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Neuropharmacological effects of carvacryl acetate on δ-aminolevulinic dehydratase, Na^+, K^+-ATPase activities and amino acids levels in mice hippocampus after seizures

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ABSTRACT

Epileptic syndromes are highly prevalent neurological conditions and can often be disabling. In order to find an alternative for treatment, this study evaluated anticonvulsant effects of carvacryl acetate (CA), a derivative of monoterpene carvacrol, after seizures induced by pilocarpine (P400), picrotoxin (PIC) or pentylenetetrazol (PTZ). We also analyzed the CA effects on Na^+, K^+-ATPase and δ-aminolevulinic acid dehydratase (δ-ALA-D) activities in hippocampus mice after seizures induced by P400, PIC or PTZ. In addition, glutamate, δ-aminobutyric acid (GABA), glutamine and aspartate levels in mice hippocampus treated with CA after seizures induced by P400, PIC or PTZ were also measured. CA produced anticonvulsant effects against seizures induced by P400, PIC or PTZ, and its effects were reversed by flumazenil, suggesting that action mechanism can be mediated by GABAergic system. CA increased GABA levels, but did not alter glutamate and aspartate concentrations in mice hippocampus after seizures induced by P400, PIC or PTZ when compared with seizures induced by P400, PIC or PTZ ($p < 0.05$), respectively, as well as decreased glutamine content in mice hippocampus after seizures induced by PIC when compared with seizures induced by PIC ($p < 0.05$). In addition, CA also increased Na^+, K^+-ATPase and δ-aminolevulinic acid dehydratase activities after seizures induced by P400, PIC or PTZ when compared with seizures induced by P400, PIC or PTZ ($p < 0.05$), respectively. This study demonstrated that CA could be a future therapeutic option for treatment of epilepsy, with a multifactorial brain action mechanism.

1. Introduction

Epilepsy is characterized by recurrent spontaneous seizures caused by abnormal discharges and corresponds to the most common serious neurological disorder [1]. In spite of a large arsenal of antiepileptic drugs, approximately 30% of patients are refractory to treatments [2]. Benzodiazepines are often used acutely in emergency units to control seizures, but, chronically, might develop physical and chemical dependence [3].

Seizures induced by pilocarpine [4–6], pentylenetetrazole [7] or picrotoxin [8,9] have been widely investigated to test new anticonvulsant agents. Thus, this study evaluated anticonvulsant properties of carvacryl acetate (CA), an acetylated derivative of carvacrol, which has already showed anticonvulsant effects [10].

CA has already suggested a Central Nervous System (CNS) action, as anxiolytic-*like* properties, probable mediated via GABAergic system. Thus, the authors tested if the probable

Abbreviations: CA, carvacryl acetate; DZP, diazepam; FLU, flumazenil; GABA, δ-aminobutyric acid; PIC, picrotoxin; PTZ, pentylenetetrazol; P400, pilocarpine; δ-ALA-D, δ-aminolevulinic acid dehydratase.

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anticonvulsant effects of CA could be mediated via GABAergic system. For this purpose, flumazenil was used as a GABAergic antagonist to clarify the probable action mechanism of action anticonvulsant [4,11].

Regarding that the pathophysiology of epilepsy may be correlated with impairments in δ-ALA-D and Na^+, Ka^+-ATPase function [12,13], we also analyzed if CA must improve those enzyme functions after seizures induced by pilocarpine, pentylenetetrazole or picrotoxin. It is important to note that some molecules derived from plants have already suggested not only anticonvulsant effects, but also antioxidant properties. Similarly, CA produced antioxidant effect in previous study [17]. These findings may, therefore, confer an advantage to use derivatives of natural products rather than drugs which have only anticonvulsant effects [4,5,7,9,10].

For the purposes, we evaluated carvacryl acetate effects after seizures induced by P400, PTZ [9], and PIC in mice, as well as, we measured the amino acid levels (glutamate, GABA, aspartate and glutamine) in mice hippocampus after seizures induced by P400, PTZ or PIC [18,19].

These three epilepsy models were applied in order to increase sensitivity to suggest clinical anticonvulsant activity, which was characterized by latency to first seizure, number of seizures and mortality rate. These models were also used to detect alterations in amino acid levels and enzymatic functions (Na^+, K^+-ATPase and δ-aminolevulinic acid dehydratase (δ-ALA-D) [4–9]. In this study, for example, there were occasions in which only one of these models showed sensitivity to detect amino acids alterations.

2. Material and methods

2.1. Preparation of carvacryl acetate

CA is chemically defined as ethyl 5-isopropyl-2-methyl-phenyl, obtained with 98% purity, molar mass of 192.26 g/mol, refractive index of 1497, boiling point of 94.56 °C at 760 mmHg; 48,414 kJ/mol of vaporization enthalpy. Its color is yellow–green. It has a pungent and astringent flavor, with a characteristic odor like oregano (*Origanum vulgare* L.). CA is found in liquid state at ambient temperature, with density of 0.994 ± 0.06 g/cm^3. It was obtained by carvacrol acetylation in which acetic anhydride was used as acylating agent and pyridine as catalyst. There were added carvacrol (5 g; 0.033 mol), pyridine (7.5 ml) and acetic anhydride (12.5 ml), in a flask (50 ml) equipped with magnetic stirrer, coupled to a Friedrich condenser in an inert atmosphere. Then, the solution was subjected to magnetic stirring and under constant reflux for 24 h [11].

Reaction mixture was poured into ice water (60 ml). The reaction product was extraction in a dropping funnel and chloroform was used as solvent extractor (3 times of 60 ml). Chloroform phases were combined and washed with saturated copper sulfate (3 times of 60 ml) and was also washed with water (3 times of 60 ml) and dried with Na_2SO_4 anhydrous. Subsequently, the solvent was evaporated in a rotary evaporator. Reaction product was subjected to column chromatography using silica gel as stationary phase and a mixture of hexane and ethyl acetate (95:5), as mobile phase. There were obtained 4.779 g (0.025 mol) of carvacryl acetate with 76% yield [20,21].

