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The effect of co-administration of pentylenetetrazole with pilocarpine: New modified PTZ models of kindling and seizure

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ABSTRACT

Background: Drug resistance is a major problem in the treatment of epilepsy. There is a critical need for new epilepsy models to evaluate antiepileptic compounds. Pentylenetetrazole- (PTZ) and pilocarpine-induced seizures are well-established models of human epilepsy. Generally, PTZ or pilocarpine has been used to produce seizures in experimental models. In this study, we explored the possibility of creating new epilepsy and seizure models by co-administration of PTZ and pilocarpine.

Methods: The protocol was divided into three parts: A) Kindling experiments: the animals received PTZ or co-administration doses of PTZ and pilocarpine every other day for a period of 26 days. B) Seizure experiments, for induction of seizure, the animals received one dose of PTZ, pilocarpine or co-administration doses of PTZ and pilocarpine. C) Evaluation of antiepileptic drugs: the animals received phenytoin or sodium valproate 20 min before injection of PTZ, pilocarpine or co-administration doses of PTZ and pilocarpine.

Results: The co-administration of pilocarpine and PTZ could induce seizure, which has behavioral similarity between electrical and chemical kindling. Pilocarpine (50 mg/kg) + PTZ (37.5 mg/kg) was the appropriate dose for kindling induction. Animals with this dose reached the stage five seizures significantly faster than those with PTZ alone. Unlike the seizure induced by PTZ, or pilocarpine, induction of seizure by PTZ + pilocarpine was resistant to phenytoin and sodium valproate treatment. As compared to the PTZ model of kindling, this model visualized the seizure behavior better and had resistance to two most popular antiepileptic drugs.

Conclusion: Our results indicated that co-administration of pilocarpine and PTZ could provide a new modified model of seizure and kindling resisting to phenytoin and sodium valproate.

1. Introduction

Epilepsy is a neurological disorder characterized by an enduring predisposition to generate epileptic seizures, and by associated co-morbidities such as the neurobiologic, cognitive, psychological, and social consequences of this condition (Fisher et al., 2014). It also affects approximately 50 million people worldwide (Van Leeuwen et al., 2003; Siebel et al., 2011; Ayati et al., 2016), with 2 million new cases annually (Bloch et al., 2014). The majority of people with epilepsy live in resource-poor countries, where up to 98% do not receive regular anti-epileptic drug (AED) treatment, and epilepsy remains a major public health problem (Kwan et al., 2013). Approximately 30% of patients with epilepsy receiving medical treatments are resistant to pharmacologic therapy despite adequate treatment (Bialer and White, 2010).

In recent years, our knowledge about the mechanism of seizures and epilepsy has expanded considerably. Advances in this field are indebted to animal models. A number of useful experimental animal models for seizure and epilepsy, such as PTZ kindling and pilocarpine-induced seizure models, are now commonly used.

Pentylenetetrazole (PTZ) induced seizure model is an experimental procedure widely used as a model of seizure and epilepsy (Löscher, 2011). PTZ acts at the picrotoxin site of the γ-aminobutyric acid type A (GABA-A), and is a cost-effective assessment for drug screening (Blanco et al., 2009).

Pilocarpine-induced seizure is another well-established rodent

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model of the temporal lobe epilepsy triggered by cholinergic hyperactivity.

Pilocarpine and PTZ models alter excitatory or inhibitory neurotransmitter systems (glutamic and aspartic acids or GABA, respectively), and increase levels of excitation or decrease inhibition (Badawy et al., 2012; Dean, 2015). Studies have suggested that many neurotransmitter systems are involved in human seizure (Bromfield et al., 2006).

Consequently, it seems that the models obtained by injection of pilocarpine or PTZ separately, are not an effective substitute model for human seizure (Engel, 1992; Stables et al., 2002; Stables et al., 2003; Bromfield et al., 2006; Blanco et al., 2009; Cremer et al., 2009; Badawy et al., 2012; Dean, 2015). Therefore, we explored the possibility of creating a new model by co-administration of PTZ and pilocarpine in male rats, and assessed the effects of phenytoin and sodium valproate on them.

