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Original article

Nicotine versus 6-hydroxy-L-nicotine against chlorisondamine induced memory impairment and oxidative stress in the rat hippocampus

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ABSTRACT

6-Hydroxy-L-nicotine (6HLN), a nicotine derivative from nicotine degradation by *Arthrobacter nicotinovorans* pAO1 strain was found to improve behavioral deficits and to reverse oxidative stress in the rat hippocampus. Rats were given CHL (10 mg/kg, i.p.) were used as an Alzheimer's disease-like model. The nicotine (0.3 mg/kg) and 6HLN (0.3 mg/kg) were administered alone or in combination in the CHL-treated rats. Memory-related behaviors were evaluated using Y-maze and radial arm-maze tests. The antioxidant enzymes activity and the levels of the biomarkers of oxidative stress were measured in the hippocampus. Statistical analyses were performed using two-way ANOVA and Tukey's *post hoc* test. *F* values for which $p < 0.05$ were regarded as statistically significant. CHL-caused memory deficits and oxidative stress enhancing were observed. Both nicotine and 6HLN administration attenuated the cognitive deficits and recovered the antioxidant capacity in the rat hippocampus of the CHL rat model.

Our results suggest that 6HLN versus nicotine confers anti-amnesic properties in the CHL-induced a rat model of memory impairment via reversing cholinergic function and decreasing brain oxidative stress, suggesting the use of this compound as an alternative agent in AD treatment.

1. Introduction

Alzheimer disease (AD) is a neurodegenerative affliction characterized by memory impairment [1] associated with three major changes in the brain [2]: i) the extra- and intracellular plaques deposits of β-amyloid peptide, (ii) the neurofibrillary tangles and (iii) death of forebrain cholinergic neurons and a significant decrease in acetylcholine (ACh) levels [3]. The search for neuroprotective therapeutics for AD has been a long time dominated mainly by the amyloid and the tau hypothesis. Unfortunately, both approaches failed so far to provide any efficient therapeutic strategy [2]. The involving of nicotinic acetylcholine receptors (nAChR) subtypes α7 and α4β2 in AD pathogenesis [4] has led to the proposal of a new approach [5]. The memory deficits and non-cognitive symptoms could be improved through using of the nAChR modulators to increase the availability of receptors for ACh and to overcome the death of the forebrain cholinergic neurons.

Nicotine is considered to be an agonist of nAChR. Its high potency as a cognition-enhancing agent and AD therapeutic strategy [6] has been explained not only by its ability to bind and modulate nAChRs but also through its anti-oxidant effects at low concentrations [7] and recently, through its ability to interact with β-amyloid peptide [8]. However, due to negative effects on human organs such as lungs [9] and bad publicity related to smoking[10], nicotine doesn't impose as a feasible therapeutic agent for AD.

The crystal structure determination of the nicotinic receptor binding domain homolog (nAchBP) with bound nicotine [11] followed by the recent solving of the α7 nAChRs structure [12] has spurred the interest of several academic and pharmaceutical laboratories in the possibility that new nicotinic drugs could be designed. From this point of view, nicotine derivatives are ideal candidates, offering a wide array of possibilities. The difficulty resides, firstly in the identification of molecules which have the beneficial effects of nicotine, but elude its side effects [13] and, secondly, in providing simple and reliable methods for production and isolation of the identified molecules. In this context, *Arthrobacter nicotinovorans* pAO1 strain through metabolizing nicotine could deliver new nicotine-derivatives with unexplored biotechnological potential.

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6-Hydroxy-L-nicotine (6HLN) is a metabolic intermediate found in the nicotine catabolic pathway encoded by *Arthrobacter nicotinovorans* pAO1 [14]. 6HLN resulted by a hydroxylation reaction mediated by nicotine-dehydrogenase (NDH, EC 1.5.99.4), a multimeric enzyme encoded by the *ndhL*, *ndhM*, and *ndhS* genes of pAO1. The compound is further dehydrogenated by 6-hydroxy-L-nicotine oxidase (6HLNO, EC 1.5.3.5) with the formation of 6-hydroxy-methylmyosmine [15]. 6HLN was repeatedly reported to temporarily accumulate in the growth medium of *Arthrobacter nicotinovorans* pAO1 [16,17].

Previously, we have shown that 6-hydroxy-L-nicotine (6HLN) a natural product obtained from nicotine degradation by *Arthrobacter nicotinovorans* enhanced behavioral response and consequently decrease oxidative stress generation in the rat hippocampus of the scopolamine treated rats [18]. Here, we further hypothesized that 6HLN versus nicotine ameliorates chlorisondamine (CHL) induced cognitive impairment through decreasing of the hippocampal oxidative stress. CHL is a known nAChR antagonist that can cross the blood-brain barrier and exerts a nicotinic blockade that lasts for months [19]. The pharmacological effects of both nicotine and 6HLN on CHL-induced memory impairment associated with brain oxidative stress were investigated, and also their possible mechanism of action underlying these effects.

