Antioxidant and Antiepileptic Activity of 1-[1-(3-Methoxyphenyl) (Tetralyl)] Piperidine as a New Derivative of Phencyclidine on Pentylentetrazole-Induced Kindling Mice

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ABSTRACT
Background and Objective: N-Methyl-D-aspartate (NMDA) antagonists such as piperidines are the most important antiepileptic drugs. Considering the fact that piperidine derivatives such as phencyclidine (PCP) and its new derivative, 1-[1-(3-methoxyphenyl) (tetralyl)] piperidine (PCP1), have different potencies, the antiepileptic effects of mentioned drugs were investigated in the present study.

Material and Methods: Fifty male mice weighing 25-30 g were randomly selected and divided into five experimental groups: 1-Control, 2- Pentylentetrazole-kindled mice, 3- Positive control group which received valproate, and groups 4 and 5, which received PCP and PCP1, respectively. Kindling was down by 11 periods injection of PTZ every second day for 22 days. At the 12th injection, all kindled group were tested for PTZ challenge dose. The exhibited phases of seizure (0-6) were observed and noted till 30 minutes after PTZ injection. Finally, the malondialdehyde, superoxide dismutase and nitric oxide levels of the animal’s brain tissues were determined and compared with others.

Results: PCP1 could have a prominent anti-convulsion effect compared to PCP, especially in the reduction of phase 2 duration time and seizure score in challenge dose. Our additional experiments showed that there was a significant reduction in NO level in PCP1 treated animals.

Conclusion: Administration of the new piperidine derivate, PCP1 could have yielded a prominent anti-convulsion effect in grand epilepsy. Regarding to the changes in conformation of PCP1 as a non-competitive antagonist of NMDA receptor, it may block the NMDA receptors potentially more effectively than phencyclidine.

Keywords: Antioxidant Effect, Antiepileptics, Phencyclidine, Mice

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Introduction

Epilepsy disease could have involved 0.5-1% of human in the world (1). It was obvious that the burst discharge activity of the neurons in the brain would result to seizure in the epileptic patients (2). However, despite the availability of different anticonvulsant drugs, about one-third of treated epileptic patients have shown epileptic seizure-induced uncontrolled neurological changes and some accompanied side effects. Such neurological changes might finally result in neuronal death (3). Glutamate and γ-aminobutyric acid (GABA) are two important excitatory and inhibitory neurotransmitters which are involved in epilepsy (4, 5).

Phencyclidine is a piperidinic derivative and non-competitive antagonist of N-methyl-D-aspartate (NMDA) receptor which binds to the receptor complex and causes to inhibit the NMDA-mediated conductance of calcium channel gating (6). Phencyclidine (1-(1-phenylcyclohexyl) piperidine), CAS 956-90-1, PCP, is a semi-rigid molecule containing a cyclohexane ring with attached aromatic and piperidine rings (7). “PCP and its analogues are highly potent and widely abused psychotomimetic drugs which influence the central nervous system” (Ahmadi, 2010, p.379). Because of the specific binding sites in the brain, PCP and its analogues display analgesic, stimulant, depressant and hallucinogenic effects (7). Furthermore, integrating phenyl group with the cyclohexane ring and the methoxy group to the aromatic ring of PCP, decreases the conversion of isomers of the drug (8) and also affects electron distribution and dipole moments (9) respectively.

Recently, some analogues of phencyclidine have been synthesized (9-12) and their pharmacological functions have been examined. To find and introduce selective and non-competitive antagonists at the PCP binding site on NMDA receptor complex, we have prepared 1-[1-(3-methoxyphenyl) (tetralyl)] piperidine (PCP-OCH3-tetralyl, III, Scheme 1) as an analogue of PCP (We will refer to it as PCP1) with a methoxy group on the aromatic ring (m-position) and a phenyl group with a cyclohexane ring (a conjugated cyclic ketone, 1-tetralone). Then we tried to examine its anti-convulsant effects on mice, using the PTZ-induced kindling model. The results have been compared to those of PCP and valproate.

