Comparative evaluation of cytotoxicity of cadmium in rat liver cells cultured in serum-containing medium and commercially available serum-free medium

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Abstract. Cadmium (Cd) is an industrial pollutant and carcinogenic metal. Most *in vitro* Cd toxicity studies have been carried out in various cell lines cultured in 10% fetal bovine serum (FBS) containing medium. In this report, we compared the toxic effect of Cd (0-300 μM) on cell growth, total RNA, total proteins, and antioxidant enzymes in rat normal liver cells cultured in medium with 10% FBS or commercially available serum-free medium for 4 or 8 hours. With Cd concentration at above 100 μM, the total levels of RNA, protein and cell growth decreased in serum-containing medium, while their levels increased in serum-free medium compared to the controls. The glutathione peroxidase and glutathione reductase levels were lower in serum-free medium than in serum-containing medium, indicating less oxidative stress in cells grown in serum-free medium. These results clearly suggest that Cd showed higher toxicity to liver cells grown in serum-containing medium in comparison to commercially available serum-free medium. It is speculated that albumin and other substances present in commercial serum-free medium chelated Cd and thereby protected these cells against Cd toxicity. Even under *in vivo* conditions, cadmium enters into various organs after passing through blood which contains serum. Based on these studies, it appears that media containing serum may be ideal for *in vivo* toxicity correlation studies with animal cells.

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Introduction

Cadmium (Cd), an abundant nonessential and carcinogenic metal (1), is a serious environmental and industrial pollutant (2). Cd salts are widely used as color pigments in paints, electroplating and galvanizing, and in batteries. Cd is also a by-product of zinc and lead mining and smelting (3). It is found in foods (vegetables, grains and cereals), water and tobacco (4). It has been shown to accumulate unevenly in human tissues, and is highly deposited primarily in lungs, liver, kidneys, brain, heart and testes (5,6).

The toxic effect of Cd has been studied in several *in vitro* and *in vivo* systems. It has been shown that Cd affects various metabolic processes, especially energy metabolism (7), membrane transport, protein synthesis and intracellular signaling networks. It has also been shown to affect DNA directly or indirectly through gene regulation at multiple levels (8-12). A variety of mechanisms have been attributed to Cd-induced toxicity. There is increasing evidence to show that the toxicity of Cd is associated with the production of reactive oxygen species (13-16). Consistent with these observations, alterations in the levels of antioxidant enzymes, such as catalase and SOD have been reported as a result of Cd treatments (13,14,17,18). Lipid peroxidation is also associated with Cd toxicity due to the formation of increasing levels of thiobarbituric acid-reactive substances (TBARS) in the lungs and liver, and urinary excretion of malondialdehyde (13,19,20).

Recently, we studied the effect of Cd on antioxidant enzymes in rat normal liver CRL-1439 cells grown in medium containing 10% fetal calf serum (13,14), where a dose-dependent effect was observed. However, there have been studies where serum proteins were shown to protect from metal toxicity in comparison with studies performed with reduced serum or serum-free media (21,22). Since, in addition to proteins, calf serum also contains several important factors necessary for cell attachment and growth, the reduction or complete withdrawal of serum in the medium in these studies could have increased stress to cells, which in turn enhanced

the metal toxicity. In order to confirm the role of serum proteins in protection against Cd toxicity, in the present study we employed commercially available serum-free medium. This contained all growth and attachment components equivalent to serum and provided non-stress conditions to the cells. The rat normal liver cells (CRL1439) were therefore cultured in medium containing 10% fetal bovine serum or commercial serum-free medium in the present study and the levels of cell growth, total RNA, total protein, and antioxidant enzymes after exposure to cadmium in serum or commercially available serum-free medium were compared.

Materials and methods

Maintenance of cell line. Rat normal liver CRL-1439 epithelial cell line was purchased from the American Type Culture Collection (ATCC) at the passage number 17 stage and cultured as per the guidelines supplied. The cells were maintained in F12K medium containing 100 units penicillin/ml, 100 μg of streptomycin/ml, 2 mM L-glutamine and 10% fetal bovine serum in T-75 cm^2 flasks at 37˚C in a 5% CO_2 incubator.

