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Effects of *Urginea sanguinea*, a traditional asthma remedy, on embryo neuronal development

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Abstract

The Southern African plant, *Urginea sanguinea* Shinz (Hyacinthaceae) (US), is a well-known traditional herbal medicine and it is used for many different ailments, including asthma. Pregnant women also use this plant and little is known regarding the toxic effects of this plant material on the developing foetus. US contains the cardiac glycoside (CG) Transvaalin; CGs are known to cross the placenta and blood–brain barrier and therefore may have a negative effect on the foetal development. To address this, in vitro cytotoxicity of this preparation as well as its effect on chick embryo neural development was investigated. Water extracts of US were shown to be cytotoxic in cell cultures of L929 cell and primary embryonic neural cell cultures. Electron microscopy studies following in ovo exposure revealed altered neuron morphology with patterns of cell damage either associated with apoptosis or necrosis. CGs are known to inhibit membrane bound Na^+/K^+-ATPase in conducting tissues, causing disruption of the calcium pathways, mitochondrial calcium overload leading to either apoptosis or necrosis or where both occur, a process of necrapoptosis. The in ovo effects observed strongly indicate that US causes necrapoptosis in chick embryonic neurons.

Keywords: *Urginea sanguinea*; Apoptosis; Necrosis; Cardiac glycosides; Electron microscopy; Cytotoxicity

1. Introduction

It is estimated that there are 27 million indigenous medicine consumers in Southern Africa and a significantly large number of these patients consult traditional healers for potentially life threatening conditions (Puckree et al., 2002; Mulholland and Drewes, 2004). The Southern African plant, *Urginea sanguinea* Shinz (Hyacinthaceae) (US) is a well-known traditional herbal medicine and the bulb of this plant is used for many different ailments (Watt and Breyer-Brandwijk, 1962; Hedberg and Stuagard, 1989; Foukaridis et al., 1995; Van Wyk and Gericke, 2000; Marx et al., 2005). The powdered bulbs are used to treat, amongst other ailments, bronchitis, asthma and influenza (Moll and Strebel, 1989), while a tea is prepared for the treatment of veneral disease, abdominal pain, backache and hypertension. However, many indigenous people taking this plant as medicine have complained of adverse health effects, and although many complaints have been noted, little scientific information regarding the toxic effects on cellular function, growth and differentiation is available. The cardiac glycoside, Transvaalin has been isolated from US (Louw, 1949, 1952). Cardiac glycosides are used to stimulate the heart muscle and act on conducting tissue (including neural tissue) by changing the functioning of the depolarisation of the membrane-linked Na^+/K^+ pump and cause an increase in Ca^{2+}. Although cardiac glycosides are beneficial in treating cardiac conditions, there is often a possible risk when used during pregnancy; likewise, US derived herbal preparations are used during pregnancy the foetus is possibly also at risk. Therefore, the purpose of this study was to determine the cytotoxicity of US in vitro using the L929 cell line and primary cultures of chick embryo neurons before studying the effects of in ovo exposure on neuron morphology by electron microscopy.

2. Materials and methods

2.1. Preparation of plant extract

Fresh US bulbs were obtained from Louis Trichardt, Limpopo province, South Africa. Prof. J.J. Meyer, from the Department of

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Botany at the University of Pretoria, confirmed the identity of the plant material. Herbalists' use four teaspoons of powdered plant material to 1 l of water prepared as tea; this is approximately 10 mg of material in 1 ml water. The extracts were prepared by stirring the plant material for 24 h before the solid material was removed by centrifugation. The supernatant, the water extract was filtered using a 0.22 cellulose acetate membrane filter and this extract was used in all experiments. Concentrations in all experiments are reported as milligrams of starting material per millilitre of water.

2.2. Cells

2.2.1. Permanent cell line

Mouse fibroblast (ATCC, CCL1 NCTC clone 929 strain designated L929) cells were obtained from Highveld Biological Company, Johannesburg, South Africa. The cells were plated at a cell concentration of 2×10^4 cells/ml in 24 flat well plates and were kept for 24 h at 37 °C in 5% CO_2 before conducting the experiments. The L929 cell line was then exposed to 0–2.4 mg/ml of US plant extracts for 48 h.

