REVERSAL OF PHENYTOIN-INDUCED IMPAIRMENT OF SPONTANEOUS ALTERNATION BY PYRITINOL IN MICE: INVOLVEMENT OF CHOLINERGIC SYSTEM

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ABSTRACT

Purpose: To study the effect of the combined treatment of phenytoin and pyritinol on seizure control, cognitive and motor functions in mice.

Material and Methods: Increasing current electroshock seizure (ICES) test was used to evaluate the effect of the combination of phenytoin and pyritinol on convulsions. Cognitive functions in mice were assessed by spontaneous alternation behaviour on a plus maze while motor functions were screened using rolling roller apparatus and by counting the number of arms entries on a plus maze. Brain acetylcholinesterase (AChE) activity was measured using the Ellman et al method.

Results: The study showed that pyritinol when co-administered with phenytoin, significantly reversed phenytoin-induced reduction in spontaneous alternation without altering the efficacy of phenytoin against ICES in both acute and chronic studies. Further, it also reversed phenytoin-induced increase in AChE activity.

Conclusion: Pyritinol alleviated the phenytoin–induced cognitive impairment without compromising its antiepileptic efficacy.

Key words: Acetylcholinesterase, cognitive functions, diphenylhydantoin, pyritinol, epilepsy, nootropic

INTRODUCTION

Cognitive deficit is one of the major problems associated with epilepsy; both the underlying pathology and drug therapy can lead to disturbances in cognitive function[1]. Nootropic agents may, to some extent, correct some of the observed cognitive deficits. Phenytoin (PHT) is one of the cheapest and widely used anticonvulsants. But, as with many other antiepileptic drugs[2], it is known to adversely affect learning and memory[3, 4]. It might be worthwhile to assess the use of nootropic agents as add-ons in antiepileptic therapy for possible protection against cognitive deficits. The antiepileptic treatment may last a lifetime in many patients. This implies that the nootropic agents may also need to be given for long periods of time. Therefore, the nootropic agents will have to be chosen very carefully. Phenytoin (PHT) is one of the low-cost and widely prescribed antiepileptic drugs (AED) known to cause cognitive impairment. Many studies have investigated the effect of PHT on learning, memory and psychomotor functions. Both acute and chronic administration of PHT has been shown to significantly impair learning and memory.[3, 4, 5.] It has been reported that PHT decreases the response of guinea pig ileum to acetylcholine (ACh) [6] and that it lowers brain ACh levels[7, 8.]. Its impairing effects on learning and memory are attributed to such alterations[4].Biochemical as well as electrophysiological evidence exists for interactions between cholinergic, noradrenergic and serotonergic systems[9].It is suggested that the behavioral effects of cholinergic degeneration can be alleviated by a reduction in noradrenergic function[10]. For an optimum antiepileptic therapy, it is desirable to have complete seizure control without interfering cognitive effect A combination of antiepileptic drugs (AEDs) with known nootropic agents appears to be a promising research area for desirable seizure control with minimal/no memory deficit. A better approach would be to use an agent that not only corrects the cognitive disturbances but also provides seizure protection. In this regard, one of the promising agents is pyritinol. Pyritinol a nootropic [13, 25] has been shown to be an effective nootropic action. It is most probably the oldest nootropic drug still in use today. The drug has been used to treat a wide range of disorders and problems from alcoholism to cerebral trauma. Pyritinol are a potent scavenger of hydroxyl free radicals[12]. It is now widely accepted that the antioxidant properties of the drug are responsible for many of the benefits of Pyritinol[12]. The discovery that Pyritinol can protect proteins in the brain against radical induced polymerization, coupled with evidence showing that the drug enhances cholinergic transmission[17,23] in the brain explains why it has been useful in the treatment of several cognitive disorders[17,23]. Trials have shown that Pyritinol is useful in protecting brain cells from hypoxia, aiding recovery from head injury and stroke, and alleviating

dementia[14, 15, 16,20,21,22]. Pyritinol is known to increase nerve activity in an area of the brain known as the locus coeruleus, which has been linked to learning and memory. Pyritinol has also been clinically proven as a treatment for rheumatoid arthritis[19,24]. The drug also enhances or normalizes glucose transport through the blood-brain barrier and increases the production of energy from glucose. In 1993, researchers found that Pyritinol enhanced the immune system by stimulating neutrophil migration, an increasing the survival time of the white blood cells[18]. Pyritinol is almost identical in structure to vitamin B6 (pyridoxine), however it does not have any actions that are similar to those of the vitamin.[11]The central cholinergic system plays an important role in learning and memory.[26, 27, 28] Phenytoin is known to reduce hippocampal Ach concentration.[2, 7, 8] In view of this we will also study the effect of this combination on the brain cholinergic system. Since the majority of AEDs including Phenytoin are known to impair motor performance, the study also evaluated this combination on motor function.

