

In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride

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Abstract

The aim of this study was to compare four in vitro cytotoxicity assays and determine their ability to detect early cytotoxic events. Two hepatoma cell lines, namely HTC and HepG2 cells, were exposed to cadmium chloride (0–300 μM) for 3, 5 and 8 h. Following exposure to the toxic metal cytotoxicity was determined with the lactate dehydrogenase leakage assay (LDH), a protein assay, the neutral red assay and the methyl tetrazolium (MTT) assay.

In HTC cells no toxicity was observed for any incubation period when the LDH leakage, the MTT and the protein assay were employed whereas the neutral red assay revealed early cytotoxicity starting after incubation of HTC cells with CdCl_2 for 3 h. In the case of HepG2 cells the MTT assay reveals cytotoxicity due to CdCl_2 exposure after 3 h whereas no such effect is seen with the other three assays. Following 5 h exposure of HepG2 cells to CdCl_2 , toxicity is observed with the MTT assay at lower concentrations compared to the ones required for detection of toxicity with the LDH leakage and the neutral red assay.

In conclusion different sensitivity was observed for each assay with the neutral red and the MTT assay being the most sensitive in detecting cytotoxic events compared to the LDH leakage and the protein assay.

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Keywords: Cadmium; LDH; MTT; Neutral red; Cytotoxicity

1. Introduction

Cytotoxicity assays are widely used in in vitro toxicology studies. The LDH leakage assay, a protein assay, the neutral red and the MTT assay are the most common employed for the detection of cytotoxicity or cell viability following exposure to toxic substances.

The LDH leakage assay is based on the measurement of lactate dehydrogenase activity in the extracellular

medium. Reliability, speed and simple evaluation are some of the characteristics of this assay (Decker and Lohmann-Matthes, 1988). It has been employed as an indicator of cytotoxicity in HepG2 cells following exposure to cadmium chloride (Dong et al., 1998) as well as in toxicity studies using rat renal proximal tubular cells (Fukumoto et al., 2001). The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage.

The MTT assay is another cell viability assay often used to determine cytotoxicity following exposure to toxic substances. It has been used in HepG2 cells (Tully et al., 2000) and in rat lung epithelial cells after expo-

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sure to cadmium chloride (Hart et al., 1999) as well as in oligodendrocytes to assess cell viability (Almazan et al., 2000).

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membranes and therefore it accumulates in healthy cells. The MTT assay was tested for its validity in various cell lines (Mossmann, 1983). More recent evidence suggests that reduction of MTT can also be mediated by NADH or NADPH within the cells and out of mitochondria (Berridge and Tan, 1992). Further modification of the initial protocol described by Mossmann (1983) were proposed (Denizot and Lang, 1986; Hansen et al., 1989) in order to improve the repeatability and the sensitivity of the assay.

The neutral red assay is also used to measure cell viability. It has been used as an indicator of cytotoxicity in cultures of primary hepatocytes (Fautz et al., 1991) and other cell lines (Morgan et al., 1991). Living cells take up the neutral red, which is concentrated within the lysosomes of cells.

Finally, the protein assay is an indirect measurement of cell viability since it measures the protein content of viable cells that are left after washing of the treated plates.

The aim of our study was to evaluate the sensitivity of the four cytotoxicity assays following exposure of HTC and HepG2 cells that were used as the test system, to cadmium chloride.

2. Materials

All the materials were from Sigma Aldrich unless otherwise stated.

3. Methods

3.1. Cell cultures and treatments

HTC cells (rat hepatoma cell line) and HepG2 cells (human hepatoma cell line) were obtained from the European collection of cell cultures. Cells were grown in DMEM (Dulbecco's modified Eagle's medium) (HTC cells) or MEM (modified Eagle's medium) (HepG2 cells) supplemented with 10% fetal bovine serum, 1% MEM non essential amino acid solution, and 1% penicillin-streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin in 0.9% NaCl) in a humidified atmosphere of 5% CO₂, 95% air at 35 °C. The passage

number range for both cell lines was maintained between 20 and 25.