One of the most important mechanisms by which neurological disorders such as epileptic seizure occur, are oxidative stress and free radicals production (13, 14). There are paradoxical reports about the role of NO in the seizure modulation in the brain. It has been introduced as an inhibitor (15, 16) and stimulator (17, 18) in different cases. The elevated level of the MDA, in the PTZ-induced kindled mice has been observed (3, 19). Since MDA is the final product of lipid peroxidation, the elevation of MDA level could be considered as an index of increased free radicals generation. SOD is an intracellular antioxidant enzyme that is able to help the conversion of peroxidase to hydrogen peroxide (H2O2) and in this way protect the cell from superoxide radicals and oxidative stress. Superoxide dismutase catalyzes the dismutation of superoxide anions to hydrogen peroxide (20).

Based on the data reporting that PCP and PCP1 are among the most important groups of antiepileptic drugs; and the fact that they have different potencies, their anticonvulsant effects are considered and compared in the present study. We have also tried to consider the antioxidant effect of PCP and PCP1; using MDA, NO and SOD assessment in a kindling method.

Materials and Methods

Animals

In this experimental research, a total of 50 male mice (NMRI) weighing 25-30 g (Razi Institute, Iran) were randomly divided into 5 groups (n=10 in each group) including: 1- Control group 2-PTZ-induced kindled mice, 3- Positive control group which beside the PTZ received valproate 100 mg/kg, i.p. (Sigma, UK) as an anti-convulsant...
drug, 4 & 5-treatment groups which beside the PTZ, received PCP and PCP1 (in doses of 5.6 mg/kg; i.p). Ten mice were housed in each cage at temperature 21±2ºC and 12 h light-dark cycling. The mice had free access to standard food and tap water ad libitum.

The experimental protocol was approved by the Ethics Committee of Shahed University.

**Kindling**

All animals but the control groups (group 1) were kindled by a total of 11 periods of PTZ injections (35 mg/kg; i.p.). PTZ (Sigma, UK) was dissolved in sterile isotonic saline. Each administration was carried out every second day and in a period of 22 days. Mice were observed for 30 minutes after the last drug administration. After an additional 30 minutes, the mice were observed for lethality before returning to the home cage. The challenge dose of 75 mg/kg PTZ was injected to the kindled mice on day 26 (test day), which could produce convulsions (clonic and tonic) and lethality (5).

In the three treatment groups (valproate, PCP and its derivative), PTZ was administrated 30 minutes after the first treatment. The exhibited phases of seizure (0-6) were observed and categorized using the following scale (21) for 30 minutes after PTZ injection. The scale introduces six phases as follows:

0: no response
1: ear and facial twitching
2: convulsive waves axially through the body
3: myoclonic body jerks
4: generalized clonic convulsions turn over into side position
5: generalized convulsions with tonic extension episode and status epilepticus
6: death

**Experimental materials**

1-Tetralone [1, 2, 3, 4 -Tetrahydro-1- naphthalenone], Cyclohexanone, Piperidine, Bromo benzene, Magnesium turning, Diethyl ether, 3-bromo anizole, and all other chemicals were purchased from Merck chemical Co. (Darmstadt, Germany). Melting points (uncorrected) were determined using a digital Electrothermal melting point apparatus (model 9100, Electrothermal Engineering Ltd., Essex, UK). 1H and 13C NMR spectra were recorded on a Bruker 300 MHz (model AMX, Karlsruhe, Germany) spectrometer (internal reference: TMS). IR spectra were recorded on a Thermo Nicolet FT-IR (model Nexus-870, Nicolet Instrument Corp, Madison, Wisconsin, U.S.A.) spectrometer. Mass spectra were recorded on an Agilent Technologies 5973, Mass Selective Detector (MSD) spectrometer (Wilmington, USA). Column chromatographic separations were performed over Acros silica gel (No.7631-86-9 particle size 35-70 micrometer, Geel, Belgium).

