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Interactions between bufadienolides derived from toad venom and verapamil in langendorff-perfused guinea-pig hearts

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ABSTRACT

Drug toxicity may occur due to dangerous drug combination. We aimed to investigate the influence of verapamil (a P-gp inhibitor) - bufadienolides interaction on cardiotoxicity and bufadienolide uptake by the isolated heart. The study was performed in Langendorff isolated perfused guinea-pig hearts by bufadienolides infusion in the absence and presence of verapamil (250, 500 ng/ml). Arrhythmia parameters were evaluated by ECG and the content of bufadienolides in heart were measured by ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS). In the present of verapamil, the wide QRS duration and lightly rapid heart rate (HR) were markedly reduced in the early stage of bufadienolide intoxication. However, the ECG changes characterized by prolonged P–R interval, and slow heart rate and low QRS amplitude in the late stage of bufadienolide intoxication were significantly enhanced. Furthermore, the contents of a variety of bufadienolide compounds in the verapamil + bufadienolide group were significantly higher when cardiac arrest occurred. Although verapamil reduced the bufadienolide-induced ventricular arrhythmias, verapamil worsened heart block and lethal bradycardia of bufadienolides partly via increasing the uptake of bufadienolides in heart tissue, which could compromise the protective effects of verapamil against bufadienolide intoxication. These results suggested that the verapamil may produce dangerous interactions with drugs containing bufadienolides.

1. Introduction

Toad venom (called "Chansu" in China) is a traditional Chinese medicine obtained from the skin and parotid venom glands of the toad, including *Bufo bufo gargarizans* Cantor and *Bufo melanostictus* Schneider. It is used for the treatment of various types of inflammation, cancer and heart disease in China (Meng et al., 2009). Toad venom is the major component of some popular traditional Chinese medicines (TCMs) (e.g., Liushenwan, Niuhuangxiaoyyanwan and Shexiangbaoxindan) and a West Indian aphrodisiac known as "Love Stone". These medicines are very popular in Asian and Western countries, and can be readily obtained as over-the-counter (OTC) drugs. The anti-inflammatory therapeutic effect of toad venom is due to its major active ingredients bufadienolides, such as bufalin, cinobufagin and bufotalin (Jiang et al., 2011; Qi et al., 2011). Bufadienolides inhibited the inflammatory proliferation of human PBMCs (T-lymphocytes and monocytes) in the pg/mL or sub-nanogram per milliliter range (Terness et al., 2001). However, bufadienolides have a steroid structure similar to digoxin (Gowda et al., 2003), and cause cardiac toxicity, including mechanical dysfunction (negative inotropy) and electrical dysfunctions (arrhythmias) (Ma et al., 2012a,b) in the ng/mL or sub-microgram per milliliter range (Bick et al., 2002; Gowda et al., 2003; Kostakis and Byard, 2009). The cardiotoxicity resulting from the use of toad venom may be due to two factors: (1) the cardiac side effects induced by bufadienolides themselves when used in high doses; (2) co-administration of toad venom with other drugs that produces the potentially dangerous drug–drug interactions.

P-glycoprotein (P-gp) is an important source of drug–drug interactions mediated at the level of inhibition or induction of the transporter (Sugimoto et al., 2011). Modulation of P-gp transport may lead to increased drug plasma and tissue concentration with resulting increases in toxicity due to reduced drug elimination (Weiss and Kang, 2002). Verapamil, a P-gp and L-type Ca^{2+} channel inhibitor, may be used together with other cardiovascular drugs. It has been reported for verapamil to increase the cardiotoxicity of digoxin via P-gp function inhibition (Arici et al., 2010; Taubert and Lafleur, 1984). In the present study, we used langendorff isolated perfused guinea-pig hearts as model to investigate the effect of coadministration of verapamil on the

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cardiotoxiciy and cardiac uptake of the bufadienolides derived from toad venom.

2. Material and methods

2.1. Drugs

Verapamil hydrochloride injection (2.5 mg/ml) was purchased from the Shanghai harvest pharmaceutical Co. Ltd., (Shanghai, China). Toad venom (Chansu) was purchased from Nanjing Medicinal Material Company and authenticated by Professor Jinao Duan of Jiangsu Key Laboratory for TCM Formulae Research as a dried secretion of *B. gargarizans* cantor. A voucher (NJUTCM-200903104) specimen is deposited in the Nanjing University of Chinese Medicine (Nanjing, China). An amount of toad venom (100 g) was dissolved in $CHCl_3$ and extracted twice. The organic extracts were combined and concentrated in a rotary evaporator at 60 °C to get the total bufadienolides (27.5 g). Further high-performance liquid chromatography ultraviolet (HPLC–UV) analysis showed that the bufadienolides contain resibufogenin (3.25%), cinobufagin (9.68%), bufalin (6.18%), bufotalin (9.69%), telocinobufagin (8.03%), arenobufagin (4.2%), gamabufotalin (3.97%), desacety-bufotalin (7.44%) and hellebrigenin (36.3%). The bufadienolides were dissolved in 0.1% dimethyl sulfoxide (DMSO).

2.2. Animals and ethics

Healthy male guinea-pigs weighing 250–300 g (12 week old) were obtained from the Experimental Animal Center of Nanjing University of Chinese Medicine. They were kept in plastic cages under standardized animal-house conditions with free access to food and water. Animal experiments were conducted in accordance with internationally accepted guidelines for the use and care of laboratory animals (guiding principles for the use of animals in toxicology as adopted by the Society of Toxicology in 1999) as well as the guidelines for animal experimentation issued by the Nanjing University of Chinese Medicine.