The cells were cultured in 75 cm² cell culture flasks. For experimental purposes, cells were cultured in 12-well plates (1 ml of cell solution/well). The optimum cell concentration as determined by the growth profile of the cell line was 10⁵ cells/ml or 2 × 10⁵ cells/ml (HTC and HepG2 cells respectively). Cells were allowed to attach for 24 h before treatment with CdCl₂.

The stock solution of cadmium chloride was made in UHQ water and filtered with Minisart Filters (0.45 μm). A working solution in the corresponding media was prepared. Cell monolayers were washed with PBS and the addition of CdCl₂ doses prepared from the working solution in media followed. Cells were treated with CdCl₂ within a range of concentrations from 0 to 2000 μM CdCl₂ for 3, 5, 8 and 24 h. The concentration range for CdCl₂ and the exposure times have been selected based on literature information on CdCl₂ toxicity in other test systems (Romero et al., 2003) as well as on preliminary studies performed in our laboratory.

3.2. LDH leakage assay

Cytotoxicity induced by cadmium was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Following exposure to the metal the culture medium was aspirated and centrifuged at 3000 rpm for 5 min in order to obtain a cell free supernatant. The activity of LDH in the medium was determined using a commercially available kit from Sigma Diagnostics (LD-L50). The assay is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD. The formation of NADH from the above reaction results in a change in absorbance at 340 nm. Aliquots of media and warm reagent were mixed in a 96-well plate (Fisher) and absorbance was recorded using a microplate spectrophotometer system (Spectra max 190-Molecular Devices). Results were analyzed with the Soft max pro software (version 2.2.1) and are presented as percentage of control values. Cadmium chloride was shown not to interfere with the determination of LDH within a range from 0 to 2000 μM.

3.3. Neutral red assay

The neutral red assay is based on the initial protocol described by Borenfreund and Puerner (1984) and determines the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells.

Following exposure to CdCl₂ cells were incubated for 2 h with neutral red dye (100 µg/ml) dissolved in serum free medium (DMEM or MEM for HTC and HepG2 cells, respectively). The pH of the neutral red solution was adjusted in all the experiments to 6.35 with the addition of KH₂PO₄ (1 M). Cells were then washed with Phosphate Buffered Saline (PBS) and the addition of 1 ml of elution medium (EtOH/AcCOOH, 50%/1%) followed by gentle shaking for 10 min so that complete dissolution was achieved. Aliquots of the resulting solutions were transferred to 96-well plates and absorbance at 540 nm was recorded using the microplate spectrophotometer system (Spectra max190-Molecular Devices). Results were analyzed with the Soft max pro software (version 2.2.1) and are presented as percentage of control values.

3.4. Protein measurement

Determination of the protein content is based on the Bradford method (Bradford, 1976) using the Coomassie Protein assay reagent (Pierce, Rockford). Cells were washed with PBS following exposure to cadmium chloride. Precipitation with 6.5% TCA for 10 min followed and dissolution of the proteins was achieved with the addition of NaOH (1 M) (overnight at room temperature). Aliquots of standard albumin or sample were pipetted in 96 polystyrene well plates and mixed with equal volume of the Coomassie reagent. The absorbance was measured at 595 nm using the microplate spectrophotometer system (Spectra max190-Molecular Devices). Results were analyzed with the Soft max pro software (version 2.2.1) and are presented as percentage of control values.

3.5. MTT assay

The MTT assay is based on the protocol described for the first time by Mossmann (1983). The assay was optimized for the cell lines used in the experiments.

Briefly, for the purposes of the experiments at the end of the incubation time, cells were incubated for 4 h with 0.8 mg/ml of MTT, dissolved in serum free medium (MEM or DMEM for HepG2 and HTC cells respectively). Washing with PBS (1 ml) was followed by the addition of DMSO (1 ml), gentle shaking for 10 min so that complete dissolution was achieved.