**Synthesis of compounds**

**PCP I**

This compound was prepared according to the reported method (22) from 1-piperidinocyclo-hexancarbonitrile (IV) and phenyl magnesium bromide (Figure 1, 2). The hydrochloride salt of I was prepared using 2-propanol and HCl and was recrystallized from 2-propanol (22).

**Fig. 1**: Structure formulas of PCP (I), Ketamine (II), PCP-OCH3-tetralyl (III) and Carbonitrile intermediates IV and V
To a solution containing 0.582 g (0.0068 mol) of piperidine in 0.253 g HCl (37%) and 1.36 g cold water, 1 g (0.0068 mol) 1, 2, 3, 4-tetrahydro-1-naphthalene (1-tetralone) was added. Then 0.465 g KCN in 1.02 ml water, 50 ml ethanol and 0.1 g tetra-n-buthylammonium bromide (0.0003 mol) were added and stirred in ambient temperature (25 °C). The progress of the reaction was controlled by TLC (7:3 ethyl acetate/n-Hexane). After one week no additional progress was seen, so the reaction was extracted with Chloroform (75 ml, 3 times). Then the organic layer was separated, dried and concentrated. The oily residue was obtained, which was passed through a silica gel column using ethyl acetate-hexane (7:3) as the eluent to afford 1.13 g of V (69 % yield).

**IR (KBr):** 3066, 2941, 2560, 1454, 1436, 1324, 1287, 1225, 764 cm⁻¹.

**1H N.M.R. (CDCl₃) (p.p.m.):** 1.5-2.85 (16H, m), 6.93-7.01 (4H, m).

**13C N.M.R. (CDCl₃) (p.p.m.):** 25.4, 26.2, 26.8, 31, 37.9, 46.7, 52.7, 117.7, 125.5, 128.1, 139.2.

**MS: m/z (regulatory intensity):** 240 [M]+ (76), 241 [M+ H]+(12).

**Sample preparation and biochemical assays**

After the injection of the challenge dose of PTZ and behavioural analysis, the mice were decapitated. The brains were removed quickly and were washed in cold saline for two times. They have been placed in freezer (-30 °C), in a glass bottle (less than 10 hours) and then the brain pieces (cutting the brain tissue using the scissors) were homogenized using ice-cold Tris-HCl buffer (50 mM, pH 7.4) four times, for two minutes at 5000 rpm. MDA and NO levels were measured at this phase. The homogenized solution was then centrifuged for 60 minutes at 5000×g to remove the debris. The supernatant solution was then extracted with a mixture of ethanol/chloroform (a volume with ratio of 5:3). After centrifuging at 5000×g for 30 minutes, the clear upper layer (the ethanol phase) was taken and used for evaluation of the SOD activity. All experiments were carried out at +4 °C (3).
MDA evaluation
The MDA concentration (thiobarbituric acid reactive substances, TBARS) in the supernatant has been measured using the following protocol. Trichloroacetic acid and TBARS reagent were added to the supernatant and were then mixed and incubated at 100 °C for 80 minutes. After cooling on ice, samples were centrifuged at 1000×g for 20 minutes and the absorbance of the supernatant was read at 532 nm (23).

NO evaluation
NO content of the supernatant was assayed by the Griess method. Since NO has a short half life and is rapidly converted to the stable end products nitrate (NO$_3^-$) and nitrite (NO$_2^-$), the principle of the assay is the conversion of nitrate into nitrite by cadmium and is followed by color development with Griess reagent (sulfanilamide and N-naphthyl ethylenediamine) in acidic medium (24). The total nitrite was measured by Griess reaction. The absorbance was determined at 540 nm with a spectrophotometer (3).

SOD activity evaluation
SOD activity measurement was carried out according to the following protocol. At first, the supernatant was incubated with xantine and xanthine oxidase in potassium phosphate buffer (pH 7.8, 37 °C) for 40 minutes and NBT was added. Blue formazan was then monitored spectrophotometrically at 550 nm. The protein level that inhibited NBT reduction to 50% maximum was defined as 1 nitrite unit (NU) of SOD activity (25).