2. Materials and methods

2.1. Drugs and chemicals

Pentylenetetrazole, phenytoin and pilocarpine were purchased from Sigma, India and sodium valproate was provided from Alhavi Pharmaceutical Company, Iran. Phenytoin was dissolved in 100 mM NaOH and then diluted with normal saline to the desired volume. Pentylenetetrazole, pilocarpine and sodium valproate were dissolved in saline. The control animals received saline.

2.2. Animals

A total of eighty five male Wistar rats (Pastor Breeding Centre Tehran, Iran) weighing 200–250 g were used in this experiment. The animals were housed under environmentally controlled conditions (12 h light/dark cycles, 7:00–19:00 light and 19:00–7:00 dark, temperature 22 ± 2 °C) at Arak University of Medical Sciences, animal facility. Food and water were supplied ad libitum. All the procedures were carried out in accordance with the EU Directive 2010/63/EU, and the Ethics Committee standards of Arak University (Arak University of Medical Sciences, Research Ethics Committee, ethical approval # 1395.65).

In this study, the effect of co-administration of pilocarpine and PTZ on induction of kindling and seizure in animals was investigated. The animals were randomly divided into eleven groups. To investigate the effect of co-administration of pilocarpine and PTZ on kindling development, the rats were divided into five groups. The rats in Group 1 received only PTZ (37.5, n = 8,) infusions. Groups 2, 3, 4 and 5 received co-administration of different doses (mg/kg, ip): pilocarpine (50,) + PTZ (30) (Group2, n = 8), pilocarpine (50) + PTZ (37.5) (Group 3, n = 8), pilocarpine (100) + PTZ (30) (Group 4, n = 8), and pilocarpine (100) + PTZ (37.5) (Group 5, n = 8). To investigate the effect of sodium valproate and phenytoin on the seizure induced by co-administration of pilocarpine and PTZ, the rats were divided into six groups. In groups 6, 7 and 8, the rats were pretreated with phenytoin (30 mg/kg, ip) 20 min before administration of PTZ (37.5, n = 13), pilocarpine (200, n = 6) and pilocarpine (100) + PTZ (37.5) (n = 8), respectively. In groups 9, 10 and 11, the rats were pretreated with sodium valproate (200 mg/kg, ip) 20 min before administration of PTZ (37.5, n = 6), pilocarpine (200, n = 6) and pilocarpine (100) + PTZ (37.5) (n = 6), respectively.

2.3. Kindling

To induce kindling, a sub-convulsive dose of PTZ (37.5 mg/kg, ip injection, Sigma, USA) and co-administration doses of PTZ and pilocarpine were administered every other day for a 26-day period (13 injections). After each injection, the animals were kept in a Plexiglas chamber (30 cm × 30 cm × 30 cm), and convulsive behavior was recorded for 30 min. Convulsive responses were classified as described previously (Davoudi et al., 2013). The rats were considered fully kindled when seizure attacks (stage five) occurred after each injection for three consecutive injections. The recording parameters were as follows: seizure stage, latency to the onset of stage two and five seizures, and stage five duration.

2.3.1. Seizure scaling

The seizure responses were observed over a 30-minute cutoff period and were classified as initially described by Racine zero: no response; one: ear and facial twitching; two: convulsive waves through the body; three: myoclonic jerks, rearing; four: tonic-clonic convulsions, turn over into side position; five: generalized tonic-clonic seizures, loss of postural control (Davoudi et al., 2013). Furthermore, seizure activity induced by pilocarpine was classified as follows; one: staring with mouth clonus; two: automatism; three: unilateral forelimb clonus; four: bilateral forelimb clonus; five: bilateral forelimb clonus with rearing and falling; 6: tonic-clonic seizure (Goffin et al., 2007). Based on our observation, the seizure induced by co-administration of PTZ and pilocarpine was similar to that in PTZ animals. Therefore, the seizure behavior induced by co-administration of PTZ and pilocarpine was classified as PTZ scaling.

2.4. Phenytoin and sodium valproate pretreatment

The effect of phenytoin and sodium valproate was evaluated on 48 animals in three doses of PTZ (37.5 mg/kg), pilocarpine (200 mg/kg) and co-administration of pilocarpine (100 mg/kg) and PTZ (37.5 mg/kg), and the effects of these drugs on seizure parameters were determined.