MATERIAL AND METHODS

Animals

Swiss albino mice of either sex (24-34 g), supplied by the Central Animal House Facility of SRM University, Chennai, used. All animals housed in cages in groups of 10, at 23-30 ^{0}C with a natural light-dark cycle. They have free access to standard pellet diet and tap water. The study approved by the Animal Ethical Committee, CPCSEA (IAEC/41/2008). Ethical norms strictly followed during all experimental procedures.

Drugs and dosing schedule

The following drugs were used: Phenytoin ('Dilantin' suspension, Parke Davis) was given p.o. in a volume of 10 ml/ kg body weight in dose of 22 mg/kg body weight 2 hr prior to each observation. Pyritinol ('Encephabol' suspension, MERK) was given p.o. in a volume of 10 ml/kg body weight in dose of 100 mg/kg body weight 1 h prior to each experiment. Control groups were given distilled water in a volume of 10 ml/kg body weight. Chronic studies were done for 21 days. All observations were made on day 21 after 2 h of phenytoin and 1 h of pyritinol administration. In chronic studies, drugs were administered between 10-12 A.M.

Increasing Current Electroshock Seizures (ICES)

The ICES as proposed by Kitano et al and modified by Marwah et al[29] was used to evaluate the anticonvulsant effect of the drugs. To start with a current of 2 mA electroshock to each mouse via ear electrodes as a single train of pulses (for 0.2 sec) was given with linearly increasing intensity of 2 mA /2 sec using an electroconvulsometer .The current at which tonic Hind Limb Extension (HLE) occurred was recorded as the seizure threshold current (STC). When no tonic HLE was observed by a current of 30 mA, electroshock was terminated.

Spontaneous Alteral Behaviour (SAB) on a plus maze

Rodents have a natural tendency to alternate. An amnesic drug impairs this behaviour and vice versa with nootropic. Hence, an increase in alternation implies improved cognition and vice versa. Cognitive functions were assessed using a plus maze proposed by Itoh et al[31] and SAB was noted following the method of Ragozzino et al[30] The maze (height—50 cm) was constructed of wood, painted gray and contained a central platform (8 X 8 cm) from which radiated four symmetrical arms (23.5 X 8 cm) with 10 cm walls. After being placed in the central platform, mice were allowed to traverse the maze freely. The number and sequence of entries were recorded during and observation period of 6 min. An alternation was defined as entry into four different arms on overlapping quintuple sets. Five consecutive arms choices within the total set of arm choices make up a quintuple set, e.g. a quintuple set consisting of arms choices A, B, A, C, B was not considered an alternation. Using this procedure percentage alternation was calculated as follows:

Percentage alternation = actual no. of alternation/possible no. of alternation X 100

Possible alternation = no. of arms entries – 4

The number of arm entries was also recorded separately to determine the motor influence on the observed effects.

Rolling roller apparatus

This method as devised and proposed by Dunham et al[32.] was used to screen the neurological deficit caused by the drugs. The speed selector was set so that the roller made

5 revolutions/minute. The animals were placed on the roller for one minute as a testing time. A normal animal can maintain its equilibrium throughout the period. Neurological deficit was indicated by the inability of the animal to remain on the roller for a one-minute test period.

Estimation of brain acetylcholinesterase (AChE) activity

The whole brain AChE activity was measured using the Ellman et al method.[23] This was measured on the basis of the formation of yellow colour due to the reaction of thiocholine with dithiobisnitrobenzoate ions. The rate of formation of thiocholine from acetylcholine iodide in the presence of tissue cholinesterase was measured

using a spectrophotometer. The sample was first treated with 5, 5'-dithionitrobenzoic acid (DTNB) and the optical density (OD) of the yellow colour compound formed during the reaction at 412 nm every minute for a period of three minutes was measured. Protein estimation was done using Folin's method. AChE activity was calculated using the following formula:

R = O.D. x Vol. of assay/E X mg of protein

Where R= rate of enzyme activity in 'n' mole of acetylthiocholine iodide hydrolyzed / minute / mg protein

δ O.D = Change in absorbance / minutes

E = Extinction coefficient = 13600 /M/cm

Statistical analysis

The data were expressed as mean±SEM The normal distributed data were subjected to one-way ANOVA followed by Dunnett's test. P values <0.05 were considered significant.

