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Original Research

Toxicity of Hoechst 33342: implication in side population analysis

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Abstract: In the current study, we discovered that Hoechst 33342 (H342) could produce significant toxicity to the human neuroblastoma SK-N-SH cells at concentrations typically used to identify side population (SP) cells. At 3.75 and 5 μg/ml, H342 induced cell death and apoptosis. Flow cytometry analysis revealed cell cycle arrest at S-phase. Our findings caution against using H342 for SP analysis without considering differential susceptibility of different cells to H342. Specifically, concentration of H342 needs to be adjusted carefully to minimize toxicity for each cell line.

Key words: Hoechst 33342, Toxicity, Side population, SK-N-SH cell line, Neuroblastoma.

Introduction

Side population (SP) cells are enriched in stem cells (1). Hoechst 33342 (H342), a fluorescent dye, is exported preferentially by stem cells. Therefore it is used widely to identify SP cells (1). At high concentration (2,3), H342 is cytotoxic binding to DNA. Then, in published data, the concentrations used for SP analysis vary from 2.5 to 20 μg/ml regarded as non-cytotoxic (4-6).

During the period of SP analysis in human neuroblastoma SK-N-SH cell line, we found incidentally that H342 could produce significant cytotoxicity in this cell line at concentrations commonly used to label SP cells. Therefore, we carried out a series of experiments to examine systematically the cytotoxicity of H342 in SK-N-SH cells. Hep-2 and HCCLM3 cells were included as controls. The underlying mechanism of H342 toxicity was also investigated.

Materials and Methods

Cell culture

Human neuroblastoma SK-N-SH cell line was obtained from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Human laryngeal squamous cancer Hep-2 cell line was kindly provided by Dr. Guanglun Wan (Eye, Ear, Nose and Throat Hospital, Fudan University, Shanghai, China). Human hepatocellular carcinoma HCCLM3 cell line was kindly provided by Dr. Guoming Shi (Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China). SK-N-SH and Hep-2 cells were cultured in RPMI 1640 medium (Gibco, Scotland, UK). HCCLM3 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA). Both RPMI 1640 and DMEM media contained 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany), 100 U/ml of penicillin and 100 μg/ml of streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

Cell morphology observation

Exponentially growing cells were detached with 0.25% trypsin/0.02% EDTA and suspended at 1×10^6 cells/ml in phosphate-buffered saline (PBS) supplemented with 2% FBS. The cells were stained with H342 (Sigma) at the following concentrations (5,7,8): 0, 1.25, 2.5, 3.75 and 5 μg/ml for SK-N-SH and Hep-2 cells (60 minutes); 0, 5, 10, 15, and 20 μg/ml for HCCLM3 cells (90 minutes) at 37°C. After the staining, cells were washed with PBS twice, and seeded in a 3.5cm plate (2×10^5 cells). Cell morphology was observed after 72 hours culture; photographs were taken under an inverted microscope. All experiments were carried out in triplicate.

CCK-8 (Cell counting kit-8) assay

After H342 staining, cells were seeded in 96-well plates at a cell density of 1×10^4 cells/well in 0.2 ml of culture medium containing 10% FBS and were treated 24h. The quantification of viable cells were recognized at 24, 48, and 72 h after treatment using CCK-8 assay (Dojindo Laboratory Co, Ltd, Kumamoto, Japan) (9). Ten microliter cell counting kit solution was added into each cell well, and then were incubated for 2 hours at 37°C. We used a microplate reader (Model 680, Bio-Rad, Hercules, Calif) to measure the absorbance value (AV) at 450 nm. We used the results as a correlate of viable cell number. The surrounding wells of the 96-plate were treated with medium. There were four wells in each group. We repeated 3 times during the whole experiment.