Chimney test
The chimney test (26) was used to quantify the effects of PCP and PCP1 on motor performance in mice. In this test, the animals had to climb backwards up a plastic tube (3 cm inner diameter, 30 cm in long), and motor performance impairment was indicated by the inability of the mice to climb backward up the transparent tube within 60 seconds (27, 28).

Statistical analysis
Data were expressed as means ± S.E.M. Statistical analyses was carried out using repeated measurement of one way analysis of variance (ANOVA) followed by post-hoc Tukey test and P values less than 0.05 were considered as significant differences.

Results
Chemistry
PCP (I) and PCP1 (III) were synthesized by reaction of substituted Grignard reagents and carbonitrile compounds (IV, V) (Fig. 3). To obtain higher electron distribution and dipole moment properties, a methyl group was substituted on the aromatic ring of the molecule (III). Known procedures were applied for the synthesis of compounds I and IV with the appropriate modifications described previously (8, 12).

![Fig. 3: Synthesis of compounds I and III](image-url)
and V. The melting points of known compounds could also confirm their identity. The purity of each compound was checked by TLC using ethyl acetate/n-hexane as the eluent.

**Pharmacology**

**General Consideration**

General consideration means mortality, morbidity, irritability and other side effects due to drugs administration. However, comparison of the motor coordination index (measured by Rota-rod apparatus, Harvard, UK) indicated no significant differences between control and treated animals.

**Effect of PCP1 on the PTZ-induced kindling intensity**

Statistical analysis of the results (Fig. 4) indicates that there are no significant differences among the experimental groups in seizure intensity till the 6th injection. However, at the 6th injection valproate administration shows significant reduction in seizure intensity relative to both PTZ-kindled and PCP-treated groups. PCP injection with 5.6 mg/kg dosage could not change seizure intensity in any period (Fig. 4). In addition, valproate (150 mg/kg) has reduced seizure intensity relative to PTZ-kindled group in most of the periods (the 6th, 8th and 9th injections) significantly ($P<0.05$) and markedly at the 11th injection ($P<0.01$). In addition, PCP1 injection with 5.6 mg/kg dosage at the 7th, 9th, 11th and 12th injections was able to reduce PTZ-induced seizure significantly ($P<0.05$).

![Fig. 4: Effect of PCP and PCP1 pre-treatment on the PTZ-induced kindling intensity. *$P<0.05$ and **$P<0.01$ indicate significant differences as compared to PTZ-kindled group. $P<0.05$ shows significant difference with PCP group. VA = valproate](image)

**Effects of PCP and its derivative on the threshold and duration time of the 2nd and 5th phases of PTZ-induced seizure**

As it could be seen in Table 1, pretreatment of animals with valproate, PCP and PCP1 do not have any significant effect on the mice’s threshold and duration time to reach phase 5 of seizure. In addition to PTZ group mortality was also observed in the PCP group. Regarding Chimney test, no motor impairment has been shown in PTZ and valproate groups. Furthermore, a low percentage of the mice (8.33%) in PCP and PCP1 groups with motor impairment were observed.
Table 1- Effect of valproate, PCP and PCP1 on the threshold and duration time of 5th phase of seizure, mortality in the 12th PTZ injection, and Chimney test analysis

<table>
<thead>
<tr>
<th>Group test</th>
<th>Phase 5 latency time (s)</th>
<th>Phase 5 duration time (s)</th>
<th>Mortality (%)</th>
<th>Chimney test analysis % of mice showing motor impairment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTZ</td>
<td>3.86 ± 0.70</td>
<td>4.51 ± 0.58</td>
<td>10.20</td>
<td>0</td>
</tr>
<tr>
<td>PTZ + VA</td>
<td>3.50 ± 0.60</td>
<td>3.15 ± 0.45</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PTZ + PCP</td>
<td>2.79 ± 0.85</td>
<td>4.15 ± 0.48</td>
<td>12.50</td>
<td>8.33</td>
</tr>
<tr>
<td>PTZ + PCP1</td>
<td>2.15 ± 0.87</td>
<td>3.86 ± 0.78</td>
<td>0</td>
<td>8.33</td>
</tr>
</tbody>
</table>

There are no significant differences between pretreated groups with PTZ group in latency and duration times. n=10 in each group.