The confirmation of chemical structure of CA (Fig. 1) was performed by infrared spectroscopic data, ^{1}H NMR and ^{13}C NMR DEPT: IR (4000–400 cm^{-1}): 3050; 2950; 2850; 1750; 1500; 850. ^{1}H NMR (200 MHz, $CDCl_3$): 7.20 (d, *J* = 7.80 Hz, 1H); 7.00 (d, *J* = 7.80 Hz, 1H); 6.90 (s, 1H); 2.95–2.75 (m, 1H); 2.30 (s, 3H); 2.15 (s, 3H); 1.26 (d, J = 6.80 Hz, 6H); ^{13}C NMR DEPT (50 MHz-$CDCl_3$): 169.1; 149.1; 147.9; 130.7; 127.0; 124.0; 119.6; 67.3; 33.4; 23.7; 20.6; 15.6.

Subsequently, CA was emulsified with 0.05% Tween 80 (Sigma Chem. Co., St. Louis, Missouri, USA), dissolved in distilled water (vehicle) and administered intraperitoneally (i.p.) at dose 100 mg/kg. This dose was selected in accordance with CA doses used in previous studies conducted in our laboratory [11,17,22]. Note that doses inferior of 100 mg/kg were tested in three epilepsy models, but they were not statistically significant when compared with negative controls in this study (*data not shown*).

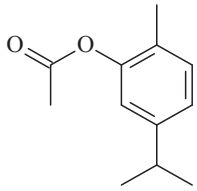

Fig. 1. Chemical structure of carvacryl acetate (ethyl 5-isopropyl-2-methyl-phenyl).

The other chemicals used in these experiments were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All doses were administered at 10 ml/kg and injected i.p.

2.2. Animals

Male *Swiss* albino mice (25–30 g; 2 months old) were used in experiments. They were maintained at a controlled temperature (25 ± 2 °C) and randomly placed in acrylic cages (maximum of six animals *per* cage) and 12/12 h light–dark cycle (light phase: 6–18 h), with free access to water and food (Purina®). Different groups were used for each test. All experiments were conducted between 8:00 and 10:00 AM, in a room with no noise.

The animals were observed during 24 h under similar ambient conditions. All experiments were previously approved by Ethics Committee on Animal Experimentation of Federal University of Piauí (# 013/2011).

In epilepsy models, seizures were identified according with previous experiments in our laboratory, which reproduces behavioral and electroencephalographic alterations which are similar to those of human temporal lobe epilepsy. Behavioral alterations were closely observed during 24 h, and included akinesia, ataxic lurching, peripheral cholinergic signs (miosis, piloerection, chromodacryorrhea, diarrhea and masticatory automatisms), tremors, staring spell, facial automatisms, stereotyped movements (continuous sniffing, paw licking, rearing and wet dog shakes that persisted for 10–15 min) and motor limbic seizures, which develop progressively within 1–2 h into *status epilepticus* [15,23,24].

2.3. Pilocarpine-induced seizures

Groups were used as follows: group 1 – vehicle (0.05% Tween 80 dissolved in distilled water, i.p., negative control; *n* = 10); group 2 – vehicle (i.p.) and, 30 min later, administration of P400 (i.p. *n* = 10; P400 group); group 3 – CA 100 mg/kg i.p. (CA100) and, 30 min later, administration of P400 (i.p., *n* = 10, CA100 plus P400); group 4 – CA100 and, 30 min later, administration of vehicle (i.p., *n* = 10, CA100 group); group 5 – diazepam 5 mg/kg (i.p., DZP5) and, 30 min later, administration of P400 (i.p., positive control, *n* = 10, DZP5 plus P400 group); group 6 – vehicle (i.p.) and, 30 min later, administration of DZP 5 mg/kg (*n* = 10; DZP5 group); group 7 – Flumazenil 5 mg/kg (i.p. FLU5) and, 15 min later, DZP5 was administered. And thirty minutes after DZP5 administration, P400 was administered (i.p., *n* = 10; FLU5 plus DZP5 plus P400 group); group 8 – FLU5 and, 15 min later, CA100 was administered. And thirty minutes after CA administration, P400 was administered (i.p., *n* = 10, FLU5 plus CA100 plus P400 group); and group 9 – vehicle (i.p.) and, 30 min later, they received administration of flumazenil 5 mg/kg (*n* = 10; FLU5 group) [6].

2.4. Pentylenetetrazol-induced seizures

PTZ (60 mg/kg; i.p.) was used to induce tonic–clonic seizures [22]. Groups were used as follows: group 1 – vehicle (0.05% Tween 80 dissolved in distilled water, ip, negative control, $n = 10$); group 2 – vehicle (i.p.) and, 30 min later, administration of PTZ (i.p., $n = 10$, PTZ group); group 3 – CA100 and, 30 min later, administration of PTZ (i.p., $n = 10$, CA100 plus PTZ group); group 4 – CA100 and, 30 min later, administration of vehicle (i.p., $n = 10$, CA100 group); group 5 – DZP5 and, 30 min later, administration of PTZ (i.p.; positive control, $n = 10$, DZP5 plus PTZ); group 6 – vehicle (i.p.) and, 30 min later, administration of DZP5 ($n = 10$, DZP5 group); group 7 – Flumazenil 5 mg/kg (i.p., FLU5) and, 15 min later, DZP5 was administered. And thirty minutes after DZP5 administration, PTZ was administered ($n = 10$; FLU5 plus DZP5 plus PTZ group); group 8 – FLU5 and, 15 min later, CA100 was administered. And thirty minutes after CA administration, PTZ was administered ($n = 10$; FLU5 plus CA100 plus PTZ group); and group 9 – vehicle (i.p.) and, 30 min later, they received administration of flumazenil 5 mg/kg ($n = 10$, FLU5 group).