Aliquots (200 µl) of the resulting solutions were transferred in 96-well plates and absorbance was recorded at 560 nm using the microplate spectrophotometer system (Spectra max190-Molecular Devices). Results were analyzed with the Soft max pro software (version 2.2.1) and are presented as percentage of the control values.

3.6. Statistical analysis

All experiments were performed three times in duplicate and data was statistically analysed with the unpaired student's *t*-test using the Prism Software.

4. Results

HTC cells were exposed to CdCl₂ (0–2000 µM) for 3, 5, 8 and 24 h and cytotoxicity was determined with the LDH leakage assay, the protein assay, the neutral assay and the MTT assay. The EC₅₀ values obtained by the four assays for these incubations times are shown in Table 1. EC₅₀ values were obtained only with the neu-

Table 1

EC₅₀ values for HTC and HepG2 cells following exposure to CdCl₂ for 3,5, 8 and 24 h based on the dose-response curves as derived from the LDH leakage assay, the neutral red assay, the MTT assay and the protein assay; (–) indicates no EC₅₀ value obtained for the corresponding incubation periods with the assay employed; data presented as EC₅₀ values (*n* = 3) ± SEM; SEM: standard error mean

| | 3 h | 5 h | 8 h | 24 h |
|-------------|---------------|---------------|---------------|----------------|
| HTC cells | | | | |
| LDH | – | – | – | 80 µM ± 16.11 |
| Neutral red | 200 µM ± 2.25 | 80 µM ± 1.26 | 40 µM ± 6.53 | 20 µM ± 3.31 |
| MTT | – | – | – | 100 µM ± 14.47 |
| Protein | – | – | – | 200 µM ± 2.25 |
| HepG2 cells | | | | |
| LDH | – | – | – | 5 µM ± 5.35 |
| Neutral red | 300 µM ± 7.88 | 100 µM ± 0.84 | 80 µM ± 1.90 | 8 µM ± 0.21 |
| MTT | 500 µM ± 1.96 | 100 µM ± 8.39 | 40 µM ± 3.53 | 15 µM ± 5.03 |
| Protein | – | – | 300 µM ± 1.01 | 10 µM ± 0.02 |