Flow cytometry (FCM) analysis of cell death and apoptosis

Cell death and apoptosis were detected by Annexin V-FITC (BD Biosciences, CA, USA) and propidium iodide (PI, Sigma) staining FCM (BD Biosciences). After H342 staining, an aliquot of 10^5 SK-N-SH cells were

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suspended in 195µl binding buffer, then mixed with 5µl of Annexin V-FITC and incubated for 10 minutes at room temperature in the dark. Moreover, cells were collected with brief centrifugation, resuspended in 190µl binding buffer, mixed with 10µl of PI, and incubated for 5 minutes at room temperature in the dark prior to FCM analysis. Finally, cell death was defined as positive PI staining and Annexin staining and apoptosis was defined as positive Annexin staining instead of PI staining.

Cell cycle distribution analysis

SK-N-SH cells were cultured for 72 hours in 10% FBS after H342 staining prior to fixation with 70% ice cold ethanol overnight at 4°C. DNA was stained with 50µg/ml PI containing 100µg/ml RNase for 30 minutes at 37°C before FCM analysis. The fraction of the cells in G0/G1, S and G2/M phase was analyzed by using cell cycle analysis software (CELL Quest version 5.1, BD Biosciences).

FCM analysis of SP cells

SK-N-SH cells were collected and suspended at 1×10^6 cells/ml in PBS supplemented with 2% FBS. Next, the cell suspension was divided into four groups and stained with 1.25, 2.5, 3.75 and 5 µg/ml H342, respectively, at 37°C for 60 minutes in the presence or absence of 50 µmol/L verapamil (Sigma). Cells were then stained with 1µg/ml PI. FCM analysis was performed using Moflo (DakoCytomation, Fort Collins, CO, USA) as described (10).

Statistical analysis

All statistical analyses were performed using SPSS 13.0 (SPSS Inc, Chicago, Ill). Data were presented as mean ± SEM. Significant differences were evaluated by Student's t test and one-way ANOVA. Significance was defined at $p < 0.05$.

Results

Cell morphology

At 1.25 and 2.5µg/ml, H342 did not produce any observable morphological change in SK-N-SH cells. At higher concentration of 3.75 and 5µg/ml, most of the SK-N-SH cells were dead and detached from the culture wall (Fig.1). The morphology of Hep-2 and HCCLM3 cells was not affected by H343 at any concentration included in this study (Fig. S1 and Fig. S2).

Cell growth

Assay with CCK-8 demonstrated that the number of viable SK-N-SH cells decreased at 24 hours after staining with 3.75 and 5 µg/ml H342 (average AV: 0.170 ± 0.008 and 0.148 ± 0.012 vs. 0.194 ± 0.019 in the blank control; $p < 0.05$ and < 0.01, respectively). Such an effect was also evident at 48 and 72 hours ($p < 0.01$ or 0.05). At 1.25 or 2.5 µg/ml, H342 did not affect the number of viable SK-N-SH cells at any time point (Fig. 2) and the number of viable Hep-2 and HCCLM3 cells was not affected by H342 at any concentration at any time point (Fig. S3 and Fig. S4).

Cell death and apoptosis

H342 concentration-dependently increased the number of dead and apoptotic cells in the SK-N-SH cell line (Fig. 3 and Fig. S5). At 1.25 and 2.5 µg/ml, cell death or apoptosis was not affected by H342. At higher concentrations of 3.75 and 5 µg/ml, H342 increased significantly cell death (25.4% and 29.2%, respectively vs. 17.5% in blank control; $p<0.01$ for both) and apoptosis (8.7% and 10.9%, respectively vs. 3.9% in blank control; $p<0.01$ for both).

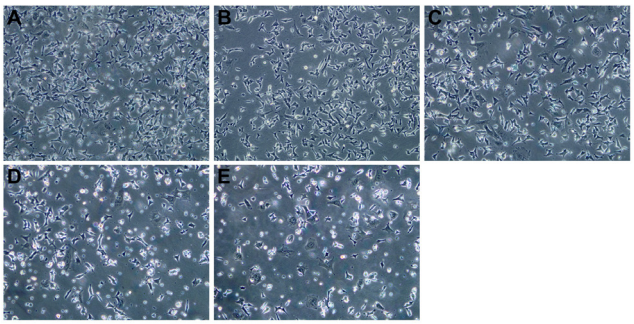

Figure 1. Morphology of SK-N-SH cells cultured in normal growth medium for 72 hours following staining with 0 (A), 1.25 (B), 2.5 (C), 3.75 (D) and 5 (E) µg/ml H342. The detachment of most of SK-N-SH cells from the culture plates was observed in 3.75 and 5 µg/ml H342-treated groups. The cell morphologies shown were representative of three independent experiments with similar findings (original magnification ×200).