2.5. Picrotoxin-induced seizures

This method has already been previously described [24–26]. Groups were used as follows: group 1 – vehicle (0.05% Tween 80 dissolved in distilled water, i.p., negative control, $n = 10$); group 2 – vehicle (i.p.) and, 30 min later, administration of PIC (i.p., $n = 10$, PIC group); group 3 – CA100 and, 30 min later, administration of PIC (i.p., $n = 10$, CA100 plus PIC group); group 4 – CA100 and, 30 min later, administration of vehicle (i.p., $n = 10$, CA100 group); group 5 – DZP5 and, 30 min later, administration of PIC (i.p., positive control, $n = 10$, DZP5 plus PIC group); group 6 – vehicle (i.p.) and, 30 min later, administration of DZP5 ($n = 10$, DZP5 group); group 7 – FLU5 (flumazenil 5 mg/kg, i.p.) and, 15 min later, DZP5 was administered. Thirty minutes after DZP administration, PIC was administered (i.p., $n = 10$, FLU5 plus DZP5 plus PIC group); group 8 – FLU5 and, 15 min later, CA100 was administered. And 30 min after CA administration, PIC was administered (i.p., $n = 10$; FLU5 plus CA100 plus PIC group); and group 9 – vehicle (i.p.) and, 30 min later, they received administration of flumazenil 5 mg/kg (i.p., $n = 10$, FLU5 group).

Due to be recognized as an antiepileptic drug, DZP was used as a positive control to compare with CA [3]. FLU was used as a GABAergic antagonist drug to test if CA exerted their action mechanism by GABAergic system [11,27].

2.6. Locomotor activity and motor coordination

Vehicle (0.05% Tween 80 dissolved in 0.9% saline) was used as negative control and diazepam 1 mg/kg (i.p.; DZP1), was used as positive control. CA (100 mg/kg; i.p.) formed the experimental group. The spontaneous locomotor activity of animals was examined in a cage of 50 cm × 50 cm × 50 cm, 30 min after drugs administration [28]. Behavioral tests were performed ($n = 10$ mice per group) to evaluate its effects on spontaneous locomotor activity (number of squares entered during 5 min) in open-field test [29] and motor coordination (ability of the mice to remain on the rota rod during 180 s and number of falls – up to three falls – were registered) in rota rod test [7].

2.6.1. Open-field test

The open-field arena was made of acrylic (transparent walls and black floor, 30 × 30 × 15 cm) divided into nine squares of equal areas [30]. Groups ($n = 10$) were treated with vehicle (0.05% Tween 80 dissolved in distilled water, i.p.), DZP1 (1 mg/kg; i.p.) or CA (100 mg/kg; i.p.). Mouse was placed individually into the apparatus and number of entries with four legs on each square (spontaneous locomotor activity) was measured during 5 min.

2.6.2. Rota rod test

In rota rod test, we evaluated motor coordination and muscle relaxation produced by drugs in animals [31]. The mice were trained before the experiment to acquire the capacity to remain for 180 s on a diameter rod, rotating at 17 rpm [29]. Two or three trials were sufficient for the animals to learn this task. The compounds were tested alone in mice to assess the ability to reproduce the performance on the next morning. DZP1 (1 mg/kg; i.p.), CA (100 mg/kg, i.p.) or vehicle (0.05% Tween 80 dissolved in distilled water, i.p.) was administered in each of the experimental groups ($n = 10$). Then, animals were placed in the four paws on the rotating bar, which has 2.5 cm in diameter is 25 cm high from the floor, and observed for 3 min. The ability of mice to remain on the rota rod for 180 s and number of falls (up to three drops) were recorded.

2.7. Evaluation of anticonvulsant effects

After seizures induced by P400, PTZ or PIC, the animals were placed in chambers of 30 × 30 cm and observed for 24 h to record the latency to first seizure (tonic–clonic seizures during 30 min intermittently), number of animals, which had seizure and number of animals which died after seizures.

Phenotypes of animals that survived 24 h after administration of P400, PTZ or PIC were selected. According with protocols described in previous studies conducted by our group, the animals, which survived were euthanized by decapitation and their brains were dissected on ice to remove the hippocampus of both hemispheres for determination of enzymatic activities and amino acid levels [4,14].

2.8. Preparation of synaptic plasma membrane of mice hippocampus

Hippocampi were homogenized in 1:10 volume (w/v) of 0.32 M sucrose solution containing 5.0 mM HEPES and 0.1 mM EDTA, pH 7.4. Synaptic plasma membranes were prepared according to the method described by Jones and Matus (1974) [32]. These membranes were isolated using a discontinuous density gradient of sucrose, consisting of successive layers of 0.3; 0.8 and 1.0 M. After centrifugation at 69,000×g for 2 h, the fraction between 0.8 and 1.0 M sucrose interface were used for the preparation of the enzyme membrane.

2.9. Determination of Na^+, K^+-ATPase activity

The reaction mixture for the assay of Na^+, K^+-ATPase contained 5.0 mM of $MgCl_2$, 80.0 mM of NaCl, 20.0 mM of KCl and 40.0 mM of Tris–HCl, pH 7.4 in a final volume of 200 ml. The reaction was started by addition of ATP to a final concentration of 3.0 mM. Controls were conducted using the difference between the two samples, as described by Wyse et al. [33]. The released inorganic phosphate (Pi) was measured by the method described by Chan et al. [34]. The specific activity of the enzyme was expressed as nmol of Pi released per minute per mg of protein.

2.10. Determination of δ-ALA-D activity

The activities of δ-ALA-D in mice hippocampus from all groups were measured by the rate of product formation of porphobilinogen [35]. An aliquot of 200 μl of synaptic plasma membranes obtained from each sample was incubated for 3 h at 37 °C. Enzymatic reaction was initiated by addition of substrate (δ-ALA-D) to a final concentration of 2.2 mM in a medium containing 45 mM

phosphate buffer, pH 6.8. Reaction was stopped by adding 250 μl of trichloroacetic acid10% with 10 mM of $HgCl_2$ and reaction product was determined using modified Ehrlich's reagent at 555 nm. Reaction was typically linear with respect to protein concentration and time of incubation. Enzyme activity was expressed as nmol of PBG/mg of protein/h. Protein concentration was measured by the method described by Lowry et al. [36].

2.11. Evaluation of amino acids levels in mice hippocampus

Animals which survived in the period of 24 h during the seizure experimental protocols were euthanized by decapitation for removal of the hippocampus to assess the amino acids levels (GABA, glutamate, glutamine and aspartate).

