Life Sciences 103 (2014) 73–78

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Evaluation of mutagenic and genotoxic activities of lobeline and its modulation on genomic instability induced by ethanol

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ARTICLE INFO

Article history:
Received 30 September 2013
Accepted 29 March 2014
Available online 12 April 2014

Chemical compounds studied in this article:
(−)-Lobeline hydrochloride (PubChem CID: 45358761)
Cyclophosphamide monohydrate (PubChem CID: 22420)
ETHANOL (PubChem CID: 702)

Keywords:
Alcoholism
Addiction
Ames test
Comet assay
Genomic
Lobeline
Micronucleus test
Mutagenicity

ABSTRACT

Aim: Lobeline is a natural alkaloid derived from *Lobelia inflata* that has been investigated as a clinical candidate for the treatment of alcoholism. In a pre-clinical trial, lobeline decreased the preference for and consumption of ethanol, due to the modulation of the nicotinic acetylcholine receptor. However, the interaction between lobeline and ethanol is poorly known and thus there are safety concerns.
The present study was conducted to evaluate the mutagenic and genotoxic effects of lobeline and assess its modulation of ethanol-induced toxicological effects.
Main methods: CF-1 male mice were divided into five groups. Groups received an intraperitoneal injection of saline solution, lobeline (5 or 10 mg/kg), ethanol (2.5 g/kg), or lobeline plus ethanol, once a day for three consecutive days. Genotoxicity was evaluated in peripheral blood using the alkaline comet assay. The mutagenicity was evaluated using both *Salmonella*/microsome assay in TA1535, TA97a, TA98, TA100, and TA102 *Salmonella typhimurium* strains and the micronucleus test in bone marrow. Possible liver and kidney injuries were evaluated using biochemical analysis.
Key findings: Lobeline did not show genotoxic or mutagenic effects and did not increase the ethanol-induced genotoxic effects in blood. Lobeline also protected blood cells against oxidative damage induced by hydrogen peroxide. Biochemical parameters were not altered, indicating no liver or kidney injuries or alterations in lipid and carbohydrate metabolisms.
Significance: These findings suggest that lobeline does not induce gene or chromosomal mutations, and that this lack of genetic toxicity is maintained in the presence of ethanol, providing further evidence of the safety of this drug to treat alcohol dependence.

Introduction

Alcohol abuse and dependence is a worldwide public health problem. Epidemiological studies have shown that alcohol increases the risks of several kinds of cancer and liver diseases (Corrao et al., 2004; Grewal and Viswanathen, 2012; Testino et al., 2012). The central cholinergic system has been implicated in the development of alcohol and/or drug abuse (Söderpalm et al., 2000; Rahman et al., 2008; Rahman and Prendergast, 2012). There is ample evidence that ethanol increases extracellular dopamine in the nucleus accumbens. In addition, particular subunits of the nicotinic acetylcholine receptor (nAChR) are believed to regulate ethanol drinking behaviors (Kamens and Phillips, 2008; Sajja and Rahman, 2012).

Lobeline is a natural alkaloid found in *Lobelia inflata* and has a long history of therapeutic use, as emetic and respiratory stimulant, tobacco smoking cessation agent, and other applications (Dwoskin and Crooks, 2002; Felpin and Lebreton, 2004).

A considerable body of evidence points to the direct interaction between lobeline and nAChRs, in a competition for binding with other cholinergic drugs (Damaj et al., 1997; Parker et al., 1998; Kaniaková et al., 2011; Roni and Rahman, 2013). Lobeline inhibits psychostimulant-induced effects mediated by its activity at nAChRs and/or its ability to

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http://dx.doi.org/10.1016/j.lfs.2014.03.034
0024-3205/

alter presynaptic dopamine storage and release, *via* the interaction with vesicular monoamine transporter-2 (VMAT2) (Teng et al., 1997; Harrod et al., 2001; Miller et al., 2001; Sajja et al., 2010; Smith et al., 2012).

Recently, lobeline has been shown to attenuate alcohol consumption and preference in rodents associated to modulation of nAChRs (Bell et al., 2009; Farook et al., 2009; Sajja and Rahman, 2011). However, the effects of interactions between lobeline and ethanol on genomic stability have not been investigated to an appropriate extent. Mutagenic and genotoxic effects of ethanol have been extensively studied (Brooks, 1997; Guo et al., 2008; de Oliveira et al., 2012). Excessive alcohol consumption is associated with an increase in reactive oxygen species (ROS), which has deleterious effects on several complex molecules, including DNA (Kido et al., 2006; Cederbaum et al., 2009; de Oliveira et al., 2012). DNA damage can lead to mutations, a primary step of cancer initiation. Alcohol metabolism also produces acetaldehyde, which has been shown to induce DNA damage, including oxidative modifications, acetaldehyde-derived DNA adducts and crosslinks (Singh et al., 1995; Blasiak et al., 2000; Lamarche et al., 2003; Brooks and Theruvathu, 2005; Mechilli et al., 2008; Balbo et al., 2012). However, few studies have assessed the genotoxicity of lobeline. Brown et al. (1992) investigated the clastogenicity (chromosome breakage) of lobeline and possible interactions between lobeline and ethanol in a mutagen-sensitivity assay on cultures of human lymphoblastoid cell lines and found mutagenicity in the combination.