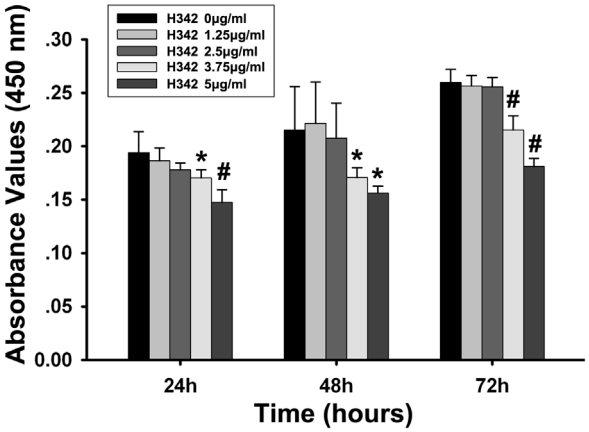

Figure 2. CCK-8 assay for SK-N-SH cells cultured in normal growth medium for 24, 48 and 72 hours following staining with indicated concentrations of H342. Data were expressed as mean ± SEM and representative of an average of three independent experiments. Significant differences from 0 µg/ml H342 group were indicated by * $p < 0.05$, # $p < 0.01$.

Cell cycle analysis

The mean percentage of cells in the S-phase was 15.2% ± 1.9% in SK-N-SH cells not exposed to H342. At 2.5, 3.75 and 5 µg/ml, H342 increased the S-phase cell population to 20.9% ± 1.8% ($p < 0.05$ for 2.5 µg/ml vs. blank control), 31.6% ± 5.4% and 40.0 % ± 1.9%, respectively ($p < 0.01$ for both 3.75 and 5 µg/ml vs. blank control) (Fig. 4 and Fig. S6).

SP cell percentage did not vary significantly among different H342 concentrations. However, the dot plot of cells in FCM was atypical at lower concentrations of 1.25 and 2.5 µg/ml (Fig. 5).

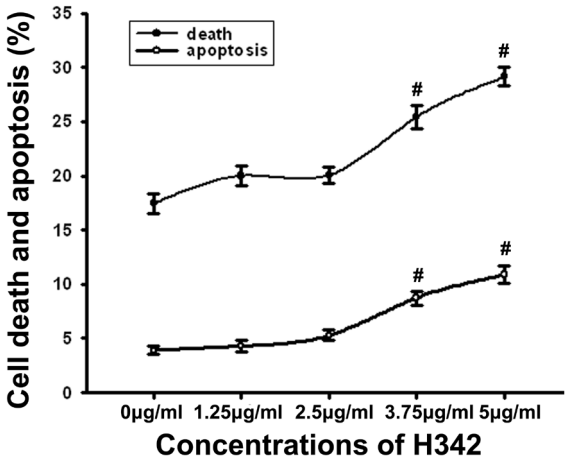

Figure 3. Annexin V-FITC/PI staining FCM analysis of cell death and apoptosis. SK-N-SH cells were treated with indicated concentrations of H342 for 60 minutes and then stained with Annexin V-FITC/PI to analyze dead and apoptotic cell populations. The dose-dependent death and apoptosis induced by H342 were observed (Data were expressed as mean ± SEM). Significant differences from 0 μg/ml H342 group were indicated by # $p < 0.01$.