Hippocampi were sonicated in $HClO_4$ for 30 s and centrifuged for 15 min in a refrigerated centrifuge at 15,000 rpm. Supernatant was separated and filtered through a membrane filter (Millipore, 0.2 mM) and combined with a solution of pre-column derivatization, to obtain fluorescence in a ratio of 1:1. One minute after the start of this association, a rate of 20 μl was removed and injected into the HPLC equipment for chemical analysis.

For amino acid analysis, CLC-ODS column (*M*) with a length of 15 cm in diameter and 4.6 mm particle diameter of 3 microns Shimadzu, Japan was used. The mobile phase was used in a gradient using two phases: A – NaH_2PO_4 (50 mM) and methanol (20% v/v) at pH 5.5, B – Pure methanol (100%). Aspartate, glutamate, glutamine and GABA were detected using a fluorescence detector (Model RF-535 from Shimadzu, Japan) with wavelengths of EX-Wavelength (370 nm) and MS-wavelength (450 nm). The chromatograms were recorded and quantified by a computer using software from Shimadzu.

The amount of amino acid levels was calculated by comparing the peak height obtained from the means of the patterns and the results were expressed in μmol/g of tissue [19].

2.12. Statistical analyzes

The data obtained on latency to first seizure and neurochemical changes were evaluated by Fisher's test followed by *t-Student–Newman–Keuls* as *post hoc* test. Percentage of tonic–clonic limbic seizures and deaths were evaluated by Fisher's test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Carvacryl acetate effects after pilocarpine-induced seizures

In CA100 + P400 group, the number of mice which died was reduced in 60% when compared with P400 group ($p < 0.001$). A reduction in percentage of seizures of 60% in CA100 + P400, when compared with P400 group ($p < 0.001$) and an increase of 340.55% in latency to first seizure in CA100 + P400 when compared with P400 ($p < 0.001$, Table 1).

In addition, FLU + CA100 + P400 group had a reversal of these three parameters when compared with CA100 + P400. The percentage of mice which died and the number of animals which had seizures in FLU + CA100 + P400 group were 100% and latency for first seizure was reduced by 76.59% when compared with CA100 + P400 ($p < 0.05$) (Table 1).

3.2. Carvacryl acetate effects after pentylenetetrazol-induced seizures

In CA100 + PTZ group, number of deaths was decreased (30% of the mice in this group died when compared with 100% in PTZ ($p < 0.0001$)). There was a reduction in percentage of seizures for 30% in CA100 + PTZ when compared with 100% of PTZ ($p < 0.0001$). An increase of 345.96% on latency for first seizure for the CA100 + PTZ was observed when compared with PTZ ($p < 0.0001$). In FLU + CA100 + PTZ group, there was a reversal of the three measured parameters when compared with CA100 + PTZ. The percentage of deaths on FLU + CA100 + PTZ was 100%; the percentage of seizure was 100% and the latency for first seizure was reduced by 77.38% when compared with CA100 + PTZ ($p < 0.0001$) (Table 2).

Table 1
Carvacryl acetate effects on pilocarpine-induced seizures in mice.

Treatments	Latency to first seizure (min)	% of seizures	% of death
Vehicle	–	0	0
P400	8.36 ± 0.34	100	100
CA100 + P400	36.83 ± 2.08[a]	60[d]	60[d]
CA100	–	0	0
DZP 5	–	0	0
DZP 5 + P400	31.62 ± 1.04[a]	40[d]	40[d]
FLU 5	–	0	0
FLU 5 + DZP 5 + P400	8.74 ± 0.42[b]	100[e]	100[e]
FLU 5 + CA100 + P400	8.62 ± 0.47[c]	100[f]	100[f]

Values are mean ± S.E.M. to 10 mice per group.
[a] $p < 0.001$ when compared with P400 group.
[b] $p < 0.001$ when compared with DZP 5 + P400.
[c] $p < 0.05$ when compared with CA100 + P400 (Fisher's test followed by *t-Student–Newman–Keuls* as *post hoc* test).
[d] $p < 0.001$ when compared with P400.
[e] $p < 0.001$ when compared with DZP 5 + P400.
[f] $p < 0.05$ when compared with CA100 + P400 (Fisher's test). Subtitles: CA: carvacryl acetate; P400: pilocarpine 400 mg/kg i.p.; DZP: diazepam; FLU: flumazenil.

3.3. Carvacryl acetate effects after picrotoxin-induced seizures

In CA100 + PIC, there was a reduction in number of deaths (50% of mice died when compared with 100% in PIC group ($p < 0.001$)). There was a reduction in percentage of seizures to 50% in CA100 + PIC when compared with 100% in the PIC group ($p < 0.001$). An increase of 163.67% on latency for first seizure in CA100 + PIC was observed, when compared with PIC ($p < 0.001$). In FLU + CA100 + PIC, there was a reversal of three measured parameters when compared with CA100 + PIC ($p < 0.001$). Percentage of deaths in FLU + CA100 + PIC was 100%; the percentage of seizure was 100% and the latency for first seizure was reduced by 61.83% when compared with CA100 + PIC ($p < 0.001$) (Table 3).

Table 2
Carvacryl acetate effects on pentylenetetrazol-induced seizures in mice.

Treatments	Latency for the first seizure (min)	% of seizures	% of death
Vehicle	–	0	0
PTZ	77.34 ± 1.65	100	100
CA100 + PTZ	344.91 ± 11.90[a]	30[d]	30[d]
CA100	–	0	0
DZP 5	–	0	0
DZP 5 + PTZ	795.12 ± 17.67[a]	30[d]	30[d]
FLU 5	–	0	0
FLU 5 + DZP 5 + PTZ	77.25 ± 1.70[b]	100[e]	100[e]
FLU 5 + CA100 + PTZ	78.03 ± 1.75[c]	100[f]	100[f]

Values are mean ± S.E.M. to 10 mice per group.
[a] $p < 0.0001$ when compared with PTZ.
[b] $p < 0.0001$ when compared with DZP5 + PTZ.
[c] $p < 0.0001$ when compared with CA100 + PTZ (Fisher's test followed by *t-Student–Newman–Keuls* as *post hoc* test).
[d] $p < 0.0001$ when compared with PTZ.
[e] $p < 0.0001$ when compared with DZP5 + PTZ.
[f] $p < 0.0001$ when compared with CA100 + PTZ (Fisher's test). Subtitles: CA: carvacryl acetate; PTZ: pentylenetetrazole; DZP: diazepam; FLU: flumazenil.

