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Cytotoxicity of HC Orange NO. 1 to L929 fibroblast cells

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ABSTRACT

HC Orange No. 1 (HCO1, 2-nitro-4′-hydroxydiphenylamine) (CAS No. 54381-08-7) is used as a colorant in commercial hair dyes. The cytotoxicity of HCO1 to L929 mouse connective tissue fibroblast cells was determined by use of a battery of endpoints that included electron transport activity determined by the MTT assay and fluorescence microscopy with Hoechst 33258, DNA agarose gel electrophoresis detected by ethidium bromide and flow cytometry. Cytotoxicity of HCO1 was found to be concentration- and time-dependent with EC_{50} values of 28.0 and 12.0 mg/L after 24 h or 48 h exposure, respectively. The mechanism of toxicity of HCO1 was more characteristic of necrosis than apoptosis. These results can be used to evaluate the relative *in vitro* toxicity of other dyes and to elucidate their mechanisms of toxicity so that accurate assessment of risks to humans can be made, especially from dermal exposure.
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1. Introduction

More than 60% of European women color their hair, as do 5–10% of men while half of all American women color their hair, and the number of men is increasing. In Asia, use of hair dye is also popular, especially for younger people. Epidemiology studies of individuals occupationally exposed to hair dyes have revealed association between hair dyes and the incidence of cancers (Rodstein et al., 1994; La Vecchia and Tavanj, 1995). The potential carcinogenic effect of hair dye products was observed (Cook et al., 1999; Nagata et al., 1999; Gago-Dominguez et al., 2001) and mutagenic carcinogens were found in hair dye products (Ames et al., 1975; Flamm, 1985). Hair dyes are applied throughout the hair including the root, which is very close to the scalp, where the blood supply is among the greatest in the human body (Nohynek et al., 2004). Thus, carcinogenic components could possibly enter the bloodstream. It has also been reported that women who dye their hair for 20 year or more have almost twice the risk of developing rheumatoid arthritis (http://www.newscientist.com). To ensure safety of hair dye products for consumers, the European Commission has banned 22 hair dye substances that could potentially cause bladder cancer if used for long periods of time. The ban takes effect on December 1, 2006. 2,4-Diaminodiphenylamine, which is one of 22 hair dye, was found to be toxic and caused methemoglobinemia (Bazin et al., 1986), renal papillary necrosis (Lenz and Carlton, 1990), and toxicity to aquatic biology Luminescent Bacterium (Drzyzga et al., 1995).

HC Orange No. 1 (HCO1, 2-nitro-4′-hydroxydiphenylamine) (CAS No. 54381-08-7) is widely used as a colorant in semi-permanent hair dyes. Product formulation data submitted to the US Food and Drug Administration (US FDA) in 1996 reported that HCO1 was used in a total of 95 hair dyes (US FDA, 1996). The greatest concentration reported in a formulation was 0.15%, but information from manufacturers suggested that greater concentrations might be used in the future (Pang and Fiume, 1998). As much as 1.28% of applied HCO1 was absorbed through cadaver skin in 24 h. Acute oral exposure for 14 d produced little mortality. However, in rats fed 0.5% HCO1 for 4 weeks, body weight was less, and liver and kidney weights greater, relative to controls. There was no evidence of reproductive or developmental toxicity in rats fed up to 1.25% HCO1, or in a multi-generation study in which the skin of rats was painted with 0.15% HCO1. The evidence (Burnent et al., 1976) suggests that HCO1 is a mild ocular irritant, does not cause skin irritation, sensitization, or photosensitization in animal trials or clinical tests with humans. Hepato-cellular and parathyroid hyperplasia were observed in the dermal carcinogenicity study (Pang and Fiume, 1998), but the overall finding indicates that HCO1 is not geno-toxic. The greatest concentration that did not cause significant sensitization in clinical tests was 3% (Burnent et al., 1976; Pang and Fiume, 1998). Other reports about the chemical are relative very few. The