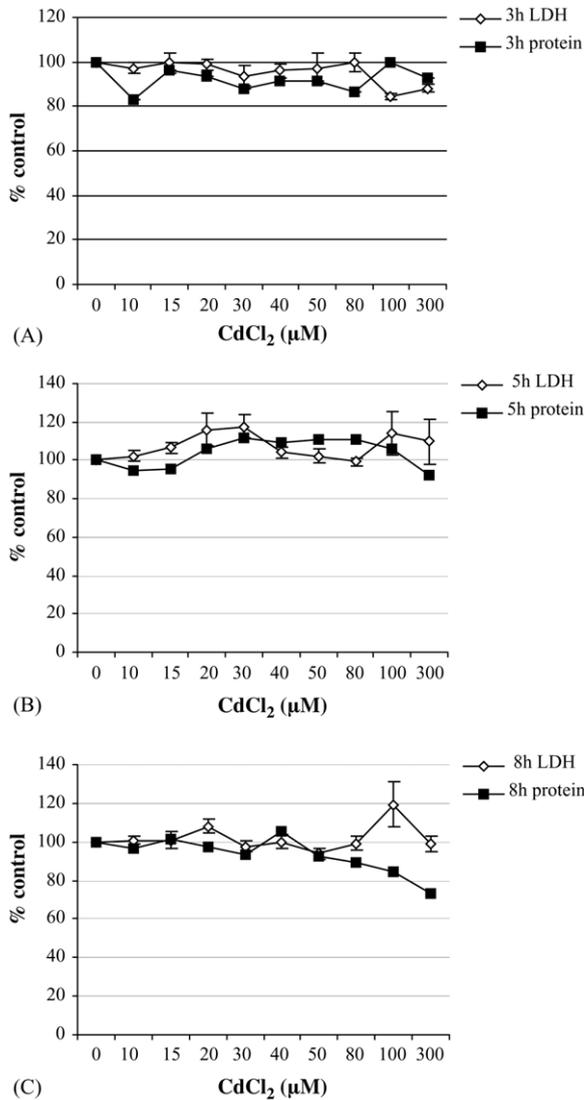


Fig. 1. Comparison of LDH leakage assay and protein assay in HTC cells after exposure to cadmium chloride for 3, 5 and 8 h; data presented as percentage of control ($n=3$) \pm SEM; SEM: standard error mean.

tral red assay when HTC cells were exposed to CdCl₂ for 3, 5 and 8 h whereas the LDH leakage assay, the MTT and the protein assay provide EC₅₀ value only after 24 h incubation period. Figs. 1 and 2 present the dose response curve for the four assays employed when HTC cells were exposed to CdCl₂ (0–300 μM) for 3, 5 and 8 h. Cytotoxicity is observed when the neutral red assay is employed (Fig. 2A–C) whereas no cytotoxicity is observed when the other three assays are employed (Fig. 1A–C). Loss of cell viability was notable even after a 3 h exposure of HTC cells to cadmium chloride (80, 100, 300 μM) and lower concentrations of CdCl₂ are required for this effect when the cells were incubated for 5 and 8 h. In contrast

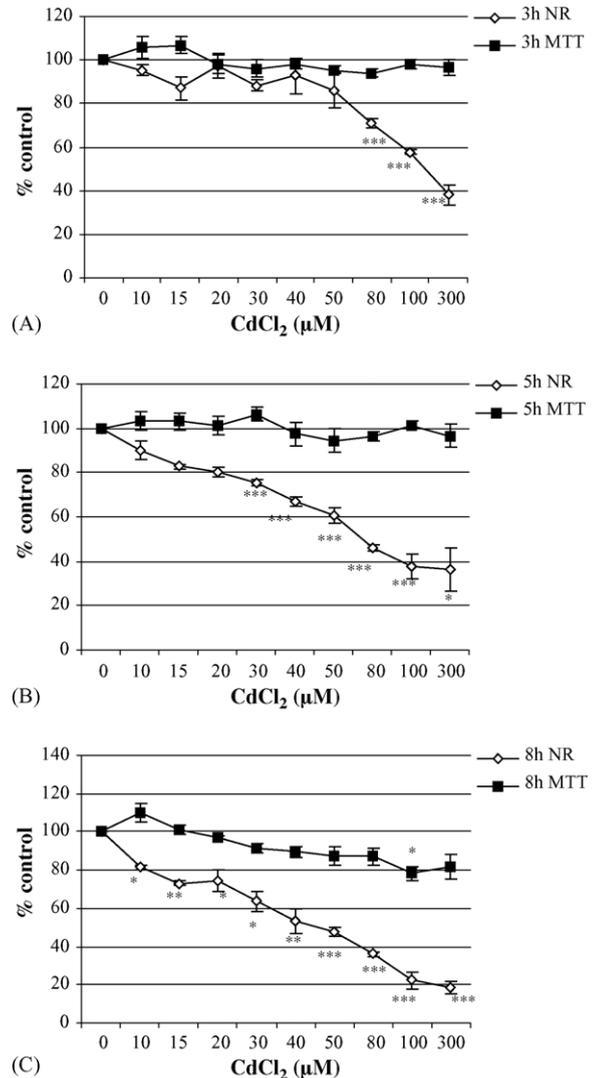
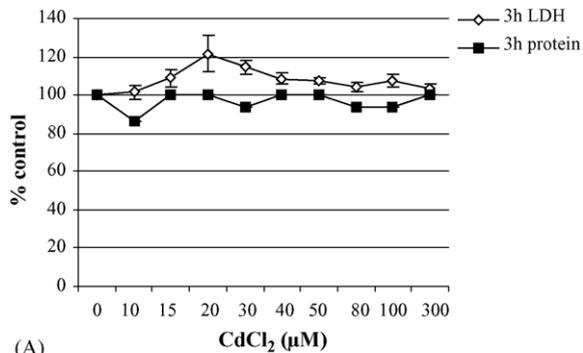