Table 3
Carvacryl acetate effects on picrotoxin-induced seizures in mice.

Treatments	Latency for the first seizure (min)	% of seizures	% of deaths
Vehicle	–	0	0
PIC	479 ± 6.97	100	100
CA 100 + PIC	1263 ± 10.12[a]	50[d]	50[d]
CA 100	–	0	0
DZP 5	–	0	0
DZP 5 + PIC	1288 ± 9.81[a]	30[d]	30[d]
FLU 5	–	0	0
FLU 5 + DZP 5 + PIC	482 ± 6.35[b]	100[e]	100[e]
FLU 5 + CA 100 + PIC	483 ± 7.14[c]	100[f]	100[f]

Values are mean ± S.E.M. to 10 mice per group.
[a] $p < 0.001$ when compared with PIC.
[b] $p < 0.0001$ when compared with DZP5 + PIC.
[c] $p < 0.001$ when compared with CA100 + PIC (Fisher's test followed by *t-Student–Newman–Keuls* as *post hoc* test).
[d] $p < 0.0001$ when compared with PIC.
[e] $p < 0.0001$ when compared with DZP5 + PIC.
[f] $p < 0.0001$ when compared with CA100 + PIC (Fisher's test). Subtitles: CA: carvacryl acetate; PIC: picrotoxin; DZP: diazepam; FLU: flumazenil.

In all experiments, none mice treated with CA alone showed significant behavioral alteration when compared with vehicle.

3.4. Carvacryl acetate effects on mice subjected on open field test

The mice subjected to DZP1 (DZP1: 35.75 ± 2.66) treatment ($n = 10$) showed a reduction of 53.76% in number of square entries when compared with negative control (vehicle: 79.00 ± 2.09, $p < 0.0001$). However, CA100 group (CA100: 73.75 ± 1.74) had no significant change in spontaneous locomotor activity when compared with negative control ($p > 0.05$) (Fig. 2).

3.5. Carvacryl acetate effects on mice subjected on rota rod test

Fig. 3A shows that DZP1 group ($n = 10$; DZP1: 2.7 ± 0.21) had number of falls increased by 123% when compared with negative control (vehicle: 1.20 ± 0.29; $p < 0.0001$). CA100 group ($n = 10$) did not alter this parameter when compared with negative control (CA100 1.60 ± 0.16, $p > 0.05$).

DZP1 group ($n = 10$, DZP1: 149.10 ± 1.33) had the time spent on rotating bar reduced by 18.27% when compared with negative control (vehicle: 176.60 ± 0.98, $p < 0.0001$) while CA100 group (CA100: 169.50 ± 1.20) had not statistical significance when compared with negative control ($p > 0.05$) (Fig. 3B).

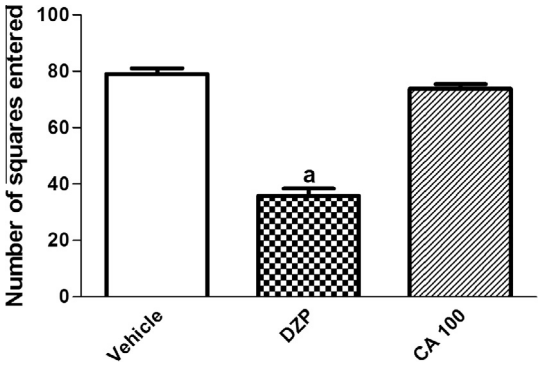

Fig. 2. Carvacryl acetate effects on number of squares entered in open field test. Values represent the mean ± the standard error of the means (S.E.M.) number of squares entered of mice ($n = 10$ per group) used in experiments. [a]$p < 0.0001$ when compared with negative control (vehicle) (Fisher's test followed by *t-Student–Newman–Keuls* as *post hoc* test). CA100: carvacryl acetate (100 mg/kg, i.p.); DZP: diazepam (1 mg/kg, i.p.).

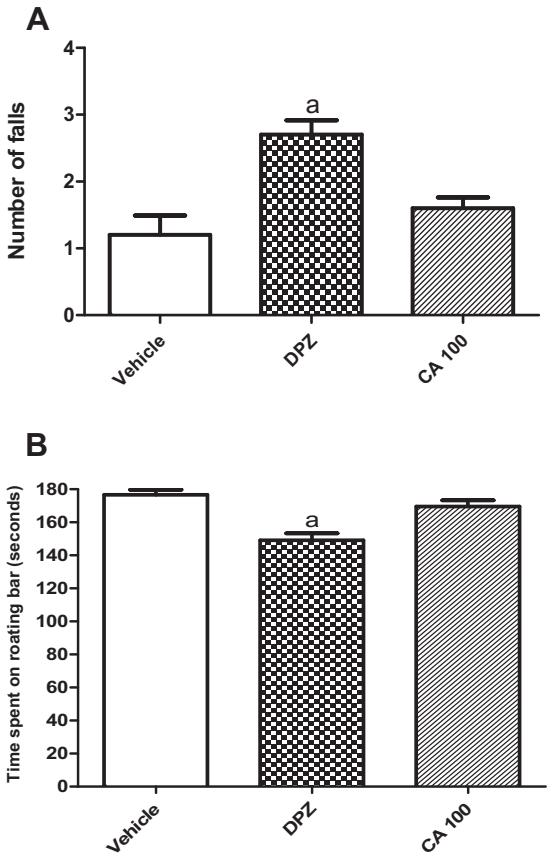

Fig. 3. Carvacryl acetate effects on number of falls (A) and on time spent on the rotating bar (B) on rota rod test. Values represent the mean ± S.E.M. of number of falls and the time spent on the rotating bar ($n = 10$ per group). [a]$p < 0.0001$ when compared with negative control (vehicle); (Fisher's test followed by *t-Student–Newman–Keuls* as *post hoc* test). CA100: carvacryl acetate (100 mg/kg, i.p.); DZP: diazepam (1 mg/kg, i.p.).