RESULTS

Increasing current electroshock seizures (ICES)

Acute studies

PHT (22 mg/kg, p.o.) showed 100% protection against ICES as evidenced by a complete abolition of HLE. PYT (100mg/kg, p.o.) at memory improving doses was found ineffective on ICES (Table 1). The combined treatment with PHT and PYT showed effects similar to those with PHT alone.

Table 1: Effect of acute phenytoin (PHT), acute pyritinol (PYT) and its combination on ICES and SAB in mice

Groups	Treatment	Dose (mg/kg, p.o.)	ICES		SAB	
			Seizure threshold Current (mA)	% Protection	% Alteration	No of arm entries
I	Control (distilled water)	10 ml/kg	14.00 ± 0.63 (6)	0	69.40 ± 2.71 (6)	16.33 ± 0.42
II	PHT	22	30 ± 0.0*(6)	100	50.55 ± 1.02* (6)	18.50 ± 0.22
III	PYT	12	13.50 ± 0.67 (6)	0	76.57 ± 0.89† (6)	21.00 ± 0.57
IV	PHT+PYT	22	30 ± 0.0* (6)	100	83.37 ± 2.57* (6)	18.17 ± 0.30
		F	414.4	F	50.41	
		df	3.20	df	3.20	
		P	<0.001	P	<0.001	

Values are mean ± SEM, Values within parentheses are number of animals, ICES- Increasing current electroshock seizure, SAB-Spontaneous alternation behaviour. Seizure threshold current values and alternation values were analysed using one-way ANOVA followed by Dunnett's test. *P<0.001, † P<0.05 Vs control.

Table 2: Effect of chronic phenytoin (PHT) and Pyritinol(PYT) on SAB

Groups	Treatment	Dose (mg/kg, p.o.)	% Alteration	No of arm entries
I	Control (distilled water)	10 ml/kg	69.38 ± 2.74 (6)	16.33 ± 0.42
II	PHT	22	50.50 ± 2.66* (6)	17.50 ± 0.22
III	PYT	100	75.10 ± 0.91 (6)	20.00 ± 0.57
IV	PHT+PYT	22+100	82.09 ± 2.79†† (6)	17.17 ± 0.30

All observations were made on 21st day starting from treatment, Values are mean ± SEM, Values within parentheses are no. of animals, SAB–Spontaneous alternation behaviour. F=31.57, df = 3,20, P<0.001 (one-way ANOVA followed by Dunnett's test).*P<0.001,††P<0.01vs control.

Table 3: Effect of acute phenytoin (PHT), acute piracetam (PIM) and its combination on AChE activity in mice

Groups	Treatment	Dose (mg/kg, p.o.)	AChE(µ moles)
I	Control (distilled water)	10 ml/kg	117.0 ± 1.55 (6)
II	PHT	22	191.2 ± 1.07 *(6)
III	PYT	100	90.83 ± 0.60 * (6)
IV	PHT+PYT	22+100	115.7 ± 1.05 (6)
		F	1897
		df	3.20
		P	<0.001

Values are mean±SEM, Values within parentheses are number of animals, AChE-whole brain AChE activity. *P<0.001 Vs control (one-way ANOVA followed by Dunnett's test.)

Spontaneous alternation behavior (SAB)

Acute studies

The administration of PHT (22 mg/kg, p.o.), has significantly reduced percentage alternation on plus maze, which accounts for the adverse effect on cognitive function. PYT (100 mg/kg, p.o.) showed significant elevation of percentage alternation. Combined treatment with PHT (12 mg/kg, p.o.) and PYT (100 mg/kg) showed effects similar to the control

group i.e. no deteriorating effect on memory without altering any effect on ICES. (Table 1)

Chronic studies

PHT (22mg/kg, p.o .X 21days) caused a significant reduction in the percentage alternation i.e. markedly impaired memory. Combination of PYT (100 mg/kg, p.o. X 21 days) with PHT (22 mg/kg, p.o. X 21 days) reversed such impairment (Table 2).