2.2.2. Culture of primary chick neurons (CEN)

Fertilized broiler hatching eggs, obtained from the National Chicks Hatchery in Pretoria, South Africa, were incubated at 37 °C in humidified air. All experiments were performed according to the international, national and institutional rules for animal experiments, clinical studies and biodiversity rights.

The brains from 7-day-old chicken embryos were removed and CEN primary cultures were prepared as follows: the brain tissue was finely cut, washed thrice with Hanks buffered salt solution (HBSS), digested with 0.025% trypsin/HBSS at 37 °C for 20 min, then washed again in Earle's minimum essential medium (EMEM) containing 5% fetal calf serum (FCS) (EMEM/FCS) before a single cell suspension was prepared by tituration. The cells were suspended in EMEM/FCS and plated onto a plastic cell culture surface to allow fibroblast attachment; after 1 h, the unattached neurons were plated at a concentration of 8×10^4 cells/ml onto a polylysine coated 24 flat well plate. The primary cultures were kept at 37 °C in 5% CO_2 and once denditic and axon formation was observed (usually after 48 h), the CEN cultures were then exposed 0–1.2 mg/ml of the US plant extract for 48 h.

2.3. Cytotoxicity assay

2.3.1. MTT assay

The MTT assay was performed to determine cell viability. A 50 μl volume of MTT (0.5 mg/ml in Dulbecco's phosphate buffered saline (DPBS)) was added to the medium in each well and incubated for 90 min at 37 °C. The medium was then discarded and the insoluble formazan was extracted with 200 μl of isopropanol:hydrochloric acid (HCl) solution (24:1 (1 M HCl) and absorbance was measured at 570 nm with a Microplate Reader (ELx800). Viability was expressed as a percentage of the control.

2.3.2. Crystal violet (CV) assay

The CV assay was undertaken to determine the effect of US on CEN cell number. The cells were fixed for 30 min by adding 100 μl of 11% gluteraldehyde to the medium and the plates were then washed with water and dried overnight. The cells were stained for 1 h by adding 300 μl of 0.1% CV solution to each well, the plates were then washed again with water, dried and the dye was extracted in a 200 μl volume of 10% acetic acid solution. Absorbency was measured at 570 nm.

2.4. Statistical analysis of in vitro studies

Results are expressed as mean ± standard deviation (S.D.) of four experiments where each experimental point is the average of four assays. Data for the toxicity of US on the L929 and CEN were statistically evaluated using one way analysis of variance (ANOVA) and p-values of 0.05 or less were considered significant.

2.5. Inoculation of fertilized eggs

In order to determine the effects of US on embryological brain development, the chick embryo model was used. Fertilized broiler hatching eggs were incubated at 37 °C in a humidified incubator and on embryonic day (E) 4, the eggs were briefly taken from the incubator and placed in an air-controlled, aseptic flow hood where a small hole was made and 1 μl of the US water extract (10 μg) was placed onto the chorioallantoic membrane before the hole was sealed with melted candle wax. This procedure was repeated on E6 and on E8 and the embryo was removed from the embryo and the mesencephalon was carefully dissected from the head for electron microscopy studies.

2.6. Electron microscopy

2.6.1. Scanning electron microscopy

The brain material was fixed for 2–4 h in 2.5% gluteraldehyde in 0.075 M sodium phosphate ($NaPO_4$) buffer with pH of 7.4. The tissue was then rinsed thrice in phosphate buffer for 5 m before being fixed for 1 h with 1% OsO_4. The samples were rinsed thrice with phosphate buffer and dehydrated serially in 30, 50, 70, 90% and three times with 100% ethanol. The dehydrated specimens were dried in a critical point drier and received a conductive coating of RuO_4 (Van der Merwe and Peacock, 1999). Coated specimens were mounted and examined with a JEOL 6000F FEGSEM, Field Emission Microscope.

2.6.2. Transmission electron microscopy

The brains of the chick embryos were fixed for 2–4 h in 2.5% gluteraldehyde in 0.075 M sodium phosphate ($NaPO_4$) buffer with pH of 7.4. The tissue was then rinsed thrice in phosphate buffer for 5 min before being fixed for 1 h with 1% OsO_4. The samples were rinsed thrice with phosphate buffer and dehydrated serially in 30, 50, 70, 90% and three times with 100% ethanol. The material was embedded in epoxy resin (Van der Merwe and Coetzee, 1992), followed by ultra-microtome sectioning. The sections were contrasted with a 4% aqueous uranyl acetate