3.6. Carvacryl acetate effects on δ-ALA-D hippocampal activity after seizures

P400, PTZ and PIC groups reduced in 33%; 40.15% and 47.8% δ-ALA-D activity when compared with negative control, respectively ($p < 0.0001$). In CA100 + P400 or PIC or PTZ groups, there was increases of 47.35%; 57.24% and 83.38% in this parameter when compared with P400, or PTZ, or PIC alone ($p < 0.0001$), respectively (Fig. 4).

3.7. Carvacryl acetate effects on Na^+, K^+-ATPase activity after seizures

P400, PTZ and PIC groups showed reductions of 37%; 32.7% and 39.8%, respectively, on Na^+, K^+-ATPase activity when compared with negative control ($p < 0.0001$). In CA100 + P400 or PTZ or PIC groups, there was increases of 30.7%; 28% and 32.6% on this parameter when compared with P400 or PTZ or PIC alone ($p < 0.0001$), respectively (Fig. 5).

3.8. Carvacryl acetate effects on amino acids levels after seizures

P400, PTZ and PIC groups showed no statistical difference in GABA concentrations when compared with negative control ($p > 0.05$). However, CA100 + P400 had an increase in GABA by 78.25% when compared with P400 group ($p < 0.001$). CA100 + PTZ increased GABA by 51.43% when compared with PTZ ($p < 0.05$).

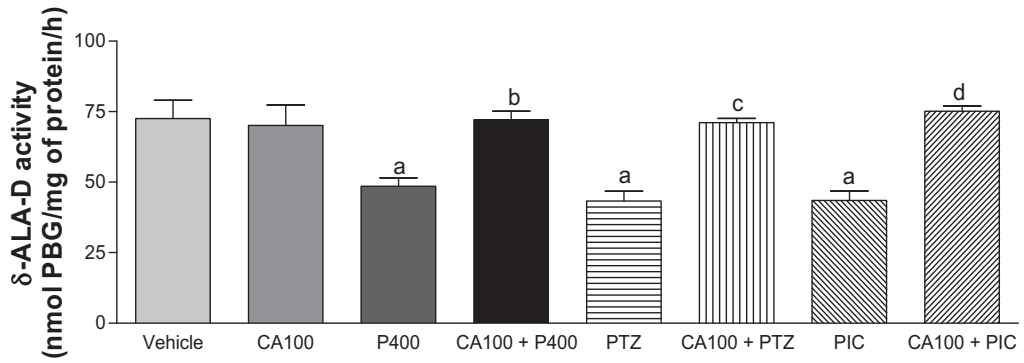

Fig. 4. Carvacryl acetate effects on δ-aminolevulinic acid dehydratase (δ-ALA-D) activity in mice hippocampus after pilocarpine-, picrotoxin- or pentylenetetrazoleinduced seizures. Differences were determined by Fisher's test followed by *t-Student–Newman–Keuls* as *post hoc* test. [a]$p < 0.0001$, when compared with negative control; [b]$p < 0.0001$, when compared with P400 group; [c]$p < 0.0001$, when compared with PTZ group. [d]$p < 0.0001$, when compared with PIC group. CA100: carvacryl acetate (100 mg/kg, i.p.); P400: Pilocarpine (400 mg/kg, i.p.); PTZ: pentylenetetrazole (60 mg/kg, i.p.); PIC: picrotoxin (7.5 mg/kg, i.p.).

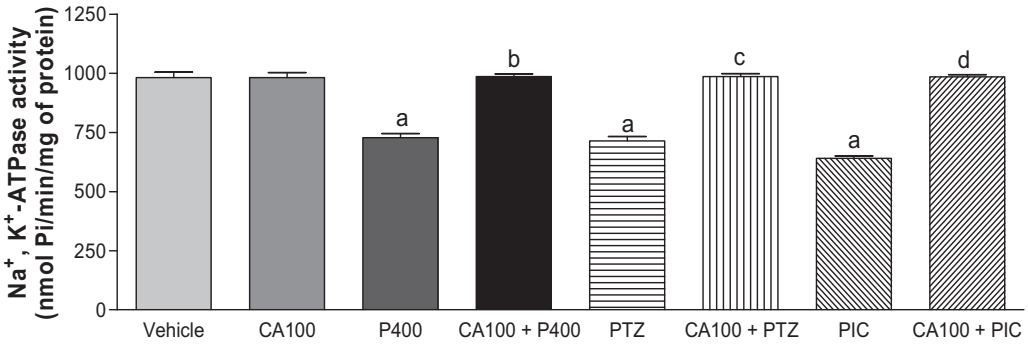

Fig. 5. Carvacryl acetate effects on Na^+, K^+-ATPase activity in mice hippocampus after pilocarpine-, picrotoxin- or pentylenetetrazole-induced seizures. Differences were determined by Fisher's test followed by *t-Student–Newman–Keuls* as *post hoc* test. [a]$p < 0.0001$, when compared with negative control; [b]$p < 0.0001$, when compared with P400 group; [c]$p < 0.0001$, when compared with PTZ; group. [d]$p < 0.0001$, when compared with PIC group. CA100: carvacryl acetate (100 mg/kg, i.p.); P400: Pilocarpine (400 mg/kg, i.p.); PTZ: pentylenetetrazole (60 mg/kg, i.p.); PIC: picrotoxin (7.5 mg/kg, i.p.).

CA100 + PIC increased GABA content in 103% when compared with PIC group ($p < 0.001$) (Fig. 6A).

There was no statistical significance when glutamate and aspartate concentrations in P400, PTZ and PIC groups were compared with negative control. CA100 + P400 or PTZ or PIC neither had statistical significance when compared with respective positive controls ($p > 0.05$) (Fig. 6B and C).