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results of previous studies (Liu et al., 2007) have shown that HCO1 is toxic to *Daphnia magna*, Zebrafish (*Brachydanio rerio*) embryos, and goldfish (*Carassius auratus*). The results of another study (Sun et al., 2006) also indicated HCO1 can be concentrated by goldfish and the bioconcentration factor is 224. Hepatic antioxidant defense parameters of fish such as the content of reduced glutathione (GSH), the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione *S*-transferase (GST) were sensitive to the HCO1 exposure, which indicates that increased oxidative stress was present in fish exposed to HCO1, possibly due to the presence of redox-active metabolites (Orhan et al., 2006). Because human skin is not an impermeable barrier for some topically applied substances, the investigation of cutaneous absorption/penetration of hair dyes and their ingredients was initiated (Steiling et al., 2001). HCO1 is widely used in semi-permanent hair dye, while a similar molecule, 2,4-diaminodiphenylamine, was banned from further use in hair dyes, on December 1, 2006 as mentioned above. Given the size of the exposed populations in addition to the large numbers of occupationally exposed professionals, we have followed these events with great interest, but also with growing unease about HCO1.

L929 cells, derived from an immortalized mouse fibroblast cell line, are internationally recognized cells that are routinely used in *in vitro* cytotoxicity assessments (Cochrane et al., 2003). In the present study, the L929 mouse connective tissue fibroblast cell line, which is a useful model for screening chemical toxicity from dermal exposure, was used to evaluate the basal toxicity of HCO1.

Thus, the toxicological properties of HCO1 on L929 cells were conducted. The effect of HCO1 on L929 cells was determined as a function of concentration and time by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Mosmann, 1983; Carmichael et al., 1987). The toxicity of HCO1 to L929 cells was also investigated by use of fluorescence microscopy, DNA agarose gel electrophoresis and flow cytometry.

2. Materials and methods

2.1. Materials

HCO1 (purity > 99%) was purchased from Nanjing King-Pharm Co., Ltd. (Nanjing, China). Stock solutions of 10,000 mg/L HCO1 were prepared in dimethyl sulfoxide (DMSO) and maintained at 4 °C. RPMI Medium 1640, supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin and MTT were purchased respectively from Gibco/BRL Company (Grand Island, NY, USA) and Sigma Company (St. Louis, MO, USA). Propidium iodide (PI), ethidium bromide (EB), 2,2-(4-hydroxyphenyl)-6-benzimidazole-6-(1-methyl-4-piperazyl) benzimidazole hydrochloride (Hoechst 33258) and proteinase K was purchased from Huashun Company (Shanghai, China). Agarose, DNA marker, RNase A were from TaKaRa Biotech. Co., Ltd. (Dalian, China). Polystyrene 96- and 24-well culture plates were obtained from Nunc Denmark Company (Nunc, Denmark). All other chemicals were commercial available products of analytical grade.

2.2. Cell culture

L929 cells, derived from the mouse connective tissue fibroblasts were obtained from American Type Culture Collection (ATCC). Cells were cultured in RPMI medium 1640, supplemented with 10% heat-inactivated FCS, 100 U penicillin/ml, and 100 μg streptomycin/ml, at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Acute toxicity test

A concentration of 1.0×10^5 cells/ml was seeded in a 96-well culture plate at a density of 1.0×10^4 cells/well in 100 μl medium. When cells had reached approximately 90% confluence, the original medium was replaced with 100 μl serum-free RPMI Medium 1640. A pilot experiment was conducted to determine the minimum concentration of 100% inhibition and the maximal concentration of HCO1 that could be used in subsequent studies. L929 cells were exposed to 5.0, 10, 15, 30, 50, 70, or 90 mgHCO1/L for 24 h or 2.5, 5.0, 7.5, 10, 15, 20, 25, or 30 mgHCO1/L for 48 h. The greatest concentration almost completely inhibited cell growth and the least concentration inhibited cell growth by only 10–15%. All experiments were performed in at least triplicate. The study stock solutions were prepared by dissolving HCO1 in less than 0.01% of DMSO to avoid co-solvent effects. Responses of cells in replicate wells were averaged so that it was based on the mean of a minimum of 10 wells. RPMI Medium 1640 and 0.01% of DMSO are set as controls. After different durations of exposure, cells were subjected to the following assays: (1) morphological images of cells were taken with XDS-1B inverted optics microscope; (2) cells were washed twice in phosphate-buffered saline (PBS), 0.2 ml of MTT solution of (0.5 mg/ml in RPMI Medium 1640) were added to each well and then incubated for 4 h at 37.0 °C to allow MTT metabolism. An aliquot of 150 μl of DMSO solvent was added to each well and the plate was incubated for 30 min at room temperature. The absorbance at 570 nm was measured with an Enzyme-Linked Immuno-Sorbent Assay plate reader

