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Assessing the genotoxic potentials of roxarsone in V79 cells using the alkaline Comet assay and micronucleus test

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ABSTRACT

Until recently, knowledge about the genotoxicity of roxarsone *in vitro* or *in vivo* was limited. This study assessed the genotoxicity of roxarsone in an *in vitro* system. Roxarsone was tested for potential genotoxicity on V79 cells by a Comet assay and a micronucleus (MN) test, exposing the cells to roxarsone (1–500 μM) and to sodium arsenite ($NaAsO_2$, 20 μM) solutions for 3–48 h. Roxarsone was found to be cytotoxic when assessed with a commercial cell counting kit (CCK-8) used to evaluate cell viability, and moderately genotoxic in the Comet assay and micronucleus test used to assess DNA damage. The Comet metrics (percentages TDNA, TL, TM) increased significantly in a time- and concentration-dependent manner in roxarsone-treated samples compared with PBS controls ($P < 0.05$), while the data from samples treated with 20 μM $NaAsO_2$ were comparable to those from 500 μM roxarsone-treated samples. The MN frequency of V79 cells treated with roxarsone was higher than that in the negative control but lower than the frequency in cells treated with 20 μM $NaAsO_2$. A dose- and time-dependent response in MN induction was observed at 10, 50, 100 and 500 μM doses of roxarsone after 12–48 h exposure time. The DNA damage in V79 cells treated with 500 μM roxarsone was similar to cells exposed to 20 μM $NaAsO_2$. The uptake of cells was correlated with the DNA damage caused by roxarsone. This investigation depicts the genotoxic potentials of roxarsone to V79 cells, which could lead to further advanced studies on the genotoxicity of roxarsone.

1. Introduction

Organoarsenic compounds, in particular 4-hydroxy-3-nitrobenzenearsonic acid (roxarsone), are regularly added to poultry feed to enhance weight gain of the animals and to improve feeding efficiency. Their allowed concentrations are regulated by authorities such as the Ministry of Agriculture in China [1] and the Food and Drug Authority (FDA) in the United States [2]. It has been estimated that approximately 70% of domestic broiler chickens and a large proportion of swine consume roxarsone [3,4].

Exposure to arsenic (As) contributes significantly to the burden of preventable diseases, especially by leading to increased risks of cancer, diabetes, and cardiovascular diseases. Arsenic has been characterized as a known human carcinogen by the International Agency for Research on Cancer [5] and by the US Environmental Protection Agency (EPA). To date, several studies have analyzed DNA damage caused by sodium arsenite ($NaAsO_2$) by using the Comet assay [6,7]. The toxicity has been shown to be associated with the cellular uptake of arsenical compounds by cells, and the uptake capabilities of arsenicals are highly dependent upon the cell type. The speciation of arsenic compounds is important in the genotoxic and cytotoxic effects in human peripheral lymphocytes [8] and the arsenic-induced genotoxic effects observed in fibroblasts are due to the high uptake of arsenicals into this cell type [9]. However, little is known the genotoxicity of roxarsone *in vitro* or *in vivo* and the capability of its cellular uptake. The alkaline Comet assay can detect inchoate DNA damage and differentiate efficiently and rapidly between genotoxins and cytoxins [10]. The purpose of this study was to evaluate the pentavalent organoarsenical roxarsone as a potentially cyto- and/or genotoxic agent compared to the trivalent inorganic compound $NaAsO_2$. The study was designed to determine by the alkaline Comet assay and the micronucleus test whether roxarsone uptake by V79 cells may have genotoxic consequences.

2. Materials and methods

2.1. Cell culture and reagents

V79 Chinese hamster cells (Shanghai Cell Institute, Chinese Academy of Sciences, China) were cultured in Dulbecco's modified Eagle's media (DMEM; Gibco, USA) that was supplemented with 10% fetal bovine serum (FBS; Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C under 5% CO_2. Solutions

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of roxarsone and $NaAsO_2$ were prepared with bidistilled water and stored at −20 °C until further use. Roxarsone was purchased from the An Da Cheng Pharmaceutical Corporation, Hubei, China, and $NaAsO_2$ was obtained from Sigma-Aldrich (Taufkirchen, Germany). Dimethyl sulfoxide (DMSO), trypsinase and Giemsa stain were obtained from TaKaRa Biotechnology Co. Ltd., China.