Additionally, there was no statistical significance on glutamine concentrations in P400, PTZ and PIC groups when compared with negative control, as well as when CA100 + P400 and CA100 + PTZ were compared with their positive controls. CA100 + PIC decreased by 42.8% glutamine concentration when compared with PIC ($p < 0.05$) (Fig. 6D).

4. Discussion

Benzodiazepines are one of the pharmacology option preconized in epilepsy treatment. GABAergic agonists, diazepam, act as an allosteric modulator of $GABA_A$ receptor, potentiating the γ-aminobutyric acid effect when bound to this receptor [37]. Thus, in this study, DZP was used as standard drug for comparison with CA effects after seizures induced by P400, PTZ or PIC.

Prior administration of DZP prevented the onset of seizures, increased latency for first seizure and reduced number of deaths, as expected. FLU reduced its anticonvulsant effect (Sections 3.1–3.3) [38,39]. CA also reduced number of seizures, deaths and increased latency for first seizure in all the three epilepsy models (Sections 3.1–3.3). Similarly with DZP, these effects were also inhibited by FLU. According with scientific literature, FLU is used as a GABAergic antagonist and does not induce seizures when administered alone. These findings are in accordance with our results (Tables 1–3). Using these references, we can propose that CA exert anticonvulsant effect via GABAergic system [4,27]. This effect may also be correlated, at least in part, with the anxiolytic-*like* effect of CA in another study conducted in our laboratory [11].

In the results, it should be noted that some animal groups had a discrepancy between the percentages of seizures and the percentages of deaths (Tables 1–3). It proposes that a direct relationship between these two parameters is not mandatory, meaning that CA could reduce the percentage of seizures, but not reduce the percentage of deaths proportionally.

Considering the open-field test, DZP impaired locomotor activity, reflected by reduction in number of squares entered. It also reduced motor coordination, verified by increase in number of falls and reduced time spent in the rotating bar in rota rod test [40]. However, CA100 did not change the number of squares entered in open field test. In trota rod test, the number of falls and the time spent on rotating bar were not significant changed (Sections 3.4 and 3.5). These findings propose that CA at dose 100 mg/kg did not present sedative action, nor muscle relaxing effect.

Muscle relaxing effect and reduction in motor coordination indicate in locomotor activity retardation, which is one of the main adverse effects observed during treatment with benzodiazepine. Decreases in the number of crossings on open field test suggest a muscle relaxing effect. And increases in number of falls, as well