Treatment of cell cultures with cadmium. Initially, the liver cells were plated at a starting density of $5x10^4$ cells/well in polystyrene, flat-bottom 24-well microtiter plates (Corning Costar, Rochester, NY) in F12K medium containing 10% FBS, and allowed to stabilize overnight in a CO_2 incubator at 37˚C. The following day, the medium was replaced either with commercial serum-free medium (Complete™, Cellgro Company, Herndon, VA) or F12K medium containing 10% FBS. Then, cells were treated with 0, 100, 200 and 300 μM of $CdCl_2$ in a final volume of 2 ml/well. Each concentration was carried out in triplicate wells. All culture plates were incubated in a CO_2 incubator at 37˚C for 4 or 8 h. All studies were repeated at least twice.

Evaluation of cell growth. At the end of each incubation period, the cell growth was evaluated by a dye uptake assay according to Badisa *et al* (24). In brief, at the end of the incubation, 400 μl of 0.25% glutaraldehyde in H_2O was added to each well and incubated for 30 min at room temperature to fix the cells. The plates were washed under tap water and dried under airflow inside the laminar hood for 5-10 min. Then 400 μl of 0.1% crystal violet in H_2O was added to each well and incubated for 15 min. The plates were washed under tap water and dried at room temperature. Later, 400 μl of 0.05 M sodium phosphate solution (monobasic) in 50% ethyl alcohol was added to each well to solubilize the dye, and the plates were read at 540 nm in a plate reader.

^{3}H uridine incorporation assay. Cell plating, treatments and incubations were performed as described above in triplicate wells containing either commercial serum-free medium or F12K medium with 10% FBS. In each well, 2 μCurie (2 μl) of ^{3}H uridine was added. The plates were incubated in a CO_2 incubator at 37˚C for 4 or 8 h. At the end of the incubation, the medium was aspirated and the cells were washed with PBS twice. The cells were trypsinized and lysed by repeated freeze-thaw cycle for 5 min. The lysed cells were transferred to a scintillation vial and 10 ml of Scintiverse Scintillation cocktail (Fisher Company, GA) was added to each vial and radioactive counts were measured in a scintillation counter.

^{3}H alanine incorporation assay. Cell plating, treatments and incubations were done as described above in triplicate wells containing either commercial serum-free medium or F12K medium with 10% FBS. In each well, 2 μCurie (2 μl) of ^{3}H alanine was added. The plates were incubated in a CO_2 incubator at 37˚C for 4 or 8 h. At the end of the incubation, the medium was aspirated and the cells were washed twice with PBS. The cells were trypsinized and lysed by repeated freeze-thaw cycle for 5 min. The lysed cells were transferred to a scintillation vial and 10 ml of Scintiverse Scintillation cocktail (Fisher Company, GA, USA) was added to each vial and radioactive counts were measured in a scintillation counter.

Preparation of enzyme extracts. Crude enzyme extracts were prepared as per the method of Ikediobi *et al* (13). For this purpose, approximately $5x10^6$ cells grown either in 10% serum-containing or serum-free media were treated with 0, 100, 200 and 300 μM $CdCl_2$ for 4 or 8 h at 37˚C in a 5% CO_2 incubator. At the end of the incubation periods, the cells were trypsinized and pelleted by centrifuging at 3,500 rpm for 10 min at 4˚C. The cell pellets were suspended in 1 ml of 50 mM phosphate buffer pH 7.0, and homogenized with a Polytron homogenizer in a glass vial on ice for 1 min at intervals of 15 sec. The homogenate was transferred to eppendorff tubes and centrifuged at 3,000 rpm for 10 min at 4˚C to remove the lysed cell membrane debris. The supernatant was transferred to different tubes and cell extracts were stored at 4˚C for protein estimation and enzyme assay studies.

Protein estimation. The protein contents of the enzyme extracts were measured using a unique method that combines the well-known reduction of Cu^{+1} by proteins in an alkaline medium (Biuret reaction) with the highly-sensitive and selective colorimetric detection of cuprous cation (Cu^{+1}) using reagent containing bicinchonic acid (25,26). Protein contents were used to calculate specific activities of the enzymes and the latter subsequently used to estimate percentage change in enzyme activities at different concentrations of $CdCl_2$.