2. Materials and methods

2.1. Strains and growth conditions

Arthrobacter nicotinovorans pAO1+ (strain ATCC 4991) was grown on citrate medium supplemented with 3 mM nicotine [20] on a rotary shaker at 28 °C/190 rpm. 100 ml medium in a 500 ml flask were inoculated with 1 ml 24 h old preculture and allowed to grow 10 h before harvest producing about 8 mg of 6HLN.

2.2. General analytical methods

Quantitative data on nicotine metabolites were obtained by high-performance liquid chromatography (HPLC) analysis by comparing the retention times and absorption spectra with literature data [21]. Supplementary, chemical synthesized 6HLN (a kind gift from Prof. Dr. Roderich Brandsch – Institute of Biochemistry and Molecular Biology, Albert-Ludwigs-University of Freiburg, Germany) was used as standard. HPLC analysis was performed as described by Tang et al. [22] with slight modifications. A Shimadzu Prominence UPLC system equipped with a Machery-Nagel Nucleodur RP C18 ec column (150 × 4.6 mm, particle size 3 μm) was used for separation of nicotine metabolites from the growth medium. The mobile phase was a mixture of 1 mM H_2SO_4: methanol (75:25 v/v) at a flow rate of 1 ml/min. The separation was performed at 30 °C using isocratic elution and 6HLN levels were monitored at 290 nm.

2.3. Animals

A total number of thirty male Wistar rats (3–4-month-old) weighing 250 ± 10 g at the start of the experiment was used. The animals were housed in a temperature and light-controlled room (22 °C, a 12 h light/dark cycle starting at 08:00 h) and were fed and allowed to drink water ad libitum. The experiments were conducted in the quiet laboratory between hours of 10:00 h–16:00 h. Rats were treated in accordance with the guidelines of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare from Romania and all procedures were in compliance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. This study was approved by the Committee on the Ethics of Animal Experiments of the Alexandru Ioan Cuza University of Iasi, Faculty of Biology (Permit Number: 2197) and also, efforts were made to minimize animal suffering and to reduce the number of animals used.

2.4. Drugs

(−)−Nicotine (free base, Nic), 6HLN and chlorisondamine (CHL) were diluted in an isotonic solution (0.9% NaCl). All reagents, except 6HLN, were purchased from Sigma-Aldrich, Germany.

2.5. Drug administration

The rats were divided into six different groups (five animals per group) as follows: (1) the Control group received saline treatment (0.9% NaCl); (2) the (−)−Nicotine (Nic, free base, 0.3 mg/kg)-alone-treated group, as positive control; (3) the Chlorisondamine (CHL, 10 mg/kg)-alone-treated group, as negative control; (4) the 6-hydroxy-L-nicotine (6HLN, 0.3 mg/kg)-alone-treated group, as positive control; (5) the CHL-treated group received Nic treatment (CHL + Nic) and (6) the CHL-treated group received 6HLN treatment (CHL + 6HLN). The drug doses used in this experiment were chosen since they have been demonstrated by our group to provide significant effects on memory formation and antioxidant profile [18,23]. CHL was injected 24 h before the experiments. Also, Nic and 6HLN were injected alone and in the CHL treated-groups, 30 min before the behavioral testing.

2.6. Y-maze task

Spontaneous alternation was examined using a three-arm Y-maze (35 × 25 × 10 cm). Rats were individually placed at the end of one arm, facing the center, and allowed to move freely through the maze for 8 min. An arm entry was counted when the hind paws of the rat were completely within the arm. The alternation score (%) of each rat was defined as the ratio of the actual number of alternations to the possible number (defined as the total number of arm entries minus two) multiplied by 100 [24,25]. At the beginning of each trial the maze was thoroughly cleaned with 10% ethanol.

2.7. Radial arm-maze task

The radial arm-maze used in the present study consisted of 8 arms, numbered from 1 to 8 (48 × 12 cm), extending radially from a central area (32 cm in diameter). The apparatus was placed 40 cm above the floor and surrounded by various extra-maze visual cues placed at the same position during the study. Prior to the performance of the maze task, the animals were kept on restricted diet and body weight was maintained of 85% of their free-feeding weight over a week period, with water being available ad libitum.

During the pre-training session (4 days), rats were individually placed in the center of the maze and were allowed to explore to habituate them to the maze for 5 min. Initially, the food pellets (50 mg, Bioserve Inc.) were placed throughout the maze but gradually restricted to the end of each arm in the food cup. Five target baited arms (nos. 1, 2, 4, 5, and 7) were selected which remained constant for a given rat throughout training. The other three arms (nos. 3, 6, 8) were never baited.

In the training session, starting with the day fifth to day twelfth, the animals were trained by performing one trial per day for seven consecutive days. Each rat was individually placed in the center of the maze and subjected to working and reference memory tasks. An arm entry was counted when a rat ate the bait or reached the end of an arm. Measures were made of the number of working memory errors (entering a baited arm, but previously entered) and