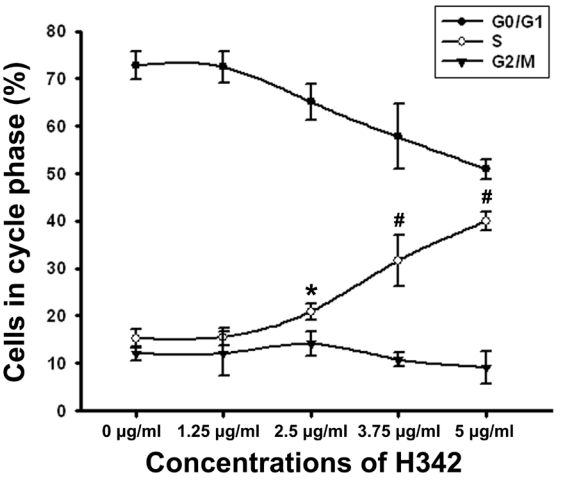

Figure 4. DNA FCM analysis on cell cycle distribution. SK-N-SH cells were analyzed by DNA FCM after culturing in normal growth medium for 72 hours following staining with indicated concentrations of H342. H342 concentration-dependent cell population increase in S-phase was observed (Data were expressed as mean ± SEM). Significant differences from 0 μg/ml H342 group were indicated by * $p < 0.05$, # $p < 0.01$.

Discussion

H342 at <20 μg/ml is generally believed to be nontoxic, and used commonly for SP analysis (4-6). Five microgram per milliliter is the most often used concentration (11,12). Our results demonstrated that H342 is not toxic to Hep-2 and HCCLM3 cells during this concentration range. SK-N-SH cells, in contrast, were susceptible to H342 toxicity at 3.75 and 5.0 μg/ml. At common concentration, significant cytotoxicity of H342 has also been reported in other cell lines, including C6, MCF7 and SK-OV3 (13,14). SP analysis reported by previous studies (15-17) using H342 staining at these concentrations, therefore, may be limited without considering potential toxicity of H342 to cells.

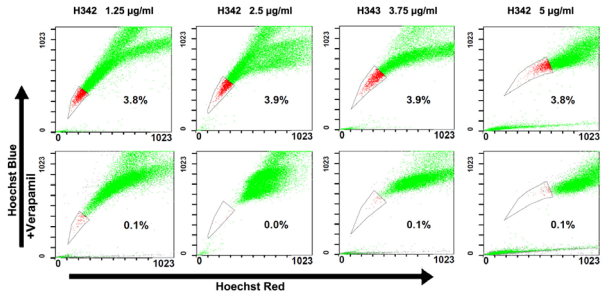

Figure 5. The percentage of SP cells detected by indicated concentrations of H342 in SK-N-SH cells. The percentage of SP cells detected by 1.25, 2.5, 3.75 and 5μg/ml H342 were 3.8, 3.9, 3.9 and 3.8% respectively (upper 4 panels). SP cells disappeared when correlated with H342 and verapamil (corresponding lower 4 panels). There were no significant differences among SP cells percentage detected by these four concentrations of H342. Data were representative of one of three similar experiments.

H342 binds preferentially to A-T rich region in DNA minor grooves and inhibits the catalytic activities of many DNA enzymes (18,19). Cell types differ with respect to their cytotoxic responses to H342 (20). We found that SK-N-SH cell line is different sensitive to the toxicity of H342 with Hep-2 and HCCLM3 cell lines further strengthened this cognition. Our experiments showed that H342 causes rapid concentration-dependent cell death as well as apoptosis in SK-N-SH cells. The exact mechanisms of this phenomenon are not understood completely, which needs further study.

Montanaro et al (6). demonstrated a decrease in the percentage of SP cells in bone marrow, muscle and skin tissues with increasing H342 concentration. But, in the current study, this phenomenon was not observed in SK-N-SH cells at a H342 concentration range from 1.25 to 5μg/ml. We recommend using 2.5-2.8μg/ml H342 to perform SP analysis in the SK-N-SH cell line for the following reasons: 1) at 2.5μg/ml, H342 did not have significant toxicity to SK-N-SH cells; 2) a previous study found that 2.8μg/ml H342 is required to stain cells with 30-60 min incubation. Validity of SP analysis using 2.8μg/ml H342 has been confirmed in our previous studies (21, 22).

In summary, our findings caution against using H342 for SP analysis without considering differential susceptibility of different cells to H342. Specifically, concentration of H342 needs to be adjusted carefully to minimize toxicity for each cell line.

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