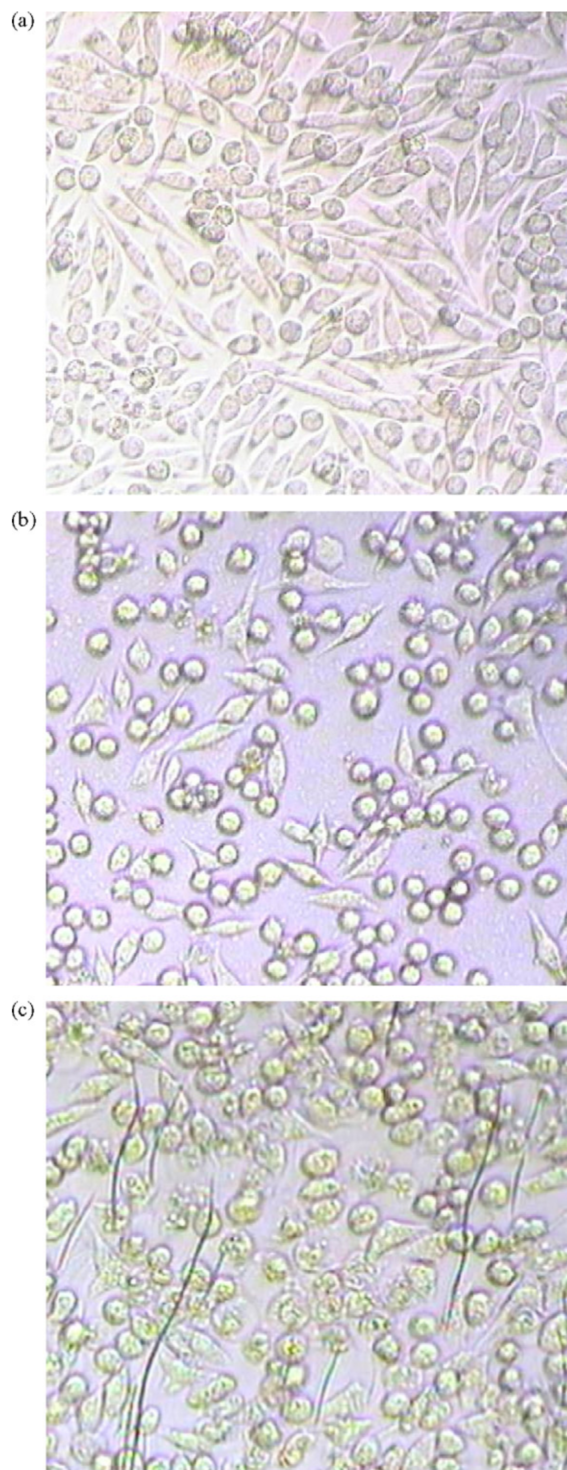

Fig. 1. Morphological changes of L929 cells exposed to HCO1 (a) Control (b) 30 mg HCO1/L for 24 h (c) 30 mg HCO1/L for 48 h. One representative experiment of three is shown.

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(TECAN A-5082, Austria) with absorbance at 630 nm used as a reference. The difference between absorbances at 570 and 630 nm, was used as an index of the cell viability. The difference between A570 and A630 from the untreated cultured cells was used as the 100% viability value. The percent viability was calculated using the following equation:

$$\left[\frac{(A570 - A630)\,\text{sample}}{(A570 - A630)\,\text{control}}\right] \times 100\% \quad (1)$$

2.4. Fluorescence microscopy

Fluorescence microscopy was used to investigate the cytotoxic effect of HCO1 on the nuclei of L929 cells. Cells (1.0×10^5) cultured on glass slides in 24-well plates were exposed to HCO1 for 24 h. After exposure cells were carefully washed with PBS and fixed in PBS buffer containing 2% glutaraldehyde at 4 °C for 30 min. Cells were stained in the dark with 50 μM Hoechst 33258 for 15 min then washed twice with PBS. Hoechst 33258 is a low molecular weight, fluorescence probe, which can easily penetrate the cell membrane and stain nuclei so that their morphology can be examined by fluorescence microscopy. Based on their fluorescent images, necrotic cells were differentiated from apoptotic cells. A minimum of 100 cells was observed in each replicate.