2.2. Cytotoxicity testing

V79 cells were treated with different concentrations of roxarsone and $NaAsO_2$ for 3 h, 6 h, 12 h, 24 h or 48 h. All experiments were performed in triplicates.

Cell viability was assessed with the commercial Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan), according to the supplier's instructions. In a CCK-8 assay, the component WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] produces a water-soluble formazan dye upon reduction in the presence of an electron carrier, for which the detection sensitivity of CCK-8 is higher than other tetrazolium salts such as MTT. The cells were inoculated at 3000 cells per well on 96-well plates and cultured overnight. They were re-cultured in FBS-free medium with different concentrations of roxarsone (1, 10, 50, 100 and 500 μM, respectively) or 20 μM $NaAsO_2$. Phosphate-buffered saline (PBS) served as negative control. At the indicated times, 10 μl of the CCK-8 reagent were added to each well, and the plates were incubated for 4 h at 37 °C. Cell counts were then determined for five wells per experimental group from the absorbance at 450 nm wavelength of the reduced CCK-8 reagent, using an automicroplate reader (Infinite M200; Tecan, Austria). Cell viability was expressed as the percentage of viable cells relative to the counts of the untreated cells.

2.3. Alkaline Comet assay

2.3.1. Slide preparation

Slides were prepared using a slightly modified version of the method described by Singh et al. [11]. Briefly, aliquots of the V79 cell suspension (5×10^4 cells/ml) were dispersed into 24-well flat bottom plates. Following a 24 h growth period to allow cell adhesion to the bottom of the wells, the cells were treated with 1, 10, 50, 100 or 500 μM of roxarsone, or 20 μM of $NaAsO_2$, in the FBS-free culture medium. Duplicate wells were employed for each concentration of the arsenicals, while PBS served as negative control. After incubation for 3–48 h, the cells were treated with 0.25% trypsinase, and the suspensions were centrifuged for 5 min at 1000 rpm. The pellets were resuspended with FBS-free culture medium and the densities of the cell suspensions were adjusted to 10^5 cells/ml at room temperature.

Aliquots of 20 μl of all cell suspensions described above were embedded in molten low-melting-point 0.7% agarose (Bio-Red, USA) in PBS and immediately pipetted onto agarose-coated slides, covered with a cover slip, and incubated at 4 °C for 10 min. The coverslips were removed, a third layer of liquefied agarose was applied, the slides were re-covered with coverslips, and re-incubated for another 10 min at 4 °C. After the coverslips were removed, the cells were lysed by incubation for 1 h at 4 °C under dark conditions in the following solution: 10 mM Tris-HCl, 2.5 M NaCl, 100 mM Na_2-EDTA, 0.25 M NaOH, 1% (v/v) Triton X-100 and 10% (v/v) DMSO, pH 12.0. Triton X-100 and DMSO were purchased from Sigma-Aldrich Co., USA.

2.3.2. Electrophoresis analysis

The slides were incubated for 20 min in an electrophoresis buffer containing 1 mM Na_2-EDTA and 300 mM NaOH, pH 12.8, to uncoil the DNA. Electrophoresis was carried out at 0.7 V/cm and 300 mA for 20 min. After NaOH neutralization and removal of the detergent by three washing steps for 5 min each with 0.4 M Tris-HCl, pH 7.5, the cells were stained with ethidium bromide (Biotech, China). All steps were conducted under dark conditions to prevent further DNA damage.

The electrophoretograms were observed at 400× magnification with a fluorescence microscope (Olympus BX40, equipped with a 515–560 nm excitation filter) connected to a Sony 3CCD-IRIS color video camera. Images of 100 randomly selected cells (50 from each replicate slide) were analyzed for each sample and the Comet parameters were evaluated.

2.3.3. Slide scoring

The cells were analyzed using the Comet scoring program CASP-1.2.2 (http://casp.sourceforge.net/index.php). The following parameters were estimated for each Comet assay: percentage of migrated DNA in the tail (% TDNA), tail moment (TM) and tail length (TL).

