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Research Article
Published: 04 Jul, 2019

Journal of Neurology Forecast

The Inhibition of Serum Cholinesterases by *Cannabis sativa* and/or Tramadol

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Abstract

We aimed to compare serum Acetylcholinesterase (AChE) and Butyrylcholinersae (BChE) in rats treated with *Cannabis sativa* resin, tramadol or both. The extract of *Cannabis sativa* was obtained from the dried resin of the plant by chloroform treatment. Δ^9-Tetrahydrocannabinol (Δ^9-THC) content of the extract was quantified using gas Chromatography-Mass Spectrometry (GC-MS). The doses of cannabis extract were expressed as Δ^9-THC content of 5,10 or 20 mg/kg. Cannabis resin (5,10 or 20 mg/kg), tramadol (20,30 or 40 mg/kg) or cannabis resin (20mg/kg) combined with tramadol (30 or 40 mg/kg) were subcutaneously administered daily for 4 weeks. Results indicated that cannabis resin extract inhibited both AChE and BChE in serum in a dose-dependent manner. Significant decrease in serum cholinesterases was also observed after treatment with tramadol and by the cannabis-tramadol combination. A significant and positive correlation was found between serum AChE and BChE. It is suggested that this inhibition of cholinesterases in serum could be a biomarker for a neurotoxic action in individuals who abuse these drugs.

Keywords: Cannabis; Hashish; Tramadol; Brain injury; Acetylcholinesterase; Butyrylcholinersae

Introduction

Hydrolysis of the neurotransmitter acetylcholine is accomplished by two cholinesterases; acetylcholinesterase, also known as true acetylcholinesterase (EC 3.1.1.7) and butyrylcholinesterase (EC 3.1.1.8), also called or pseudo or plasma cholinesterase. Both enzymes hydrolyze acetycholine but with differing specificity and are distributed ubiquitously [1,2]. Acetylcholinesterase (AChE) is found in neuronal synapses, neuromuscular junction, and cerebrospinal fluid, on the outer membrane of erythrocytres, lymphocytes and platelets. The enzyme is important in the termination of cholinergic neurotransmission by degrading acetylcholine in the synaptic left. Butyrylcholinesterase is found together with AChE in neuronal synapses, motor endplate, muscle fibers, heart and plasma but its exact physiological function is not yet fully established [3]. Cholinesterases are target for carbamates and organophosphorus pesticides and nerve gas agents and inhibition of AChE results in accumulation of acetylcholine and cholinergic excitation with the emergence of the symptoms and signs characteristic of excessive central and peripheral cholinergic activity [4,5]. Measuring cholinesterases in plasma is widely used as a reliable measure for exposure to these chemicals and other cholinergic toxicants [6].

Cannabis sativa, the most commonly used illicit substance Worldwide [7] is well known for its recreational usage, causing mild euphoria, relaxation, a sense of well-being, and intensification of sensory experiences [8]. These effects of cannabis are mediated by its main psychoactive constituent delta-9-Tetrahydrocannabinol (Δ^9-THC) acting on cannabinoid CB1 receptors [9]. The long-term use of cannabis, however, is associated with memory problems and cognitive decline and these appear to persist after abstinence [10-12]. There are also grey matter volume changes in brain of cannabis users [13,14]. Tramadol is a centrally acting analgesic, possessing weak μ-opioid receptor agonist effect. The agent also inhibits serotonin and noradrenaline-reuptake [15]. The drug has gained recent interest in view of its popularity as a drug of abuse among adolescents [16,17]. The neurotoxic effects of tramadol are not well known, but neuronal degeneration and decreased astrocytic cells in cerebral cortex have been found in rats after 30 mg/kg of tramadol [18].

Previous studies investigating the effect of cannabis and/or tramadol on brain AChE and serum BChE found inhibitory effect for these agents on serum BChE but not on brain AChE [19]. The effect of cannabis on AChE in serum is, however, not known. The present study was therefore designed to investigate the effect of *Cannabis sativa* and/or tramadol on their ability to inhibit serum AChE

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Received Date: *08 Jun 2019*
Accepted Date: *28 Jul 2019*
Published Date: *04 Jul 2019*

Citation: *Abdel-Salam OME, Khadrawy YA. The Inhibition of Serum Cholinesterases by Cannabis sativa and/or Tramadol. J Neurol Forecast. 2019; 2(1): 1005.*

Table 1: Acetylcholinesterase (AChE) and Butyrylcholinerase (BChE) in cannabis and/or tramadol-treated rats.