2.5. DNA fragmentation assay

DNA fragments (180–200 bp) were observed by use of EB, which is a type of fluorescence probe staining after separation by agarose gel electrophoresis. To further analyze the cytotoxic effects of HCO1, cells at a density of 1.0×10^6 grown in 30 cm^2 culture flasks were treated with HCO1 of different concentrations for 24 h. Both attached and unattached cells were harvested and washed with ice-cold PBS, then lysed with 20 μl dissolve-buffer containing 20 mM EDTA, 100 mM Tris–HCl (pH 8.0) and 0.8% (w/v) SDS for 1 h. Next, the lysate was incubated with 20 mg/ml RNase A at 37 °C for 1 h, then proteinase K was added to a final concentration of 5 mg/ml. The mixture was incubated for another 3 h at 50 °C. An aliquot of 5 μl loading buffer (0.01 ml 1 M Tris, pH 7.5; 0.04 ml 0.5 M EDTA, pH 7.5; 0.5 ml glycerol; 0.8 mg bromophenol blue; added H_2O to 1 ml) was added to the sample. Samples were subjected to electrophoresis in a 1.5% agarose gel for 4 h at room temperature at 40 V. The DNA fragmentations were made visible by EB staining, and photographed under UV light at 254 nm.

2.6. Flow cytometry analysis

Cytotoxic effects by HCO1 were also examined by flow cytometric analysis (Darzynkiewicz et al., 1997). Both attached and detached L929 cells were collected after treated with HCO1 for 24 h and suspended with ice-cold PBS. Cells were fixed in 70% ethanol at 4 °C for at least 1 h. After fixation, cells were washed twice with PBS, and stained with 1 ml 50 μg/ml PI for 30 min. Fluorescence analyses were performed on a FACSCalibur flow cytometer (Becton Dickenson, San Jose, CA). PI fluorescence were collected through 630 nm filter and displayed by logarithmic amplification. The percentages of cells in the different phases (G_0/G_1, S, and G_2/M) of the cell cycle as well as apoptotic cells or necrotic cells were estimated from DNA forward light scatter (cell size) histograms using the CellQuest program.

2.7. Statistical analysis

Each experiment was performed at least three times. Data were expressed as mean ± S.D. and analyzed using the Sigmoidal software with three parameters. Analysis of variance (ANOVA) was used to determine if there were differences among treatments. If the overall ANOVA indicated the presence of statistically different treatment effects, Dunnett's *t*-test was used to determine which groups were significantly different from the control. The EC_{50}, defined as the concentration required reducing the response to 50% of the control, was calculated using probit analysis regression techniques on untransformed values.

3. Results

3.1. Cytotoxicity of HCO1 to L929 cells

HCO1 caused morphological anomalies of L929 cells that could be observed under light microscopy (Fig. 1). Normal L929 cells were fairly transparent, attached, and well spaced in RPMI Medium 1640 (Fig. 1a). When exposed to 30 mg/L HCO1 for 24 h, L929 cells became more round and not touching adjacent cells (Fig. 1b) and when exposed 48 h, become swollen and lost membrane integrity (Fig. 1c). Also transparency of cells became less and the amount