Cell damage was visually scored according to the % TDNA parameter [12] into five categories:

(1) C0: undamaged, $0 \leq$ % TDNA ≤ 5%.
(2) C1: low damage, 5% < % TDNA ≤ 20%.
(3) C2: minor damage, 20 < % TDNA ≤ 40%.
(4) C3: intermediate damage, 40% < % TDNA ≤ 60%.
(5) C4: high damage, % TDNA > 60%.

For each exposure time point at least 300 Comet assays were examined for % TDNA, TM, and TL.

2.4. Determination of the cellular uptake of arsenic

Cells harvested in the Comet assays were washed three times with PBS and suspended in 2 ml of fresh culture medium. After cell counting, the suspensions with 10^6 cells in 2 ml were centrifuged for 5 min at 1000 rpm, and the pellets were resuspended in 2 ml of triple-distilled water for at least 30 min to lyse the cells. Total arsenic concentrations in the cell extracts were determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500a, Agilent Technologies, Germany).

The spectrometer was operated at 1260 W rf-power, with argon flows of 15 L/min (plasma gas), 0.98 L/min (carrier gas), and 0.9 L/min (auxiliary gas). The sample solutions (up to 1:100 in dilution) were delivered at 0.3 ml/min to a Babington nebulizer and routed through a double-pass Scott-type spray chamber maintained at 2 °C. The signals for ^{75}As (1000 ms), ^{77}Cl (500 ms), and ^{115}In (1000 ms) were monitored. Apart from the signals obtained at $m/z = 75$ for the arsenic analyte and at $m/z = 115$ for the internal indium standard, the signal at $m/z = 77$ was monitored in order to control arsenic chloride interference. Quantification was performed by external calibration with an arsenate standard solution (Merck, China) and validated by analyzing a certified reference material (SERO B2 SERO AS, Billingstad, Norway). Both blank levels and sensitivity were controlled every ten analyzes in order to correct for drifts in instrument background and sensitivity levels.

2.5. In vitro micronucleus test

Cells harvested in the Comet assays were washed three times with PBS and centrifuged at $250 \times g$ for 10 min at room temperature. The pellets were dissolved in 10.0 ml of 0.075 M KCl solution and incubated at 37 °C for 20 min. After a second centrifugation step the pellets were resuspended in 10.0 ml of freshly prepared fixative solution (methanol:acetic acid/3:1). The centrifugation and fixation steps were repeated for three more times and the pellet was resuspended in 0.5 ml of fresh fixative solution. Smears of the suspensions were administered on clean slides and dried in laminar flow for 2 h. The slides were stained with 5% Giemsa solution for 10–15 min at room temperature, followed by drying in a laminar hood for 1–2 h. Five hundred cells were scored for micronuclei under light microscope, as described by Fenech et al. [13].

2.6. Statistics

Statistical evaluation was carried out using the SPSS software package (SPSS, Chicago, IL, USA). ANOVA with Tukey's post-hoc test was performed on the cytotoxicity and Comet assay data. Differences in the numbers of micronuclei were analyzed using Fisher's exact test.

3. Results

3.1. Cell viability

The viability of cells treated with roxarsone and $NaAsO_2$ were found to be concentration- and/or time-dependent, with the exception of cells incubated at a concentration of 1 μM roxarsone, where the survival was similar to the vehicle control. The viability of cells exposed to various concentrations of roxarsone was higher than that of cells in $NaAsO_2$ at similar exposure times. The lowest cell viability exceeded 85% after 48 h exposure to $NaAsO_2$; the data are summarized in Fig. 1.

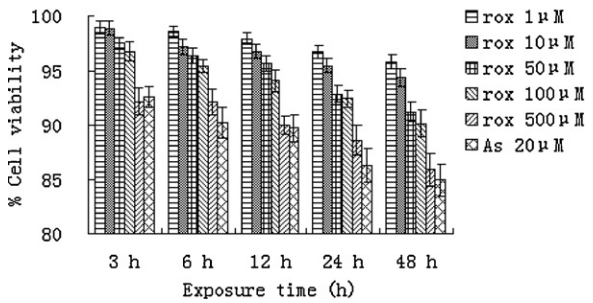

Fig. 1. The cytotoxicity of roxarsone (rox) and $NaAsO_2$ (As). The percentage of cell viability is given in relation to the untreated control (SD < 5%). Each column represents the mean ± SD of triplicate assays.