2.3. Cardiac arrhythmias in ex vivo isolated guinea-pig heart

Male guinea-pigs were anaesthetized with pentobarbital (45 mg/kg, i.p.) after injectioned of 1000 U sodium heparin. Hearts were quickly excised and arrested in ice-cold standard perfusion buffer (Krebs–Henseleit solution) equilibrated with 95% O_2 and 5% CO_2. The composition of solution was as follows (mM): NaCl 120, KCl 4.6, $MgSO_4$ 2.6, KH_2PO_4 1.2, $CaCl_2$ 1.3, $NaHCO_3$ 20, and glucose 8.8. The ascending aorta was cannulated and perfused with 37 °C Krebs solution. After stabilization for 15 min, normal Krebs solution was replaced with the Krebs solution containing bufadienolides (2 μg/mL), bufadienolides + verapamil (2 μg/mL + 250 ng/ml) and bufadienolides + verapamil (2 μg/mL + 500 ng/ml). Hearts were excluded from the study if they were mechanically unstable or arrhythmic. Bufadienolides or/and verapamil in Krebs solution with DMSO (0.1% v/v) were given by continuous infusion for 30 min. Limb lead II ECGs was recorded on a recorder polygraph (Model MPA 2000, Shanghai Alcott Biotech Company Limited, Shanghai, China) by placing two electrodes on the right atrium and apex. The alone treatment of verapamil (250 and 500 ng/ml) did not markedly induce heart block and other ECG changes during 25 min observation period. The outflow samples were collected continuously during the course of the study. The heart samples were kept frozen at −70 °C until analysis of bufadienolides by UPLC–MS.

2.4. Sample preparation

The hearts were homogenized in three volumes of homogenizing medium for 5 min. The homogenate was then centrifuged at 3000 rpm for 15 min at 4 °C. Two milliliters supernatant sample was extracted by 3 ml ethyl acetate three times. The organic layer was transferred, and evaporated to dryness by N_2 and stored at −20 °C until analysis. The residue was dissolved in 200 μL methanol and an aliquot of 5 μL was injected into the column for UPLC–MS/MS analysis after centrifugation at 13,000 rpm for 10 min.

2.5. Instrumentation and UPLC–ESI–QTOF/MS conditions

Chromatographic separation were performed on a Waters ACQUITY™ BEH C_{18} column (2.1 × 100 mm, 1.7 μm particle size) using a Waters ACQUITY™ UPLC™ system (Waters Corp., Milford, MA, USA). The column was maintained at 35 °C. The mobile phase consisted of (A) water containing 0.1% (v/v) formic acid and (B) acetonitrile using a gradient elution of 15–30% B at 0–10 min, 30–40% B at 10–25 min, 40–60% B at 25–30 min, 60% B at 30–35 min. The flow rate was 0.25 mL/min, injection volume was 5 μL.

The MS instrument consisted of a Waters Synapt™ Q-Tof/MS (Waters Corp., Milford, MA, USA). Ionization was performed in the positive electrospray (ESI) mode. The mass range was set at m/z 100–1000 Da with a 0.5 s scan time. For analysis, the electrospray source parameters were fixed as follows: electrospray capillary voltage was 3.0 kV, source temperature was 120 °C and desolvation temperature was 350 °C. The cone voltage was set at 30 V. Nitrogen was used as cone and collision gases and the cone and desolvation gas flows were 50 and 700 L/h, respectively. Argon was employed as collision gas. The collision energy varied from 25 to 30 eV. The first-order full-scan mass spectra covered the range 50–1000 m/z. Compounds were identified by MS characterization and UV absorption against external standards of gamabufotalin, arenobufagin, hellebrigenin, telocinobufagin, bufotalin, bufalin, resibufogenin and cinobufagin. All the analyses were acquired using the Lock Spray™ to ensure accuracy and reproducibility; leucine–enkephalin was used as the lock mass (m/z 556.2771). The raw data were analyzed by Masslynx (version 4.1; Waters, UK).

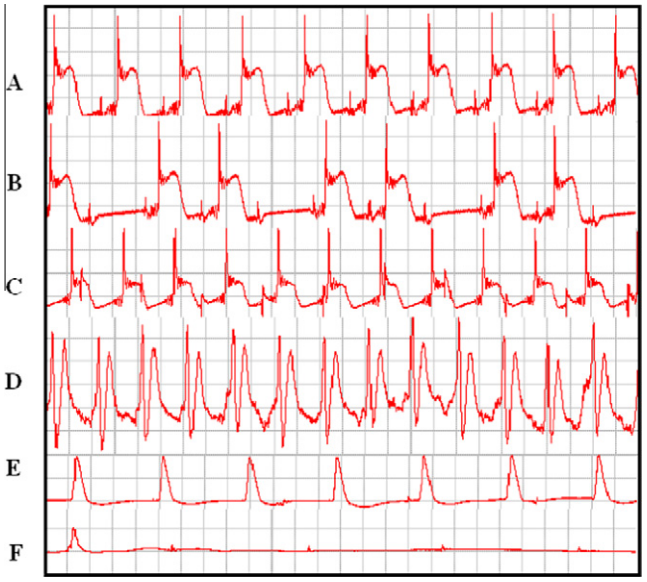

Fig. 1. ECGs showing examples of cardiac disorders induced by direct perfusion of bufadienolides (2 μg/ml). Trace A: before treatment; trace B and F: heart block; trace C: junctional ectopic beats; trace D: ventricular tachycardia; trace E: ventricular bradycardia.