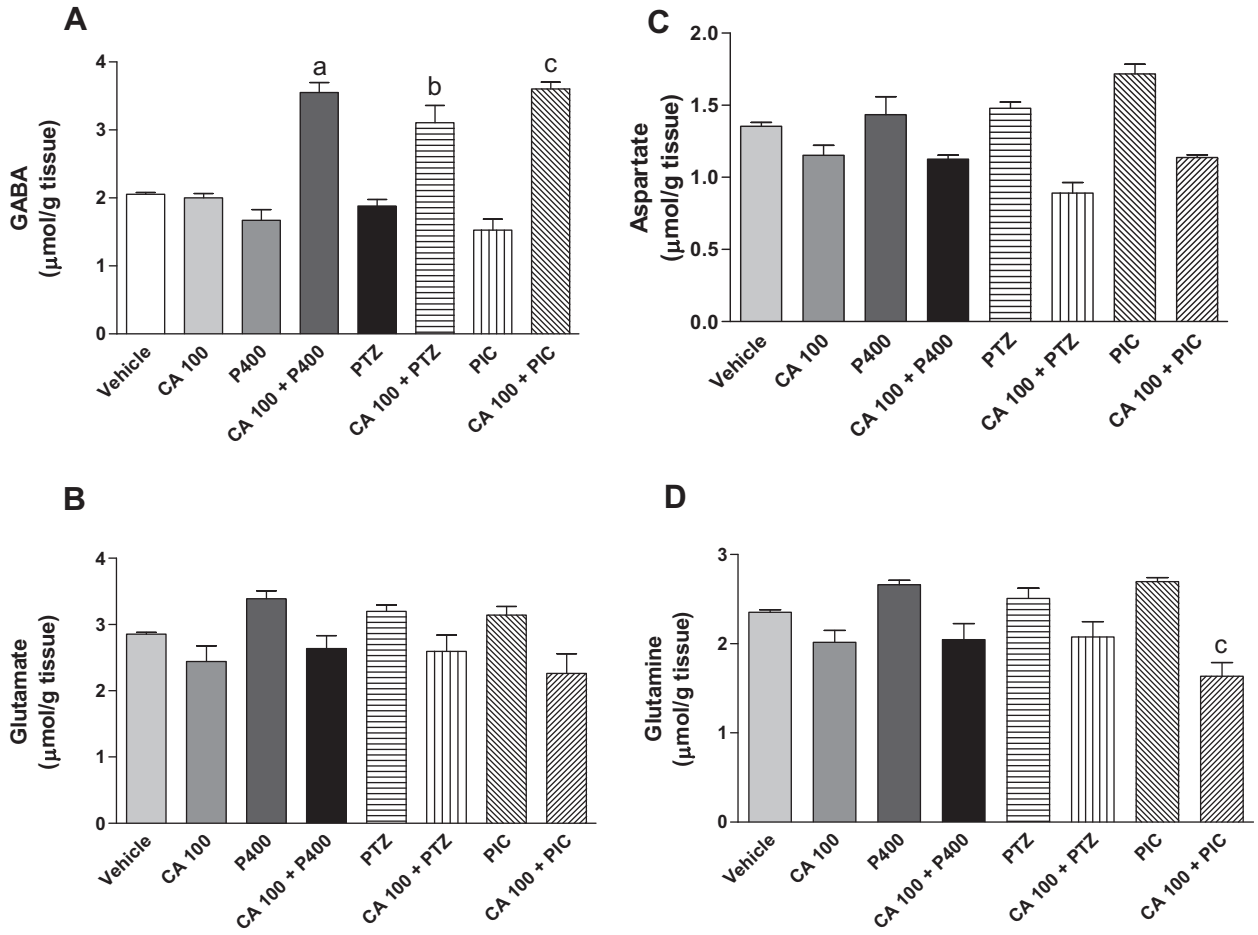

Fig. 6. Carvacryl acetate effects on δ-aminobutyric acid (GABA) (A), Glutamate (B), Aspartate (C) and Glutamine (D) levels in mice hippocampus after pilocarpine-, picrotoxin- or pentylenetetrazole-induced seizures. Differences were determined by Fisher's test followed by *t-Student–Newman–Keuls* as *post hoc* test. $^{a}p < 0.001$, when compared with P400 group; $^{b}p < 0.05$,when compared with PTZ group. $^{c}p < 0.05$, when compared with PIC group. CA100: carvacryl acetate (100 mg/kg, i.p.); P400: Pilocarpine (400 mg/kg, i.p.); PTZ: pentylenetetrazole (60 mg/kg, i.p.); PIC: picrotoxin (7.5 mg/kg, i.p.).

as decreases in time spent in rotating bar in rota rod test suggest a muscle relaxing effect and a reduction in the motor coordination [7,40]. Considering the findings of Sections 3.4 and 3.5, CA showed a potential to be more tolerated than DZP, the standard drug to treat seizures, since CA did not significantly change the parameters analyzed in open field and rota rod tests.

In neurochemical assays, we observed that CA100 improved δ-ALA-D and Na^+, K^+-ATPase activities, which was previously reduced by P400, PTZ or PIC (Sections 3.6 and 3.7). δ-ALA-D is a sulfhydryl-containing enzyme essential for aerobic organisms that catalyzes the synthesis of tetrapyrrolic compounds such as billins and hemes. δ-ALA-D contains sulfhydryl (−SH) groups and zinc, which are essential for its activity. Therefore, δ-ALA-D is inhibited by substances that compete with zinc and/or that oxidize the −SH groups and is sensitive to situations associated with oxidative stress. Furthermore, enzyme inhibition can lead to accumulation of substrate 5-aminolevulinate in the blood, which in turn can intensify oxidative stress by generating carbon-centered reactive species or by releasing iron from proteins such as ferritin [41].

Na^+, K^+-ATPase is present at high concentrations in brain cellular membranes, consuming about 40–50% of ATP generated in this tissue. It is a membrane bound enzyme known to play a pivotal role in cellular ionic gradient maintenance and is particularly sensitive to reactive species. The overproduction of free radicals may lead to the development of epileptic focus by disruption of antioxidant activity and by oxidative inactivation of Na^+, K^+-ATPase [16,42–45].

Thus, considering the findings of Sections 3.6 and 3.7, we can propose that, in addition to increase latency to first seizure, reduce the number of seizures and the number of deaths, CA also suggested a capacity to improve these two enzymatic functions, which may be involved in pathophysiology of epilepsy.

Regarding amino acid levels, it is reported that an extracellular aspartate and glutamate accumulation space can lead excitotoxicity. However, some antiepileptic drugs inhibit these excitatory amino acids and increase GABAergic action. Additionally, hippocampal glutamine levels could be increased in some epilepsy models, whereas levetiracetam reduces this increase [46–49]. CA demonstrated a similar tendency to those antiepileptic drugs, since increased GABA level in P400, PTZ and PIC models and reduced glutamine content in PIC model (Fig. 6).

However, that tendency was not observed for all the other amino acids concentration. In some occasions, only one epilepsy model had sensibility to detect alterations in some amino acid level, but the other epileptogenic substance did not. As cited above, although reduction on glutamine levels by CA100 is consistent with antiepileptic drug properties [47], this finding existed only for PIC model. It justifies, at least in part, why we applied three epilepsy models, considering that P400 and PTZ had not sensibility to detect these changes.

It has been published that glutamate, aspartate and glutamine levels are increased during seizures [46,47], instead the results obtained in this study were different, showing no increase in these amino acid levels in seizures induced by P400, PTZ and PIC when compared with negative control.

However, it seems there is not a complete consensus about these amino-acid levels in epilepsy models. On studying frontal and temporal foci, Van Gelder and co-workers found a decrease in aspartate in all the examined areas, and a decrease in glutamate levels in excitable regions [48]. Conversely, another study reported a 28% increase in aspartate concentration in surgical specimens from patients with temporal epilepsy [48,49].

Nevertheless, there are a greater number of studies showing the tendency of animal in epilepsy models suffer an increase in GABA levels when treated with anticonvulsant agents, which is in accordance with our results [46–50].

The addition of an ester group to carvacrol theoretically could confer CA an additional lipophilic property, which would allow an easier penetration on blood–brain barrier and exerts its possible therapeutic potential more safely and effectively. However, there are not any study comparing these parameters between carvacrol and CA [51]. Additionally, it is not possible to compare anticonvulsant activity of CA with carvacrol yet, since the CA doses used in our experiment were different from carvacrol doses used by Quintans-Júnior et al., 2010 [10].

This study showed significant anticonvulsant effects of CA at dose 100 mg/kg. Beyond this anticonvulsant effect, CA improved Na^+, K^+-ATPase and δ-ALA-D enzymatic activities. These findings can be correlated with the antioxidant effect suggested previously, in which CA at different concentrations and doses tested, including at dose 100 mg/kg, reduced lipid peroxidation content and nitrite levels (*in vitro* and *in vivo*) and hydroxyl radical formation *in vitro*, as well as increased reduced glutathione levels and improved glutathione peroxidase and catalase activities *in vivo* [17]. Taken all these findings together, CA could suggest multifactorial action mechanisms that can contribute to their anticonvulsant effects.

5. Conclusions

Although the anticonvulsant activity of CA was not superior to diazepam in this study, but CA did not alter locomotor activity and suggested that improve Na^+, K^+-ATPase and δ-aminolevulinic acid dehydratase activities. Some amino acid concentrations in mice hippocampus after seizures induced by P400, PTZ or PIC, which were altered by seizures, were also regulated after CA treatment. In summary, CA anticonvulsant property seems to be multifactorial, demonstrating a significant potential in epilepsy models.

Conflict of Interest

The author states no conflict of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

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