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RESEARCH ARTICLE

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Evaluation of the inhalation toxicity of arecoline benzoate aerosol in rats

Ming Chu[1,2] | Guofeng Fu[1,3] | Jingjing Deng[1] | Ruoxi Wang[2] | Qiming Fan[4] | Zuxin Chen[5,6] | Jin Lu[1,3] | Xin-an Liu[2,6]

[1]Laboratory of Life and Health Sciences, Shenzhen First Union Technology Co., Ltd., Shenzhen, China

[2]Guangdong Provincial Key Laboratory of Brain Connectome and Behavior, CAS Key Laboratory of Brain Connectome and Manipulation, Brain Cognition and Brain Disease Institute (BCBDI), Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen-Hong Kong Institute of Brain Science-Shenzhen Fundamental Research Institutions, Shenzhen, China

[3]Laboratory of Life Sciences, Shenzhen Icybetel Biotechnology Co., Ltd., Shenzhen, China

[4]Guangdong Zhongke EnHealth Science and Technology Co., Ltd., Foshan, China

[5]Shenzhen Key Laboratory of Drug Addiction, Shenzhen Neher Neural Plasticity Laboratory, Brain Cognition and Brain Disease Institute, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences (CAS); Shenzhen-Hong Kong Institute of Brain Science-Shenzhen Fundamental Research Institutions, Shenzhen, China

[6]University of Chinese Academy of Sciences, Beijing, China

Correspondence
Jin Lu, Laboratory of Life and Health Sciences, Shenzhen First Union Technology Co., Ltd., Shenzhen 518103, China.
Email: 13842062044@163.com

Xin-an Liu, Guangdong Provincial Key Laboratory of Brain Connectome and Behavior, CAS Key Laboratory of Brain Connectome and Manipulation, Brain Cognition and Brain Disease Institute (BCBDI), Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen-Hong Kong Institute of Brain Science-Shenzhen Fundamental Research Institutions, Shenzhen 518055, China.
Email: xa.liu@siat.ac.cn

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Abstract

Arecoline is a pharmacologically active alkaloid isolated from *Areca catechu*. There are no published data available regarding the inhalation toxicity of arecoline in animals. This study aimed to evaluate the inhalation toxicity of arecoline in vitro and in vivo. For this purpose, arecoline benzoate (ABA) salt was prepared to stabilize arecoline in an aerosol. The MTT assay determined the half-maximal inhibitory concentration values of ABA and arecoline in A549 cell proliferation to be 832 and 412 μg/ml, respectively. The toxicity of acute and subacute inhalation in Sprague–Dawley rats was evaluated using the guidelines of the Organization for Economic Cooperation and Development. For acute inhalation, the median lethal concentration value of ABA solvent was >5175 mg/m^3. After the exposure and during the recovery period, no treatment-related clinical signs were observed. In the 28-Day inhalation toxicity test, daily nose-only exposure to 2510 mg/m^3 aerosol of the ABA solvent contained 75 mg/m^3 ABA for male rats and 375 mg/m^3 ABA for female rats, which caused no observed adverse effects, except for the decreased body weight gain in male rats exposed to 375 mg/m^3 ABA. In this study, the no observed adverse effect level (NOAEL) for the 28-day repeated dose inhalation of ABA aerosol was calculated to be around 13 mg/kg/day for male rats and 68.8 mg/kg/day for female rats, respectively.

KEYWORDS
28-day inhalation toxicity, acute inhalation toxicity, aerosol, Arecoline benzoate, cytotoxicity, NOAEL

Ming Chu and Guofeng Fu contributed equally to this study.

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1 | INTRODUCTION

Areca catechu is a palm species found throughout Southeast Asia, the East African seaboard, and the Western Pacific. Chewing betel nuts, which are the seeds of the *A. catechu* tree, is still popular in these areas (Peng et al., 2015). Following tobacco, alcohol, and caffeine, betel nut is now considered the fourth most commonly consumed psychoactive substance, affecting over 600 million people worldwide (Hussain et al., 2018; Warnakulasuriya & Peters, 2002). Jahns was the first to isolate the active alkaloid arecoline (methyl-1,2,5,6-tetrahydro-1-methyl-nicotinate) from betel nut (Jahns, 1888). Arecoline hydrobromide (AH) salt is widely used commercially because arecoline is unstable and can be hydrolyzed by alkali (Jahns, 1888; Jahns, 1891).