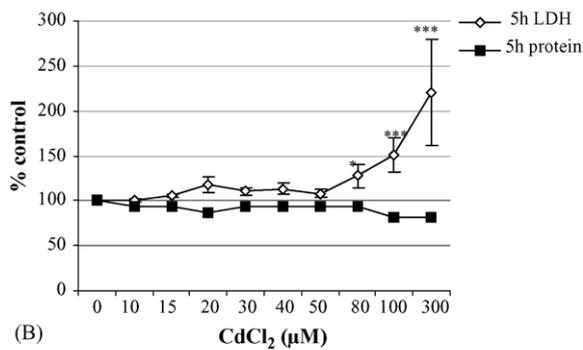
Fig. 2. Comparison of neutral red (NR) assay and MTT assay in HTC cells after exposure to cadmium chloride for 3, 5 and 8 h; data presented as percentage of control ($n=3$) \pm SEM; SEM: standard error mean; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

there is no apparent cytotoxicity when the MTT or the LDH leakage and protein assay were used for the same incubation periods.

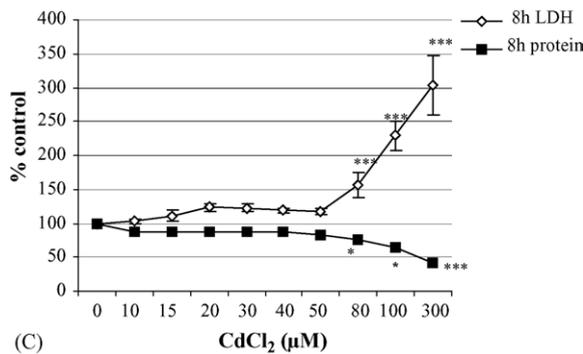
As shown in Table 1 in the case of HepG2 cells, the Neutral red and the MTT assay provide an EC₅₀ value following a 3 and 5 h incubation period of the cells with CdCl₂ whereas no EC₅₀ values were obtained when the LDH leakage assay or the protein assay were employed for the same exposure times. The protein assay provides an EC₅₀ value (300 μM) after 8 h incubation of HepG2 cells with CdCl₂ whereas only after 24 h incubation period the LDH leakage assay provides and EC₅₀ value (5 μM).



(A)



(B)

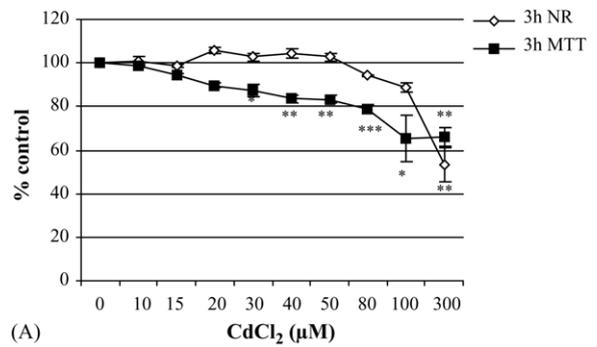


(C)

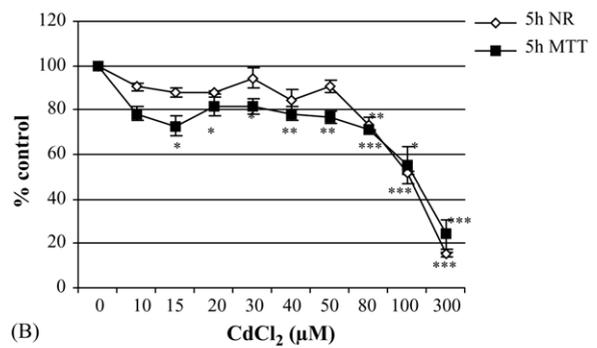
Fig. 3. Comparison of LDH leakage assay and protein assay in HepG2 cells after exposure to cadmium chloride for 3, 5 and 8 h; data presented as percentage of control ($n=3$) \pm SEM; SEM: standard error mean; * $p<0.05$, *** $p<0.005$.