Furthermore, Table 2 indicates that pretreatment of mice with PCP, PCP1 and valproate 150 mg/kg is able to reduce the period that mice remain in phase 2 of seizure significantly ($P<0.05$). Duration of phase 5 has not shown any significant reduction with PCP and its derivative pretreatment. This is while valproate pretreatment has significant reductive effect on phase 5 duration time ($P<0.05$). However, none of the experimental groups have shown significant differences in phase 2 and phase 5 latency times relative to PTZ group.

Table 2- Effect of valproate, PCP and PCP1 on the threshold and remaining time in the phases 2 and 5

<table>
<thead>
<tr>
<th>Group test</th>
<th>Phase 2 latency time (s)</th>
<th>Phase 2 duration time (s)</th>
<th>Phase 5 latency time (s)</th>
<th>Phase 5 duration time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTZ</td>
<td>4.41 ± 0.52</td>
<td>27.19 ± 2.19</td>
<td>3.33 ± 0.86</td>
<td>4.11 ± 0.48</td>
</tr>
<tr>
<td>PTZ + VA</td>
<td>3.66 ± 1.17</td>
<td>9.50 ± 1.55 *</td>
<td>2.12 ± 0.60</td>
<td>2.15 ± 0.45 *</td>
</tr>
<tr>
<td>PTZ + PCP</td>
<td>5.81 ± 0.55</td>
<td>9.72 ± 0.74 *</td>
<td>4.75 ± 0.90</td>
<td>4.15 ± 0.64</td>
</tr>
<tr>
<td>PTZ + PCP1</td>
<td>5.51 ± 0.69</td>
<td>10.11 ± 1.70 *</td>
<td>_</td>
<td>_</td>
</tr>
</tbody>
</table>

$*P < 0.05$ indicates significant differences as compared to PTZ-kindled group. N=10 in each group

Effects of PCP and PCP1 on the biochemical indexes of stress oxidative and antioxidant

Table 3 indicates the brain levels of biochemical factor changes that are usually indices of stress oxidative in tissues, among kindled and non-kindled groups with or without pretreatment with valproate, PCP and PCP1. The NO level of brain tissues in none of PTZ and PCP groups did not show any significant differences relative to controls. In addition, PCP could not change NO level in the brain tissue of PTZ-induced mice. Nonetheless, valproate and PCP1 had significant reductive effect on NO level of brain tissues in comparison with the control group mice ($P<0.01$). In addition, a reductive effect on NO level in valproate and PCP1 pretreated animals’ brain tissue relative to kindled mice was also observed ($P<0.05$ and $P<0.01$ respectively). PTZ-induced kindling has increased the MDA level in the brain tissue of kindled mice relative to control group significantly ($P<0.05$). Pretreatment with valproate, PCP and PCP1 had not any significant effect on PTZ-induced MDA increments. However, the significant reductive effect of PCP on the SOD level in the brain was also observed ($P<0.05$) as compared to the control mice. Pre-
Brain levels of NO, MDA and SOD are compared in five groups. *$P < 0.05$ and **$P < 0.01$ indicate significant differences as compared to the control group. $P < 0.05$ and $$P < 0.01$ show significant differences as compared to the PTZ-kindled group. In each group $n$ is equal to 10.

### Discussion

Our results indicate that administration of PCP1 can diminish the intensity, improvement and duration time of PTZ-induced seizure especially in the reduction of phase 2 duration time. Meanwhile, PCP1 can yield a marked antiepileptic effect in its challenge dose. However, PCP did not have a reductive effect on seizure intensity in kindled mice. These results are in accordance with previous results about antiepileptic effects of non-competitive NMDA receptor antagonists, especially phencyclidine (29). One of the PTZ-induced seizure mechanisms is attributed to the activation of NMDA receptor, especially phencyclidine (30). At normal resting potentials, NMDA receptors are conducting channels for Na$^+$ and Ca$^{2+}$ and can blocked by magnesium ion (1). PCP and its derivatives are non-competitive antagonists of NMDA receptor and by binding to the receptor, block Na$^+$ and Ca$^{2+}$ channels (6). It is reported that the action site of phencyclidine on the NMDA receptors is distinct from glutamate ligand and ligands of PCP receptors (31). It is observed that NMDA receptor agonists have high affinity to the PCP receptor (32). Therefore, probably PCP and PCP1 have anti-seizure effect by blocking the NMDA receptor via Na$^+$ and Ca$^{2+}$ channel inhibition, and in this way PCP1 is more powerful than PCP.