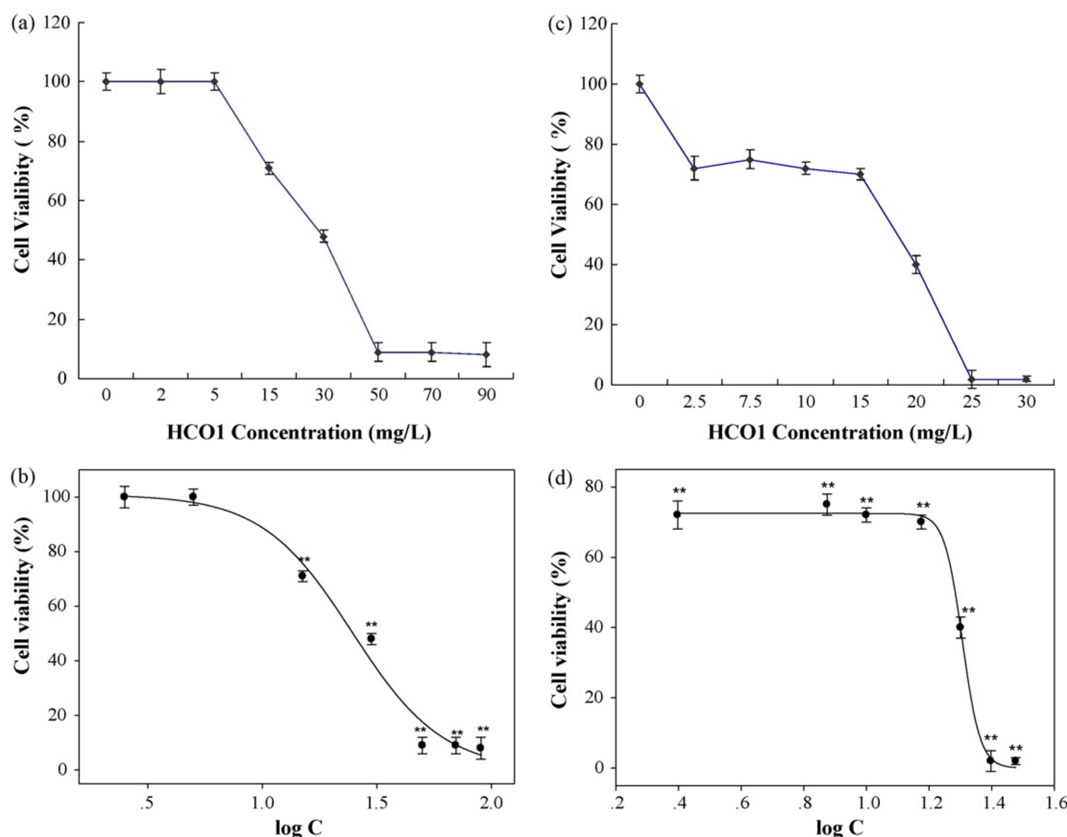

Fig. 2. Viability (determined by MTT assay) of L929 cells exposed to HCO1 (a,b) 24 h (c,d) 48 h. Data are expressed as means ± S.D. ($n = 3$). (b) and (d) (**) highly significantly different from control, $p < 0.01$.

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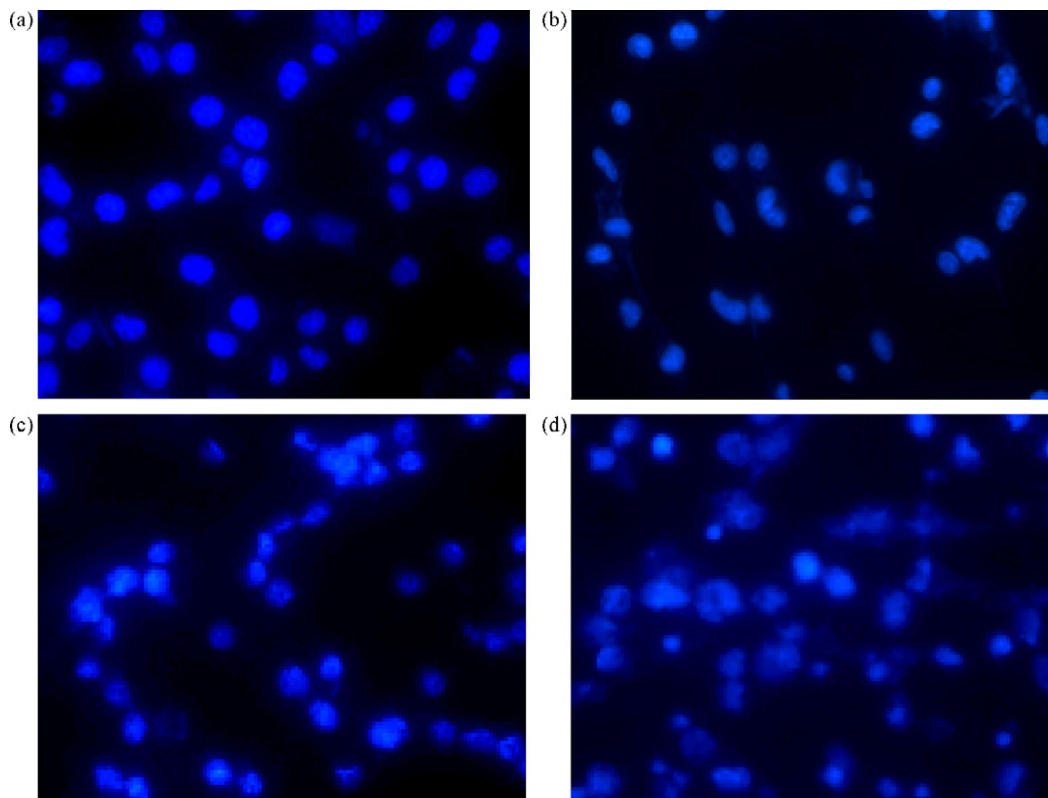