Traditionally, arecoline has been used as an anthelmintic drug to treat tapeworm infestation (Feng et al., 1999). The various pharmacological activities of arecoline have been studied in relation to the nervous, cardiovascular, endocrine, and digestive systems, in addition to its antiparasitic effects (Liu et al., 2016). In folk medicine, arecoline claim to treat schizophrenia and depression, and chewing betel nut can help with the symptoms of schizophrenia (Bales et al., 2009; Sullivan et al., 2000). Additionally, clinical research has shown that arecoline can improve cognitive function and memory in patients with Alzheimer's dementia (AD) (Maiese et al., 1994), and chewing betel nut can improve concentration, increase work capacity, and relaxation (Peng et al., 2015). Cardiovascular pharmacodynamics of arecoline has been reported to have an antithrombotic effect by activating endothelial target for acetylcholine (Chen et al., 2002) and an antiatherogenic effect by regulating cytokines and mRNA expression (Duan & Wang, 2006; Yao et al., 2009). Furthermore, many studies have shown that arecoline can stimulate endocrine activity, including endorphin (Risch et al., 1982), adrenal (Lim & Kim, 2006), and testosterone (Wang et al., 2008). Arecoline may also improve digestion functions by stimulating the muscarinic receptor, which promotes smooth muscle activity (Xie et al., 2004).

The toxicity of arecoline is being increasingly explored. Recently, arecoline was classified as 'possibly carcinogenic to humans' (Group 2B) by the International Agency for Research on Cancer (IARC) (Marques et al., 2021). Previous studies have reported that the maximal tolerable dose (LD_0) value and the median lethal concentration (LD_{50}) value of arecoline in mice were 70 and 174.71 mg/kg, respectively (Zhou et al., 2010). Additionally, the LD_0 and LD_{50} values of AH in mice were 460 and 691.83 mg/kg, and in rats were 1500 and 2054 mg/kg, respectively. Dasgupta et al., (2006) have reported that injecting AH into the peritoneal cavity of mice could induce immunosuppression, hepatotoxicity, and a decrease in the antioxidant status. A 14-day repeated oral administration of AH at a high dose had significant toxic effects as indicated by body weight, organ coefficient, hematological parameters, and histopathological indexes in Wistar rats, and the no observed adverse effect level (NOAEL) was <100 mg/kg/day (Wei et al., 2015). According to Chatterjee and Deb (1999), arecoline, administered orally or intraperitoneally, caused chromosomal aberration, micronucleus formation, and sister-chromatid exchange in the bone marrow of mice. Recent research has shown that arecoline's oral toxicities are the most serious adverse effects (Liu et al., 2016). According to Xia et al. (2009), arecoline can affect the collagen metabolism of the oral keratinocyte fibroblast co-culture system, indicating that arecoline may be a key etiological factor in the development of oral submucous fibrosis (OSF). Arecoline can also induce epithelial-mesenchymal transition-related factors in primary human buccal mucosal fibroblasts (Chang et al., 2014). In reality, the majority of research has focused on the impact of betel nut on human health, particularly as a potential cause of oral cancer. According to the studies, multiple factors, including constituents of betel nut such as alkaloids, polyphenols, as well as natural or added components such as betel leaf, slaked lime, and additives, have been linked to OSF and oral cancer from betel nut uses (Sharan et al., 2012).

Arecoline has shown promise in the treatment of a variety of disorders, including AD-related diseases, cardiovascular diseases, and tapeworms. Arecoline may one day be developed into new drugs to treat these diseases. Additionally, arecoline still has some toxicity, particularly in the case of oral diseases induced by chewing betel nut, necessitating the development of alternate drug delivery routes (Volgin et al., 2019). Besides its pharmacological activities, more research should be focused on the potential toxicities of arecoline in various organs after long-term exposure. The study on the effects of arecoline on the respiratory system, on the other hand, was limited and lacked inhalation data. Only one study (Taylor et al., 1992) found that inhaled arecoline caused dose-related bronchoconstriction in patients with asthma, provocative concentration causing a 20% fall in forced expiratory volume in 1 s (PC_{20} FEV1) at a concentration of 5.2 mg/ml. The lack of response to inhaled arecoline in all, except one healthy subject at a concentration of 34.2 mg/ml pointed to a low risk of bronchoconstriction in healthy betel chewers.

This study aimed to investigate the acute and subacute toxicity of arecoline when inhaled, to provide information about the effect of arecoline on the respiratory system for its future drug use. Because arecoline is chemically unstable when dissolved in common aerosol forming agents such as propyl glycol and glycerol, and extremely irritating to the respiratory tract of animals, arecoline benzoate (ABA) was prepared, which was found to be more stable during inhalation administration. The toxicity of ABA was systematically studied in vitro and in vivo in Sprague–Dawley (SD) rats. To our knowledge, this report was the first comprehensive inhalation study on arecoline aerosol that includes endpoints for respiratory and other systemic toxicity.

2 | MATERIALS AND METHODS

2.1 | Preparation of arecoline benzoate (ABA)

The reagents and starting materials were purchased and used without any further purification. Two medicinal grade aerosol carrier solvents, propyl glycol (PG) purchased from Dow (USA) and vegetable glycerol (VG) purchased from Procter&Gamble (USA), were used without further purification. The arecoline was extracted from *A. catechu* using super-critical CO_2 extraction and arecoline benzoate (ABA) was

prepared mixing calculated amounts of arecoline and benzoate acid. The ABA thus prepared was a viscous yellow liquid, with a structure shown in Figure 1. NMR spectra were recorded on a Bruker 600-MHz spectrometer. Chemical shift values (δ) were expressed in parts per million using residual solvent protons as the internal standard (^{1}H NMR, δ[H] is 7.26 ppm for $CDCl_3$, ^{13}C NMR, δ[C] is 77.16 ppm for $CDCl_3$). Mass spectra were recorded on a Thermo Scientific OrbitrapExploris™ 120 mass spectrometer.