Considering the therapeutic potential of lobeline for the control of alcohol abuse and addiction, the aim of this study was to evaluate the genotoxic and mutagenic effects of lobeline alone and combined with ethanol, using the comet assay in peripheral blood and the micronucleus test in bone marrow of mice. The findings were supplemented with measurements of DNA oxidative damage in blood tissue using hydrogen peroxide as a DNA strand-break inductor. Liver and kidney injuries and metabolic alterations were evaluated using biochemical analyses. In addition, we studied the mutagenic effect of lobeline using the *Samonella*/microsome assay. Thus, the procedures to evaluate mutagenicity were those internationally recommended in drug approval protocols; the micronucleus test is able to detect chromosomal mutations (Krishna and Hayashi, 2000), while the *Salmonella*/microsome assay detects gene mutations (Mortelmans and Zeiger, 2000).

Material and methods

Drugs

(−)-Lobeline hydrochloride (PubChem CID: 45358761) and cyclophosphamide monohydrate (PubChem CID: 22420) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (PubChem CID: 702) was of analytical grade and purchased from Merck (Darmstadt, Germany). All solutions were prepared immediately prior to administration.

Animals and experimental design

In total, 30 CF-1 male mice, weighting from 35 to 40 g, were obtained from the State Foundation for Production and Research in Health (FEPPS), Porto Alegre, RS, Brazil. Mice were housed in plastic cages, with *ad libitum* access to water and food, under a 12-h light/dark cycle and at a constant temperature of 22 ± 3 °C. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the consent of the Ethics Committee of the Lutheran University of Brazil (CEP-ULBRA number: 2010010A).

The animals were divided to form groups with 5 individuals per group, and received an intraperitoneal injection once a day composed of saline solution (NaCl 0.9%), lobeline (5 and 10 mg/kg), ethanol 2.5 g/kg, or lobeline 5 mg/kg + ethanol 2.5 g/kg, in a volume of 10 mL/kg body weight, for three consecutive days. All animals were euthanized on the 4th day. Total blood samples were collected from each mouse for biochemical analyses, and to perform the comet assay. Bone marrow was collected from femurs and used to evaluate mutagenicity by the micronucleus test. A positive control group treated once with cyclophosphamide 50 mg/kg was included to perform a micronucleus test as previously described (Mavournin et al., 1990). The lobeline doses of 5 and 10 mg/kg were chosen because they have been proved to attenuate the preference for ethanol intake in rodents (Bell et al., 2009; Farook et al., 2009; Sajja and Rahman, 2011, 2012). Ethanol at 2.5 g/kg has shown genotoxic effects in previous studies (Guo et al., 2008) and the modulation of this effect could be evaluated in the combination with lobeline.

Comet assay

The alkaline comet assay was carried out according to a specific guideline (Tice et al., 2000). A 50 μL-blood sample of each mouse was placed in 15 μL anticoagulant (heparin sodium 25,000 IU—Liquemine®); 5.0 μL of each sample in heparin was embedded in 95 μL 0.75% low melting point agarose (Gibco BRL). The mixture (cell/agarose) was spread on a fully frosted microscope slide coated with a 300-μL layer of normal melting agarose (1%) (Gibco BRL). After solidification, slides were transferred to either PBS or 0.25 mM freshly prepared hydrogen peroxide (H_2O_2) solution (*ex vivo* treatment) for 5 min, at 4 °C as described by da Silva et al. (2012). Slides were washed 3 times with PBS and then placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, freshly added 1% Triton X-100 and 10% DMSO, pH 10.0) for 48 h at 4 °C. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min, at 4 °C. Electrophoresis was performed at 300 mA and 25 V (0.90 V/cm) for 15 min. The slides were then neutralized (0.4 M Tris, pH 7.5), stained with silver, and inspected using a microscope. Images of 100 randomly selected cells from each animal (50 cells from each of two replicate slides) were analyzed. Cells were also scored visually according to tail size into five classes, ranging from undamaged (0), to maximally damaged (4), resulting in a single DNA damage score for each animal, and consequently for each group studied. Therefore, the damage index (DI) can range from 0 (completely undamaged, 100 cells × 0) to 400 (with maximum damage, 100 × 4). The damage frequency (DF) was calculated based on the number of cells with tail *versus* those with no tails. The percentage of reduction in DI was calculated as: $R\% = [DI\ Saline + H_2O_2 - DI\ lobeline + H_2O_2] / [DI\ saline + H_2O_2 - DI\ saline] \times 100$ (Flores et al., 2011).

Micronucleus test

The micronucleus test was performed according to the US Environmental Protection Agency Gene-Tox Program (Mavournin et al., 1990). Bone marrow extracted from both femurs of the mice was suspended in fetal calf serum and smeared on clean glass slides. Slides were air-dried, fixed in methanol, stained in 10% Giemsa and coded for a blind analysis. To avoid false negative results and to obtain a measure of toxicity on bone marrow, the polychromatic erythrocyte: normocromatic erythrocyte (PCE:NCE) ratio was scored in 1000 cells. The incidence of a micronucleus (MN) was observed in 2000 PCEs for each animal (Picada et al., 1997).

Biochemical assay

All mice were given no food for 4 h prior to euthanasia. Blood samples were collected from each mouse into tubes without an anticoagulant. The serum was separated and was assayed for glucose, creatinine, total protein, albumin, alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and cholesterol using commercially available kits (Labtest® kits, Lagoa Santa, Minas Gerais, Brazil) in a Thermoplate®, model TP Analyzer Basic automated analyzer.