Rolling roller apparatus

Dose of PHT and PYT in both acute and chronic studies, as well as in combination, not produced any motor deficit.

Whole brain AChE activity

The whole brain AChE activity with PHT (22 mg/kg, p.o.) was differ from the control. PHT (22 mg/kg, p.o.) demonstrated a significant rise in AChE activity as compared to control. PYT at a dose of 100 mg/kg, p.o. altered brain AChE activity significantly. PYT at a dose of 100 mg/kg, p.o. lowered AChE levels significantly. A combination of PHT (22 mg/kg, p.o.) and PYT (100 mg/kg, p.o.) exhibited AChE levels similar to control (Table 3).

DISCUSSION

The results of the present study show that PHT in doses 22 mg/kg, p.o., adversely affected the cognitive function in both acute and chronic studies. These doses were ED100 against ICES. This result is in agreement with the previous studies of PHT on cognitive functions.[1-3] Pyritinol, a well-known nootropic agent[13, 25] exhibited significant nootropic effect on spontaneous alternation behavior, a model specific for measuring spatial memory in rodents. At the nootropic doses used, it was found to be ineffective against ICES. The present study was based on the assumption that co-administration of PYT with clinically used AED might be useful in reducing some of the cognitive adverse effects of antiepileptic therapy. Our study showed that PYT, when co-administered with PHT, significantly reversed PHT induced cognitive impairment without altering the efficacy of PHT against ICES. In the chronic study, PYT at dose of 100 mg/kg enhanced the percentage alternation but it was not statistically significant. This does, however, reverse the PHT-induced impairment of SAB.

To study the effect of motor influences on observed effects, the rolling roller apparatus was used but PYT alone, as well as in combination with PHT, did not exhibit any significant effect on motor functions. Further, the number of arm entries remained unaffected in SAB, thus ruling out the involvement of motor functions in the observed cognitive effects.

The precise mechanism by which PYT exerts its nootropic effect is not known. Multiple mechanisms have been suggested such as a potent scavenger of hydroxyl free radicals[12]. It is now widely accepted that the antioxidant properties of the drug are responsible for many of the benefits of Pyritinol[12]. The discovery that Pyritinol can protect proteins in the brain against radical induced polymerization, coupled with evidence showing that the drug enhances cholinergic transmission[17,23] in the brain. Later explains why it has been useful in the treatment of several cognitive disorders. Trials have shown that pyritinol is useful in protecting brain cells from hypoxia, aiding recovery from head injury and stroke, and alleviating dementia[14, 15, 16,20,21,22]. Pyritinol is known to increase nerve activity in an area of the brain known as the locus coeruleus, which has been linked to learning and memory. The drug also enhances or normalizes glucose transport through the blood-brain barrier and increases the production of energy from glucose. In 1993, researchers found that pyritinol enhanced the immune system by stimulating neutrophil migration, an increasing the survival time of the white blood cells[18]. Pyritinol is almost identical in structure to vitamin B6 (pyridoxine), however it does not have any actions that are similar to those of the vitamin.[11]

In our study, PHT, (22 mg/kg, p.o.) significantly elevated the 'brain AChE activity'. PYT (100 mg/kg, p.o.) on the other hand significantly lowered this activity indicating the counteracting action of the two drugs on the cholinergic system. The impairing effects of PHT on learning and memory have been attributed to alternations in the cholinergic system.[2,16,17] It has been reported that PHT lowers brain ACh levels.[2,16,17] Our results on AChE are thus consistent with these reports. In our study PYT alters the AChE activity of the brain. But, more importantly, in this context, it is interesting to note that when co-administered with an effective dose of PHT, PYT significantly alleviated the PHT-induced sharp rise in total brain AChE level, indicating the counteracting action of PYT and PHT on the cholinergic system.

CONCLUSION

To conclude, PYT when co-administered with therapeutic doses of PHT, significantly alleviated the adverse effects of PHT on cognitive function without compromising its antiepileptic efficacy, the effect possibly mediated by an action on the cholinergic system. However, clinical studies are required to explore the full potential of PYT in correcting PHT induced cognitive deficits and finding a place in the current AED therapy.

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