Our study has shown a non-significant increase in the NO level of PTZ-kindled mice. In addition, PCP1 compensated the increasing effect of PTZ on the NO level, showing a reduction in the NO level in the PTZ kindled mice in comparison with control and PTZ groups significantly. PTZ-induced seizure is caused by activation of glutamate NMDA receptor. This activation induces Ca$^{2+}$ influx and consequently NO is produced (19, 33). In parallel with this report, it is observed that glutamate, by activation of NMDA receptor has an important role in seizure induction that is accompanied by NO synthesis (34). Therefore, possibly PCP1 as a powerful antagonist of NMDA receptor is able to inhibit PTZ-induced seizure due to suppressing of NO synthesis. However, in a research in contrast with our study, PTZ-induced kindling reduced NO level, and the researchers justify this response based on the proconvulsant effect of NO (19). Therefore, the role of NO in the pathophysiology of seizure induction is not exactly clear and more research should be carried out on it.

MDA is a final product of lipid peroxidation and its increased level in the tissue indicates the induction of oxidative stress and free radical
generation (35). It is reported that PTZ-induced kindling has an increasing effect on MDA level that is in consistence with our results (3, 36). However, in our study, PCP and its derivative could not change the PTZ-induced increased level of MDA in the brain tissue, so either piperidines are not involved in the inhibition of oxidative stress, or the dosage and other parameters of our study and Jain’ report are not the same. Furthermore, as it is reported earlier (37), although there is an overlap between NMDA and PCP receptors, yet all of PCP receptors do not work exactly coupled with NMDA receptors and thereby NMDA antagonists are not able to act exactly at the same site of the NMDA receptor.

SOD activity in all groups except for valproate and PCP-treated group, has non-significant reduction relative to the control group. Interestingly, PCP had an accumulative effect on the diminishing effect of PTZ on the SOD activity in PTZ-induced kindled mice. It means that PCP and PCP1 are unable in reducing stress oxidative and antioxidant enzyme in PTZ-kindled mice. Moreover, PCP1 is acting similar to PTZ on the brain tissue. Therefore, considering our results on MDA level and SOD activity one can conclude that possibly piperidins may affect the seizure in a different way from stress oxidative and lipid peroxidation inhibition. In consistence with our report, SOD enzyme activity has not been changed in all areas of the brain (38).

However, in contrast to our study, Ilhan and colleagues observed that brain SOD activity had decreased in PTZ-induced kindled mice (3). In addition, it has also been observed that PTZ administration at a single convulsive dose reduced the SOD activity and increased lipid peroxidation, therefore suppressing the antioxidant defence systems (39). Anyway, the unchanged SOD activity in our study and Erakovic’ work might be explained by their hypothesis that oxidative stress caused simultaneous up-regulation of SOD and enzyme degradation (38). Considering the increased levels of NO and MDA and the decreased level of SOD in PTZ-induced kindled mice in our result, and with regard to the previous hypothesis about epilepsy (40), one can conclude that free radical generation and stress oxidative are among the most reasonable explanations of the mechanism by which epilepsy happens. In addition, PCP1 is more efficient than PCP in reducing PTZ-induced kindling via antioxidant effect.

Conclusion

A new derivative of the PCP has a marked anticonvulsant effect in epilepsy. Furthermore, PCP1 is more effective than PCP in suppressing convulsion and probably does this via the antioxidant effect.

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Zahra Kiasalari, et al. 147