	Saline	Cannabis 5 mg/kg	Cannabis 10 mg/kg	Cannabis 20 mg/kg	Tramadol 20 mg/kg	Tramadol 30 mg/kg	Tramadol 40 mg/kg	Cannabis 20 mg/ kg + tramadol 30 mg/kg	Cannabis 20 mg/ kg + tramadol 40 mg/kg
AchE	1.34 ± 0.05	1.08 ± 0.3 (-19.4%)	0.88 ± 0.09* (-34.3%)	0.87 ± 0.06* (-35.1%)	1.40 ± 0.07 (4.5%)	0.98 ± 0.09* (-26.9%)	0.80 ± 0.07*+ (-40.3%)	0.71 ± 0.07*# (-47.0%)	0.64 ± 0.08*+# (-52.2%)
BChE	956.09 ± 29.51	615.44 ± 44.55* (-35.6%)	581.37 ± 29.79* (-39.2%)	526.87± 43.87* (-45.1%)	647.23 ± 33.77* (-32.3%)	620.0 ± 28.56* (-35.1%)	608.63 ± 34.22* (-36.3%)	585.92 ± 34.68* - (-38.7%)	535.95 ± 33.12* - (-43.9%)

Units for AChE: μmol SH/ml/min. Units for BChE: U/l. Asterisks indicate significant change from saline control or from the tramadol 10mg/kg treatment group (p<0.05). The plus sign indicates significant change from the tramadol 40mg/kg treatment group. The # sign indicates significant change from the cannabis 5mg/kg treatment group. The percent inhibition from the saline control group is shown in parenthesis.

compared with BChE.

Materials and Methods

Animals

Male Sprague-Dawley rats, weighing between 130-140g were used. Rats (from Animal House of the National Research Centre, Cairo) were group-housed under temperature-and light-controlled conditions with free access to standard laboratory rodent chow and water. The experimental procedures were performed in compliance with the institutional Ethics Committee and with the guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No.85-23, revised 1985).

Drugs and chemicals

Cannabis sativa resin (hashish) and tramadol were kindly provided by the Laboratory of Forensic Sciences of the Ministry of Justice (Cairo, Egypt). Other chemicals and reagents were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A).

Preparation of cannabis resin extract

Cannabis resin extract was prepared from the dried resin of *Cannabis sativa* (Family *Cannabaceae* L). The extraction was performed using chloroform according to the method of Turner and Mahlberg [20] with modification. In brief, 10g of the resin was grounded in a mortar, subjected to oven heat (100°C) for 1h to decarboxylate all its cannabinolic acids content. The resin was extracted in chloroform overnight and then filtered. The filtrate was evaporated under a gentle stream of nitrogen, stored at 4°C and protected from light in an aluminium-covered container. One gram of the residue (dry extract) was suspended in 2% ethanol-saline. Δ^9-Tetrahydrocannabinol (Δ^9-THC) content was quantified using Gas Chromatography-Mass Spectrometry (GC-MS). The resin contained ~ 20% Δ^9-THC and 3% CBD.

Study design

Rats were randomly allocated into ten different treatment groups (six rats each). Group 1 received the vehicle (0.2 ml saline) daily. Group 2-4 received *Cannabis sativa* resin at the doses of 5,10 and 20 mg/kg, subcutaneously daily. Groups 5-7 received tramadol at doses of 20, 30 and 40 mg/kg subcutaneously daily. Groups 8, 9 received *Cannabis sativa* resin at 20 mg/kg in combination with tramadol at 30 or 40 mg/kg.

Determination of acetylcholinesterase activity

At the end of the experiments, blood samples were obtained from the retro-orbital venous plexus, under ether anaesthesia. Acetylcholinesterase activity was determined by a modification of the method of Ellman *et al* [21]. As described by Gorun *et al* [22]. The principle of the method involves measurement of the thiocholine produced as acetylthiocholine is hydrolyzed. The color was read immediately at 412nm.

Determination of butyrylcholinesterase activity

Butyrylcholinesterase activity was measured using a commercially available kit (Biodiagnostic, Egypt). Cholinesterase catalyzes the hydrolysis of butyrilthiocholine into butyrate and thiocholine. The thiocholine reacts with Dithiobis-Nitrobenzoic acid (DTNB) forming a colored compound. The increase in absorbance in the unit time at 405nm is proportional at the activity of the cholinesterase in the sample [21].

Statistical analysis

Data are expressed as mean ± SE. Data were analyzed by one-way analysis of variance, followed by Duncan's multiple range tests for post hoc comparison of group means. Correlation between AChE and BChE was done using Pearson's correlation coefficient. Effects with a probability of p<0.05 were considered to be significant.

Results

Results are shown in Table 1.

Acetylcholinesterase

Both agents significantly inhibited AChE in a dose-dependent manner, although cannabis-tramadol combination was more effective than cannabis or tramadol alone in decreasing serum AChE. Cannabis resin extract at doses of 10 or 20 mg/kg produced a significant inhibition of serum AChE (-34.3 for 10 mg/kg, -35.1% for 20 mg/kg, as compared to saline control). Meanwhile, tramadol given at 30 or 40 mg/kg caused 26.9% and 40.3% inhibition of AChE. On the other hand, treatment with cannabis 20mg/kg in combination with either 30 or 40 mg/kg tramadol resulted in more significant attenuation of AChE by -47.0% and -52.2%, respectively compared with the saline control group (Table 1).

Butyrylcholinesterase

Cannabis resin alone at a dose of 5,10 and 20 mg/kg caused significant inhibition in serum BChE activity by -35.6%, -39.2% and -45.1% compared to the saline control group. Serum BChE activity was also significantly decreased by tramadol at 20,30 or 40 mg/kg by -32.3%, -35.1%, and -36.3%, respectively and following treatment with both cannabis resin and tramadol by -38.7%, -43.9%, respectively (Table 1).