2.5. Statistical analyses

Statistical analyses were conducted using Graph Pad software (Version 6). A one-way analysis of variance (repeated measures for time rows) was conducted, followed by a Tukey's test for multiple comparisons. An unpaired Student's *t*-test was used to compare two different animal groups. The criterion for statistical significance was $p < 0.05$. Results are reported as mean ± standard error of the mean (SEM).

3. Results

3.1. Seizure behavior

The specific feature of seizure behavior in rats subjected to co-administration of pilocarpine and PTZ seems to show the seizure of stages three (myoclonic jerks, rearing) and four (tonic-clonic convulsions, turn over into side position). In most cases, in the PTZ induced seizure model, the animals did not exhibit stages three and four, and this seizure behavior is similar to that in the electrically-induced amygdala kindling model. Furthermore, the co-administration of pilocarpine and PTZ, induced clonic-seizure without any tonic contraction (this seizure behavior has been seen in all animals). This is in contrast to the chemical seizure models (PTZ or pilocarpine-induced seizure models) that are largely characterized by generalized tonic-clonic seizures.

The above results demonstrated that the seizure behavior induced by co-administration of pilocarpine and PTZ exhibited combination characteristics of electrical and chemical kindling properties, therefore; this model may be considered a new modified seizure model.

3.2. Seizure parameters in combined doses

Table 1 presents the percentage of animals showing stage two, stage five seizure and percentage of mortality following a single dose of co-administration of pilocarpine and PTZ. These parameters for doses of

Table 1
The percentage of animals showed stage two, stage five, and mortality, in the co-administration of pilocarpine and PTZ.

	Stage two seizure (%)	Stage five seizure (%)	Mortality (%)
PTZ	0	12.5	100
50Pilocarpine* + 30PTZ*	0	0	0
50Pilocarpine + 37.5PTZ	25	62.5	75
100Pilocarpine + 30PTZ	37.5	37.5	62.5
100Pilocarpine + 37.5PTZ	75	75	75

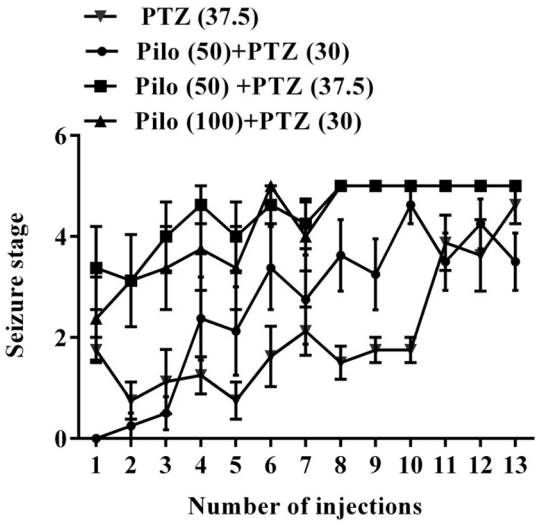

Fig. 1. Effect of PTZ or co-administration of PTZ and pilocarpine on the rate of kindling development.

50 + 30, 50 + 37.5, 100 + 30 and 100 + 37.5 (mg/kg), were 0-0-0, 75-62.5-25, 62.5-37.5-37.5, 75-75-75%, respectively. Following a single dose of pilocarpine (200 mg/kg), 83% of the animals showed stage two and three of seizures. Moreover, the rate of mortality in this group was 17%.