Fig. 3. Effects of HCO1 on L929 cells observed by fluorescence microscopy (magnification 400×). Cells exposed to HCO1 for 24 h were stained by Hoechst 33258. (a) Control; (b) 5 mg HCO1/L; (c) 10 mg HCO1/L; (d) 30 mg HCO1/L.

of cell fragments significantly increased relative to that of the control.

Exposure to HCO1 caused cytotoxicity expressed as changes in MTT (Fig. 2). L929 cells in RPMI Medium 1640 and DMSO are all growing well. There was little effect on cell viability at HCO1 concentrations between 2.5 and 5 mg/L, for 24 h (Fig. 2a), but concentrations of HCO1 from 5 to 50 mg/L caused a decrease in viability index of 10%. Then we use the Sigmoidal software to regress the data (Fig. 2b) ($R^2 = 0.9836$, $R^2_{adj} = 0.9754$, $F = 119.9$). The effective concentration for half maximal response (EC_{50}) for viability index of L929 cells exposed for 24 h was 28.0 mg HCO1/L. Cell viability was inversely proportional and dose-dependent when exposed to concentrations of HCO1 from 0 to 15 mg/L for 48 h (Fig. 2c). However, between concentrations of 15–25 mg HCO1/L, cell viability was inversely proportional to concentration and ranged from 70 to 5%, respectively. In the same way, the EC_{50} value for the MTT cell viability index for cells exposed to HCO1 for 48 h was 12.0 mg/L (Fig. 2d) ($R^2 = 0.9979$, $R^2_{adj} = 0.9969$, $F = 957.4$). These results demonstrate that the toxicity of HCO1 to L929 cells was concentration- and time-dependent. The results of the MTT assay were consistent the results of the light microscope observations.

3.2. *Effects of HCO1 on nucleus*

Morphological anomalies in nuclei were observed when cells were exposed to 5 mg/L, 10 mg/L, or 30 mg HCO1/L for 24 h (Fig. 3). The nuclei of the control cells are stained with uniform color blue and well-defined edges in RPMI Medium 1640 (Fig. 3a). When exposed to 5 mg HCO1/L, nuclei shrank and became more crescent-shaped with more fragmented nuclei (Fig. 3b). This result suggests that cells were undergoing apoptosis. Whereas, exposure to 10 mg/L