Serum AChE was positively correlated with BChE in rats treated with cannabis, tramadol or their combination (r = 0.507; p<0.001).

Discussion

In this study, we have shown that treatment with cannabis resin, tramadol or both cannabis and tramadol significantly attenuated serum AChE and BChE, suggesting an inhibitory action for the two agents on the activity of serum cholinesterases in the rat. The study thus confirms and extends our previous findings of an inhibitory effect for these gents on serum BChE [19]. In the present work,

however we investigated the effect of higher doses of tramadol alone or combined with high dose cannabis. The significance of the present findings is yet to be established. In blood, BChE activity is restricted to serum, while AChE is attached on the outer membrane of erythrocytres [23]. Because of the ease and accuracy of the assay, plasma or serum cholinesterase measurement is routinely used as a reliable marker for exposure to organophosphorus insecticides and a variety of environmental toxicants [24,25]. It is also largely acceptable that a significant inhibition in blood cholinesterases in subjects exposed to a chemical represents a potentially hazardous event and/or denotes toxicity of this chemical [2,23]. The physiological functions of serum and erythrocyte cholinesterases are unclear [23]. Serum BChE degrades drugs e.g., succinylcholine, physiostigmine, cocaine, amitriptyline, scavenges and subsequently detoxify a number of naturally occurring and synthetic anti-cholinesterases e.g., organophosphate and carbamate inhibitors [25]. A role in protection from natural and synthetic anti-cholinesterases has thus been suggested; scavenging these toxicants by BChE would protect AChE from inhibition [3,23,26]. It follows that inhibition of serum cholinesterases by drugs of abuse as shown in this study would render subjects susceptible to low concentrations of these anti-cholinesterases, and possibly enhancing their toxicity. One notable example is the link between exposure to organophosphate insecticides and the increase in the risk for developing Parkinson's disease. It is thus possible that a decrease in the activity of serum cholinesterases by these drugs of abuse could result in increased neurodegeneration in subjects exposed to insecticides. Another example is cocaine toxicity where mice lacking carboxylesterase and BChE showed increased cocaine toxicity [27]. Marijuana results in a significant increase in peak cocaine levels in plasma of recreational drug users by increasing the absorption of cocaine [28]. It could be also that inhibition of the cocaine degrading enzyme BChE in serum that accounts, at least partly, for the increase in the plasma level of cocaine by smoking marijuana. It is also worthy to mention that the depression in serum AChE and BChE does not necessarily imply the development of cholinergic toxic manifestations, nor the extent of damage to the nervous system [24] and it is inhibition of AChE at the cholinergic synapse that results in the toxicity effects seen in organophosphate and carbamate poisoning [4]. It is also suggested that organophosphorus pesticides have direct action on post-synaptic ACh receptors [29].

Several terpenoids e.g., pulegone, limonene, and limonene oxide in the cannabis plant have been reported to inhibit AChE *in vitro* [30]. Interestingly, the main cannabinoid and psychoactive constituent in cannabis, Δ^9-THC has been shown to cause competitive inhibition of AChE by binding to the anionic site of the enzyme [31]. This latter effect of Δ^9-THC could explain the unexpected finding of cannabis extract protecting against the deleterious effects of the organophosphate pesticide malathion in the rat [32], possibly by competing with malathion at the AChE enzyme.

On the other hand, drugs that inhibit cholinesterase, have therapeutic roles in several human aliments e.g., Alzheimer's disease, Down's syndrome, and myasthenia gravis. Alzheimer's disease, the most common cause of age-related dementia worldwide is associated with brain cholinergic hypofunction. Thus, drugs with cholinesterase inhibiting properties e.g., donepezil, galantamine and rivastigmine are in use in these patients with the aim to enhance the cholinergic brain function by increasing the amount of acetylcholine available for the post-synaptic acetylcholine receptors [33]. It is not clear, however, if the inhibition of serum cholinesterases by cannabis or tramadol could be extended to the brain. The effect of cannabis or Δ^9-THC on cholinergic neurotransmission is important in view of the ability of the herb or its main psychoactive constituent on memory and cognitive functions. Studies have reported variable effects for cannabis or Δ^9-THC on brain acetylcholine. Thus Δ^9-THC was reported to increase acetylcholine [34], inhibit 3H-ACh synthesis [35] or reduce acetylcholine release in rat brain [36]. Moreover, we have found increased AChE activity in rat brain after cannabis resin, but not tramadol [19]. Nevertheless, cannabis or tramadol have been shown to impair memory and to cause neuronal degeneration [18,36-39], suggesting no benefit from the alterations in brain acetylcholine by these agents. On the contrary, it is suggested that alterations in cholinergic neurotransmission by these drugs of abuse could be one factor underlying their memory impairing effects [19,38].

Funding

This research was supported by NRC Research Project number 10001004.

Competing Interests

The authors declare that they have no competing interests.

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