Figs. 3 and 4 present the dose response curve for the four assays employed when HepG2 cells were exposed to CdCl₂ (0–300 µM) for 3, 5 and 8 h. Following exposure of HepG2 cells to CdCl₂ for 3 h the MTT assay appears to be more sensitive in detecting loss of viability compared to the other three assays. Increasing incubation time results in increased toxicity, which is observed more accurately with the MTT assay (Fig. 4B) and the MTT and neutral red assay (Fig. 4C).

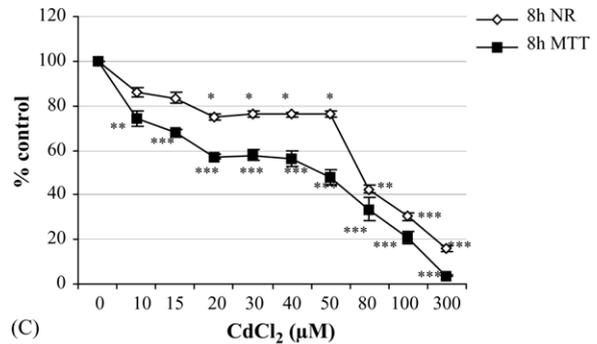
The results obtained from both cell lines show that the LDH leakage assay and the protein assay are the



(A)



(B)



(C)

Fig. 4. Comparison of neutral red (NR) assay and MTT assay in HTC cells after exposure to cadmium chloride for 3, 5 and 8 h; data presented as percentage of control ($n=3$) \pm SEM; SEM: standard error mean; * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

least sensitive when compared to the neutral red and the MTT assay.

5. Discussion

The results obtained from the cytotoxicity assays indicate that there are differences between the two cell lines concerning their sensitivity to CdCl₂. HepG2 cells appear to be more sensitive as indicated by the LDH leakage assay. Therefore, rupture of the cell membrane occurs when lower concentrations of cadmium chloride or shorter incubation times are employed in HepG2 cells than in HTC cells. This difference could be due to differ-

ent uptake mechanisms of CdCl₂ by the two cell lines. Studies performed in our laboratory show that higher levels of cadmium were detected in HepG2 cells than in HTC cells (data not shown). We have also found that glutathione levels are lower in HepG2 cells compared to HTC cells (data not shown), which would make them more sensitive to cadmium chloride.

The cytotoxicity assays employed revealed different profiles, with the neutral red and the MTT assay being the most sensitive cytotoxicity assays showing statistically significant difference between the treated cells and the controls. The LDH leakage assay and the protein assay revealed toxicity following exposure to CdCl₂ either at high concentrations of the metal or at long incubation times.

When the four cytotoxicity assays, employed to assess cadmium toxicity *in vitro*, are compared it is obvious that the results obtained are not in agreement. This observation can be explained by the nature of each assay. The LDH leakage assay is based on the release of the enzyme into the culture medium after cell membrane damage whereas the MTT assay is mainly based on the enzymatic conversion of MTT in the mitochondria. The neutral red assay is a colorimetric assay measuring the uptake of the dye by functional lysosomes. As shown, in HTC cells the neutral red assay reveals toxicity following exposure to cadmium chloride before any toxicity is observed when the LDH leakage assay, the MTT assay or the protein assay are employed.