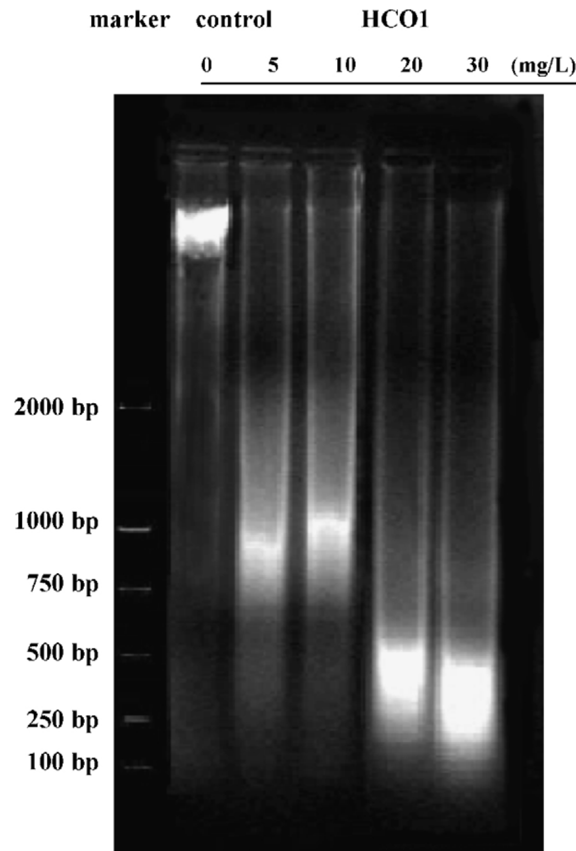

Fig. 4. Agarose gel electrophoresis analysis of DNA fragmentations in L929 cells after exposure to HCO1 for 24 h. One representative experiment of three is shown.

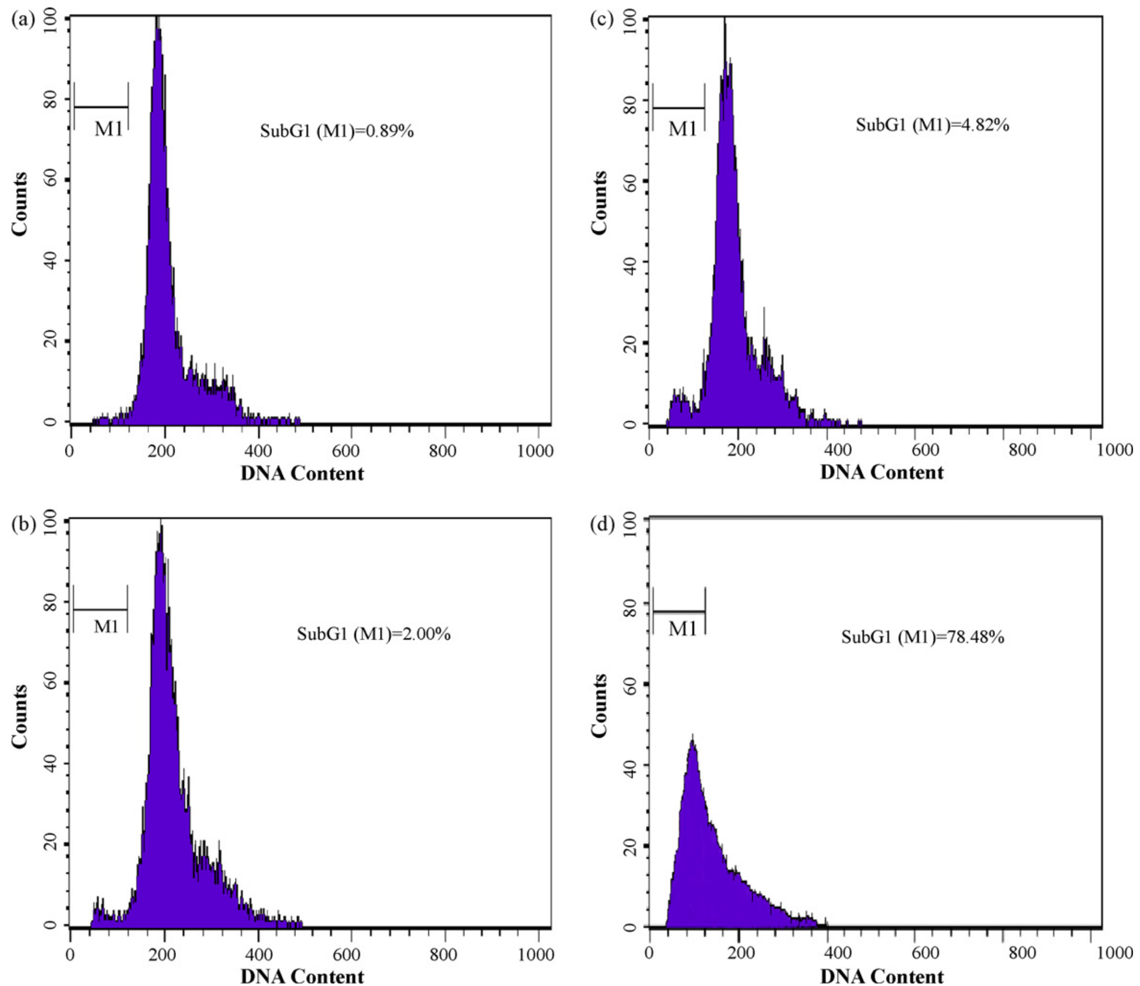

Fig. 5. Proportion (%) of L929 cells exposed to HCO1 for 18 h that were in the subG_1 phase. (a) Control; (b) 14 mg HCO1/L; (c) 28 mg HCO1/L; (d) 42 mg HCO1/L.

or 30 mg HCO1/L, most of the nuclei swelled and lost membrane integrity, which is typical of necrotic cells (Fig. 3c and d).

