuronal loss in limbic systems CA1, CA3, dentate gyrus of hippocampus,
amygdala and entorhinal cortex [33,34](Cavazos and Satula 1990; Pitkänenet al. 1998). An increased activity of the glutamatergic transmission has also been found to play a crucial role in neuronal cell death of the PTZ kindling in rats due to free radicals generation [35]. In the present study, PTZ kindled rats showed significant learning and memory deficit and depression behavior in rats. Herein, Morris water maze (MWM) was used to evaluate spatial learning and memory [36]. Most importantly, spatial learning in general and MWM performance in particular appear to be depending upon the coordinated action of different brain regions constituting a functionally integrated neural network [37]. PTZ-kindling disrupts performance in the MWM partially parallels the results of Lamberty and Klitgaard [38], who reported that PTZ-induced kindling disrupts spatial memory for place but not spatial learning. In contrast to this report, however, we found that spatial learning is also affected by PTZ kindling. Our findings are consistent with other reports showing significant spatial learning deficits following PTZ-induced kindling [39, 40]. Whereas, object recognition task (ORT) is a commonly employed test of memory that relies on rodents inherent preference for exploration of novel versus familiar object and required very little training and hippocampal formation mediates several aspects of cognitive function, including spatial learning, formation of new memories and retrieval of stored memories. The PTZ administration in rats produced significant impairment in learning and memory object recognition task (ORT). Depression, one of the most debilitating psychiatric comorbidity of epilepsy, generally examined in rodents using various experimental models viz., tail suspension test (TST) [41,31]. In the present study, PTZ kindled animal showed significant depressive behavior indicated by increased immobility period in TST. Whereas, Vinpocetine attenuated PTZ-induced learning and memory deficit and depressive behavior in rats. Vinpocetine treated rats showed significant improvement in acquisition and memory consolidation and were able to discriminate between familial and novel objects. Moreover, Vinpocetine treated rats showed significant decrease in immobility duration indicating anti-depressant effect. Vinpocetine significantly reduced the level of oxidative stress and other neuroinflammatory mediators which confirm the neuroprotective potential of Vinpocetine in PTZ induced comorbid conditions.

Preclinical studies of Vinpocetine in rodents against corazol, strychnine and thiosemicarbazide induced convulsions has proved it as a potent anticonvulsant. Anticonvulsive action was suggested to be mediated by GABA and serotonergic mechanisms [42]. Moreover, Vinpocetine has been reported to be 100 fold more efficient than phenytoin (a prototype Na+-channel blocker) in causing inhibition of veratridine evoked cell death, by Na+ channel activation [43]. Na+ channel activation is one of the possible factors responsible for release of excitatory neurotransmitters; therefore Na+ channel inhibition could be responsible for the anticonvulsant action of Vinpocetine [22, 43]. Vinpocetine has been reported to attenuate different types of seizures in rats and was found to be effective in PTZ-, amygdala- and neocortically kindled rats [44]. The very first molecular target recognized for Vinpocetine was phosphodiesterase (PDE) enzyme [45]. Phosphodiesterase (PDE) enzyme inhibition is a way to enhance level of second messenger and consequently influence the pathways involved in learning and memory [22]. PDE1 has been reported to show significant expression in neurons of the hippocampus. Further cAMP and cGMP related effects of Vinpocetine have been related to its inhibitory action on Na+ conductivity [46]. Vinpocetine has been further reported to improve cerebral blood flow [47]. Vinpocetine has also been demonstrated to inhibit veratridine induced opening Na+ channel activity and glutamate release [48, 49]. Further cAMP[50] and cGMP[51] dependent effects of Vinpocetine have been related to its action on Na+ conductivity. The observed behavioral changes well corroborated with biochemical impairment, as kindled animals showed depleted monoamine and exacerbated nitrosative stress level in the brain. These biochemical changes have been suggested as a pathogenic feature of depression [52]. The Vinpocetine treatment, in this study, elevated the norepinephrine and serotonin level, possibly via blocking monoamine oxidase A and attenuated the elevated nitrosative stress level in the brain.

In the present study, PTZ induced kindling cause increased oxidative stress. MDA is an end product of lipid peroxidation [53] and glutathione as an endogenous antioxidant plays an important role in protecting cells against oxidative damage. In the present study, PTZ kindling increased the level of MDA and decreased the GSH level in the rat brain. PTZ thus caused an imbalance between antioxidant and free radical generation which may be atleast partially responsible for seizures and cognitive impairment [28]. As oxidative stress is known to contribute to the deficits of cognitive function [54], the observed increase in oxidative stress by PTZ may be one of the factors responsible for the cognitive impairment seen with chronic seizures. Administration of Vinpocetine prevented the rise in brain MDA levels in a dose-dependent manner. In addition, Vinpocetine has been shown to inhibit a cyclic GMP phosphodiesterase, this inhibition enhances cyclic GMP levels in the vascular smooth muscle, leading to reduced resistance of cerebral vessels and increase of cerebral flow. Vinpocetine has been shown to enhance cerebral oxygen and glucose utilization uptake and enhance neuronal ATP bio-energy production, even under hypoxic (low oxygen) conditions, makes it suitable to overcome the factors which irradiates brain ageing [45]. Moreover Vinpocetine has been reported to be a potent antioxidant and demonstrated to scavenge hydroxyl free radicals [22].
Conclusion

Thus, the observed beneficial effects of Vinpocetine against PTZ induced behavioural and biochemical abnormalities may be due to its ability to improve cerebral cyclic nucleotide signaling, inhibition of Na+ channel activity and through its observed antioxidant mechanisms. Nonetheless, our results support the earlier observations and provide a rationale for the therapeutic application of Vinpocetine in the management of epilepsy and associated comorbidities such as depression and cognitive deterioration.

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References


Na+, Ca 2+ and glutamate release in hippocampal isolated nerve endings. *Neurochemistry international*, 49(1), 55-61.