On the other hand during incubation of HepG2 cells with CdCl₂ for 3, 5 and 8 h the MTT assay reveals loss of viability at concentrations that no significant difference with the LDH leakage assay or the protein assay are observed. Neutral red assay also reveals less cytotoxicity when HepG2 cells were treated with CdCl₂ than the MTT assay, although it appears to be more sensitive than the LDH and the protein assay when HepG2 cells were treated with CdCl₂ for 8 h.

Romero *et al.* (2003) reviewed the effective concentrations (EC₅₀) after exposure of different cell lines to cadmium chloride for 24 h, indicating variation between the cell lines examined and the tests employed to assess cytotoxicity. The EC₅₀ values obtained were in the range of 8.3–180 μM when the neutral red assay was employed for cytotoxicity assessment. The results from our studies as shown in Table 1 are in accordance with these data concerning the 24 h exposure of both cell lines to CdCl₂.

Although the EC₅₀ shown above show good correlation with the values reported by Romero *et al.* (2003), this was not the case for shorter incubation times. The Neutral red and the MTT assay appear to be more sensitive in detecting early toxicity compared to the LDH

leakage assay and the protein assay as indicated by both the EC₅₀ values presented in Table 1 as well as by the dose response curves presented in Figs. 1–4. These differences among the cytotoxicity assays indicate intracellular effects due to exposure to cadmium before any permanent cell membrane damage occurred.

In vitro cytotoxicity assays can be used to predict human toxicity and for the general screening of chemicals (Clemedson and Ekwall, 1999; Scheers *et al.*, 2001). It has been previously reported that different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed (Weyermann *et al.*, 2005). In cadmium-induced cytotoxicity, the respiratory impairment appears to precede the plasma membrane breakdown (Koizumi *et al.*, 1996). In that study changes in nuclear staining with propidium iodide occurred at an early stage while the LDH leakage occurred later. It is thought that inhibition of the mitochondrial respiration induces active oxygen related cell death. Reactive oxygen species that can be generated within the mitochondria can also damage mitochondrial components (Koizumi *et al.*, 1996) and therefore a cytotoxicity assay based on mitochondrial respiratory activity would give early signs of toxicity following exposure to a mitochondrial toxicant. The XTT (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide) assay, which is similar to the MTT assay, also gave greater sensitivity than the LDH leakage assay in other test systems (Mitsuyoshi *et al.*, 1999). Moreover, cadmium chloride caused structural alterations in LLC-PK1 cells before any cytotoxicity was observed with LDH leakage assay (Gennari *et al.*, 2003).

It appears that in HTC cells CdCl₂ has a profound effect on lysosomes without affecting the cell membrane or the mitochondria. This localized effect on lysosomes could account for the low cell viability observed in HTC cells with the neutral red assay. The lysosomal effect of cadmium chloride in HTC cells was further characterized (Fotakis *et al.*, 2005) and it appears that it is one of the primary effects in this cell line and may account for the DNA damage observed at a later stage. Cadmium is among the heavy metals that have the potential to cause lysosomal damage (George and Pirie, 1979; Etxeberria *et al.*, 1994). Therefore, neutral red assay is a useful tool to detect lysosomal damage when used in conjunction with other tests in order to distinguish between cytotoxicity and organelle damage.

The fact that in HepG2 cells the MTT assay revealed toxicity before any effect with the LDH leakage assay or the neutral red assay is observed can be attributed to the fact that CdCl₂ may have an effect on mitochon-

dria in this cell line. It has been suggested that cadmium disrupts mitochondrial function both in vivo (Belyaeva et al., 2002) and in vitro (Pourahmad and Brien, 2000). In addition there is lysosomal damage in HepG2 cells before any significant LDH leakage is observed.

To conclude it has to be noted that in order to avoid overestimation or underestimation of the toxicity of a substance, incubations with various concentrations at many time points are required to be able to distinguish between effects on specific organelles or general cytotoxicity. More than one assay should be used to determine cell viability in in vitro studies, as this would increase the reliability of the results obtained.

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