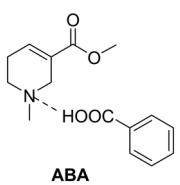
2.2 | In vitro experiment

2.2.1 | Cell culture

Human epithelial lung carcinoma cell lines (A549, ATCC No. CCL-185) were used to assess cytotoxicity of ABA and arecoline. Cells were cultured in sterile, 55-cm^2 (D × H: 100 × 20 mm; Corning, USA) cell culture dish with Dulbecco's Modified Eagle's Medium (DMEM: contain 4.5 g/L glucose, L-glutamine and sodium pyruvate; Corning, USA) supplemented with 10% (v/v) fetal bovine serum (FCS; Corning, USA) and 1% (v/v) antibiotic (10,000 Penicillin and 10-mg streptomycin per ml; Beyotime, China). In cytotoxicity experiments, A549 cells were cultured at 37°C in a humidified 5% CO_2 incubator. Cell layers at 80%–90% confluency were rinsed with phosphate-buffered saline (PBS; Corning, USA) and incubated with Trypsin/EDTA (0.25% Trypsin, 0.1% EDTA; Corning, USA) for 2 min at room temperature to allow cell detachment from the cell dish surface, and the cell pellets were resuspended in culture medium for the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) cytotoxicity experiments. Cell viability was assessed by vital staining with trypan blue (0.4% [w/v]; Sigma-Aldrich, USA), and cell number was determined using a light microscope (Soptop ICX41, Ningbo Sunny Instruments Co., Ltd., China).

2.2.2 | MTT assay

The cytotoxicity of ABA and arecoline was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Phygene Life, China), which was based on the protocol described by Mosmann (1983). A549 lung epithelial cells were first seeded into 96-well plates in a density of 10^4/well and incubate at 37°C in a humidified 5% CO_2 incubator for 24 hours. The MTT assay was performed 3-fold dilutions of ABA and arecoline at the concentrations of 2222, 741, 247, 83.2, 27.4, and 9.1 μg/ml, respectively. The cells were incubated with ABA or arecoline with serial dilutions in culture medium for 24 h, then 10 μl of 5 mg/ml MTT dissolved in PBS were added to each well and incubated for 4 h at 37°C. Solutions were removed, and 150-μl dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) were added to each well and the outcome was quantified by measuring absorbance at 490 nm using an Epoch micro-plate reader (Biotek Synergy H1, USA). Three replicate experiments were performed respectively, and data were statistically analyzed with the SPSS Statistics (USA).



ABA

FIGURE 1 The molecular structure of arecoline benzoate (ABA)

2.3 | In vivo experiment

2.3.1 | Electronic aerosol generator and exposure system

The efficacy of an inhaled drug was both related to the total amount of drug deposited in the respiratory tract and the lungs, and also the amount deposited in some specific lung region (Pritchard, 2001). The main inhalation drug delivery methods for medicinal or toxicity studies include ultrasonic or resistive atomization; the former generates larger particles with median particle size over 3 μm, unsuited for high efficient drug delivery. In this work, an electronic aerosol generator (EAG) device was used generated inhalable aerosol particles containing the target ABA. Three automated 6-port EAG devices were purposely built (Icybetel Biotechnology, China) with software control program. The working power of atomizer was 11.5 W, charging current for 5 V/500 mA. The interface between the EAG and the nose-only inhalation exposure system (HRH-MNE 3026, Huironghe Technology, China) are shown in Figure 2. The aerosol concentrations in the animal exposure chamber were controlled by the number of activated electronic atomizer and the dilution air-flow rate. Each EAG device produced controlled aerosol delivery by following the ISO 3308 (International Organization for Standardization, 2012) standard protocols, which produce a rectangular-shape puff flow profile at 55 ± 0.3 ml puff volume, 3 s ± 0.1 s puff duration and 30s ± 0.5 s puff frequency. A near continuous atomization concentration was achieved by asynchronous activation of the six atomizers. The levels of O_2, CO_2, temperature, relative humidity and chamber airflow were monitored at 1 Hz during the exposure period. The O_2 concentration was at least 19% and CO_2 concentration did not exceed 1%. The chamber temperature was maintained at 22 ± 3°C and the relative humidity at 30%–70%. The aerosol particle size distribution was measured for 1-min duration with 28.3 L/min airflow rate using a cascade impactor (Promo 2000, PALAS GmbH, German), which was used to determine the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD). The amount of the aerosol produced was measured with a gravimetric filter analysis method, collected at once per 30 min, by measuring the weight difference of a Cambridge filter pad fixed inside a filter holder at 1 L/min airflow rate for 4 min. The chemical constituents of aerosol deposited on the Cambridge filter pad were extracted, and then analyzed by gas