3.3. Effect of PTZ or co-administration of PTZ and pilocarpine on kindling development

Fig. 1 shows the rate of kindling development in PTZ and different doses of co-administration of PTZ and pilocarpine. Repeated injections of PTZ significantly increased the seizure stage, and the mean of seizure stage reached stage four at the end of the thirteenth injection ($F(12, 84) = 8.759$, $p \leq 0.0001$, seizure stage in repeated injections in PTZ (37.5)). Induction of kindling using repeated injections of PTZ (30) + pilocarpine (50) showed that kindling started from stage zero, and the animals reached stage four after the thirteenth injection ($F(12, 84) = 8.218$, $p < 0.0001$, seizure stage in repeated injections in the PTZ (30) + pilocarpine (50) group). Kindling induction using PTZ (37.5) + pilocarpine (50) showed that kindling started from stage three, and the animals reached stage five after the eighth injection ($F(12, 84) = 2.261$, $p < 0.0155$, seizure stage in repeated injections in the PTZ (37.5) + pilocarpine (50) group). Administration of PTZ (30) + pilocarpine (100) produced stage two seizure in the first injection, and reached stage five after the eighth injection ($F(12, 84) = 3.816$, $p < 0.0001$, seizure stage in repeated injections in the PTZ (30) + pilocarpine (100) group) (Fig. 1). Comparison of the rate of kindling in different groups with two-way repeated measure ANOVA showed that the animals receiving combined doses of pilocarpine (50) and PTZ (37.5) ($p < 0.0036$) and pilocarpine (100) and PTZ (30) ($p < 0.0001$) had a significantly faster rate of kindling development than those receiving the PTZ kindling ($F(36, 252) = 1.986$, $p = 0.0013$).

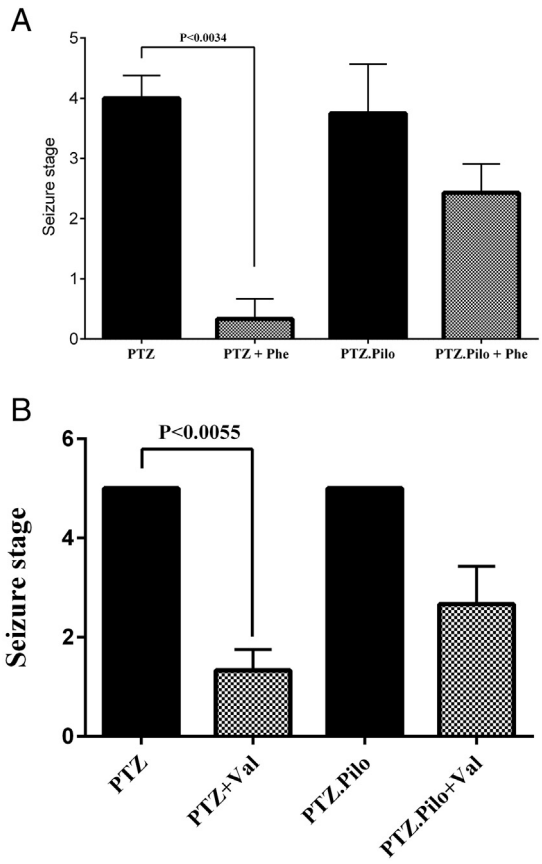

Fig. 2. Effect of phenytoin (A) and sodium valproate (B) on seizure induced by administration of PTZ, pilocarpine or co-administration of PTZ and pilocarpine. The data are presented as mean ± SEM.

3.4. Effect of pretreatment with phenytoin and sodium valproate on seizure induced by PTZ, pilocarpine and co-administration of PTZ and pilocarpine

Comparison of the effect of phenytoin (30 mg/kg) on seizures induced by PTZ (37.5), pilocarpine (200) and co-administration of PTZ (37.5) and pilocarpine (100) with Kruskal-Wallis test showed that although phenytoin significantly inhibited seizures induced by PTZ ($p = 0.0034$, Dunn's test) and pilocarpine ($p = 0.048$, Dunn's test), it had no significant effect on the seizure induced by co-administration of PTZ and pilocarpine (Fig. 2A). Likewise, although administration of sodium valproate (200 mg/kg) reduced seizure stage in both PTZ ($p = 0.0055$, Dunn's test) and pilocarpine ($p = 0.015$, Dunn's test) groups, it did not have a significant effect on the seizure induced by co-administration of PTZ and pilocarpine (Fig. 2B).

4. Discussion

The results indicated that co-administration of pilocarpine and PTZ could create new models for seizure and kindling showing some of the features of electrical and chemical models (PTZ). Additionally, this model was resistant to phenytoin and sodium valproate.