3.3. *DNA fragmentation*

HCO1 caused fragmentation of chromosomal DNA of L929 cells (Fig. 4). DNA ladder fragmentations of approximately 200 bp which is characteristic of apoptosis, however, they were not observed in this study. Rather, DNA smears with series of low molecular weight were observed. The average molecular weight of DNA smear was inversely proportional to HCO1 concentrations from 5 to 30 mg/L, with a decrease from 900 to 300 bp. This result is characteristic of random cleavage of DNA during necrosis.

3.4. *Flow cytometry*

Exposure to HCO1 caused changes in relative proportions of L929 cells in G_0/G_1 and G_2/M phases of the cell cycle as determined by FACS (Fig. 5). FACS can also determine the hyplo-diploid cells in subG_1 phase, which are caused by the loss of small DNA fragments from chromosome DNA during apoptosis (Fig. 5a). The ratio of subG_1 cells in control cells was approximately 0.19%. After treatment for 18 h with 14 or 28 mg HCO1/L, the subG_1 population of L929 cells was 2.0 and 4.8%, respectively (Fig. 5b and c). When exposed to 42 mg HCO1/L the subG_1 feature was not observed in L929 cells and the nominal subG_1 ratio of 78.5% observed with flow cytometry was due to random DNA fragments (Fig. 5d). The result is in good agreement with those found in the fluorescence microscope and DNA fragmentations analysis (Masoud et al., 2003).

4. Discussion

The time- and concentration-dependent decrease in the MTT viability index by HCO1 on L929 cells observed in this study is consistent with our previous studies (Liu et al., 2007), showing that the toxic effects of HCO1 to *Daphnia magna*, Zebrafish (*Brachydanio rerio*) embryos, and goldfish (*Carassius auratus*).

The fact that the magnitude of effects was greater as a function of time is likely due to the fact that chemicals must enter the body of organisms before they can exert toxic effects. Hence, uptake rates and bioaccumulation levels of substances within tissues also affect the toxicity of HCO1. This was demonstrated with goldfish (Sun et al., 2006). In some cases, chemical cytotoxicity *in vitro* may help to deduce the toxicity for individual mammals. However, when the concentration of HCO1 in organism bodies would be measured, the EC_{50} values might be greater than the observed ones in this study because of the bioaccumulation effect and the time-accumulation effect (Sun et al., 2006). L929 cells, which are derived from mouse subcutaneous connective tissue fibroblast cells, express little cytochrome P450 activity

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(Chen et al., 2004). Thus, HCO1 is not easily metabolized by these cells.

Necrosis and apoptosis are distinct ways in the cellular response to certain toxins. Necrosis is caused by catastrophic toxic or traumatic events. In contrast, apoptosis is an active process of cell destruction with specific defining morphologic and molecular features (Kerr, 1991). In necrosis cells, leakage of cellular contents causes a pro-inflammatory response in the neighboring cells. Furthermore, conditions that either enhance or inhibit normal apoptotic rates have been associated with many disease states (Thompson, 1995). In carcinogenesis, inhibition of apoptosis has been correlated with tumor promotion (Bursch et al., 1992). The distinct morphological differences of cells after treatment with HCO1 under optical and fluorescence microscopy, the effects on DNA fragmentation suggest that the toxicity of HCO1 is due to necrosis, rather than apoptosis. This result may be due to the fact that strongly lipophylic HCO1 tends to deposit inside cells, stimulating cells and inducing DNA random cleavage. Although no detail mechanisms regarding HCO1 L929 cells cytotoxicity have been reported, further studies on necrosis will provide new approaches to the risk assessment of hair dyes to humans from dermal exposure.

Conflicts of interest

Nothing Declared.

Acknowledgements

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