The point differentiating the combined model from the PTZ model and the pilocarpine model is the better visualization of the seizure behavior, and therefore the more accurate measurement of the seizure parameters. Our results demonstrated that rats that received co-administration of PTZ and pilocarpine showed the seizures of stage three (clonus of the front limb) and stage four (standing on the hind legs and clonus of the front limbs), which is similar to the electrical kindling

(Castel-Branco et al., 2009). However, the rats in the PTZ model did not show stages three and four (Lüttjohann et al., 2009). Moreover, unlike the PTZ or pilocarpine-induced seizure that has tonic contractions (Velíšková, 2006; Mandhane et al., 2007), stage five seizure after co-administration doses has not any tonic contraction in all animals. This result could indicate that the model derived from the combined dose has some characteristics of electrical and chemical models.

To determine the proper combination doses, different doses of pilocarpine and PTZ were used to induce seizure and kindling. In this regard, parameters, such as seizure behavior, rate of kindling, response to phenytoin and sodium valproate and mortality rates after seizures, were considered important criteria to select an appropriate dose. In the combined dose of pilocarpine (100 mg/kg) + PTZ (37.5 mg/kg), most animals exhibited stage five seizures at the first injection (75%), but all of them died after the seizure. Depending on the high incidence of stage five seizures, it seems that this combined dose can be suitable for seizure evaluation. The combined dose of pilocarpine (50 mg/kg) + PTZ (37.5 mg/kg) has low mortality rate (25%) and high incidence of stage five seizures (62.5%) after each injection. Considering the facts that kindling requires repeated injections, and death after seizure is an important factor in the kindling model, this combined dose can be suitable for a new kindling model. In addition, comparison of the kindling rate in the combined dose and PTZ kindling model revealed that rate of kindling was faster in the combined dose than in the PTZ kindling model.

The use of co-administration of PTZ and pilocarpine for induction of seizures was also reported in previous studies. Pontes et al. showed that PTZ injection six months after the status epilepticus induced by injection of pilocarpine in marmosets created a manageable and reliable model of seizure (Pontes et al., 2016). In addition, Blanco et al. reported that administration of PTZ one month after pretreatment with pilocarpine in rats produced an effective and valuable method for the treatment of anticonvulsants (Blanco et al., 2009). The difference between our results and previous studies is the time interval between injections of pilocarpine and PTZ to create seizure. In contrary to the two previous studies, our results indicated that there was no need for a time interval between injection of PTZ and pilocarpine, and the simultaneous use of the two drugs could create seizure.

Nicola Marchi et al. showed that pilocarpine's penetration across the blood brain barrier (BBB) was significantly lower than expected based on its structural properties (Marchi et al., 2007). They suggested that peripheral activation of the immune system might lead to changes in neuronal excitability or focal BBB damage observed in the pilocarpine model might contribute to development of seizures. However, the results of our study showed that co-administration of pilocarpine and PTZ (immediately after injection) produced severe seizures, which were resistant to anti-seizure drug, indicating that pilocarpine induced seizure has not an inflammatory mechanism. Future studies are needed to find the molecular and precise mechanisms involved in this process.

Another finding in this study is the phenytoin and sodium valproate resistance. Our results demonstrated that although phenytoin and sodium valproate inhibited the seizure induced by PTZ (Löscher et al., 2004) or pilocarpine (Leite et al., 2002), and co-administration of pilocarpine and PTZ produced a model of seizure resistant to both anticonvulsant drugs. Along with these results, Blanco et al. reported that administration of PTZ one month after pretreatment with pilocarpine would lead to resistance to phenytoin in rats (Blanco et al., 2009). Resistance to both classical antiepileptic drugs can introduce this model as a suitable model for drug resistance researches. The use of other doses of phenytoin and sodium valproate, and perhaps the dose response of these drugs, as well as the assessment of sensitivity of this model to other antiepileptic drugs, could improve the results of this study.

Finally, there are some limitations in the seizure induction by co-administration of PTZ and pilocarpine, and the most important of them was the high rate of mortality in the co-administration group as compared to PTZ or pilocarpine received animals.

5. Conclusion

Our results demonstrated that co-administration doses of pilocarpine and PTZ could create a new model of seizure and epilepsy, which clearly exhibits seizure parameters and has resistance to phenytoin and sodium valproate.

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Ethical approval

Ethical approval for the study was provided by the Arak University of Medical Sciences Research Ethics Committee # 1395.65.

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Declaration of Competing Interests

The authors declare that they have no competing interests with respect to the authorship and/or publication of this article.

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