Glutathione reductase. Glutathione reductase activity was assayed as reported in our previous paper (13). This assay is based on the following reaction:

$$NADPH + H^+ + GSSG \xrightarrow[\text{reductase}]{\text{glutathione}} NADP^+ + 2GSH$$

$$GSH + DTNB \longrightarrow GSTNB + TNB$$

The change in absorbance at 412 nm due to the formation of TNB was measured. The specificity of this assay allows quantification of glutathione reductase without purification. The assay volume (2 ml) contained 1 ml of 0.2 M potassium phosphate with 1 mM EDTA buffer pH 7.5, 500 μl of 3mM 5'5-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.01 M phosphate buffer pH 7.0, 250 μl water, 100 μl of 2 mM NADPH in water, 50 μl cell extract, 100 μl of 2.4 U/ml

glutathione reductase and 100 μl of 20 mM oxidized glutathione. The increase in absorbance at 412 nm was monitored for 3 min in a Beckman DU 7500 Spectrophotometer at 24˚C. The enzyme activity was calculated using the extinction coefficient of TNB (E_{412}=13.6 l $mmol^{-1}cm^{-1}$). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmole NADPH per minute.

Glutathione peroxidase (GPx). Glutathione peroxidase activity was assayed as reported in our previous paper (13). Briefly, the reaction mixture (0.5 ml) contained 3.2 mM GSH, 0.32 mM NADPH, 1 unit glutathione reductase (GR), 1 mM sodium azide and 0.82 mM EDTA in 0.16 M Tris-HCl pH 7.0. When H_2O_2 was used as a substrate, 1 mM sodium azide was added to the reaction mixture to inhibit endogenous catalase activity. The reaction mixture was pre-incubated with 50 μl of sample at 37˚C for 5 min. The reaction was started by the addition of H_2O_2 at a final concentration of 100 μM in the reaction mixture. The rate of NADPH consumption was monitored at 340 nm for 3 min. One unit of GPx activity was defined as the amount of enzyme required to consume 1 μmole of NADPH/min in the coupled assay. Se-dependent GPx was calculated from the change in absorbance using only H_2O_2 as substrate.

Statistical analysis. The experimental results were presented as mean ± SD (n=6). The data were analyzed for significance by one-way ANOVA, and then compared by Dunnett's multiple comparison test, using GraphPad Prism Software, version 3.00 (San Diego, CA). The test values $p<0.05$ and $p<0.01$ were considered significant and highly significant respectively in comparison to the respective untreated control.

Results

Comparison of cell growth in serum and serum-free media. The difference in rat normal liver epithelial cell growth due to Cd exposure in serum-containing or serum-free media at 4 and 8 h is shown in Figs. 1 and 2 respectively. In serum-containing medium, the cell growth after 4 h exposure with Cd was not affected significantly at any concentration ($p>0.05$, Fig. 1) in comparison to control. On the other hand, in serum-free medium, a 4-h Cd exposure caused a statistically significant increase in cell growth at all concentrations ($p<0.01$). In this case, the percentage of increase in cell growth at 100, 200 and 300 μM Cd was 28±2.4, 41±2.1 and 41±1.4% respectively.

An 8-h treatment with Cd in serum-containing medium resulted in a significant decrease in cell growth by 26.8±5.1 and 27.4±3.5% at 200 and 300 μM, respectively ($p<0.01$, Fig. 2). Contrary to this, in serum-free medium, Cd caused statistically significant increase in cell growth by 22.9±2.69, 24.5±0.42 and 28.35±3.18% at 100, 200 and 300 μM Cd, respectively, in comparison to control ($p<0.01$). These results clearly suggest that Cd promotes cell growth in serum-free medium, but inhibits cell growth in serum-containing medium.

Comparison of RNA levels in serum and serum-free media. Fig. 3 shows the difference in RNA levels by ^{3}H uridine incorporation in cells grown in serum-containing or serum-free media for 4 h at 0, 100, 200 and 300 μM Cd. It was observed that the RNA levels in serum-containing medium decreased significantly by 15.1±1.06 and 64.2±0.64% at 200 and 300 μM Cd respectively, in comparison to control cells ($p<0.01$). On the other hand, in serum-free media RNA was increased significantly at higher levels by 62±12.7 and 228±16.3% at 200 and 300 μM Cd respectively, in comparison

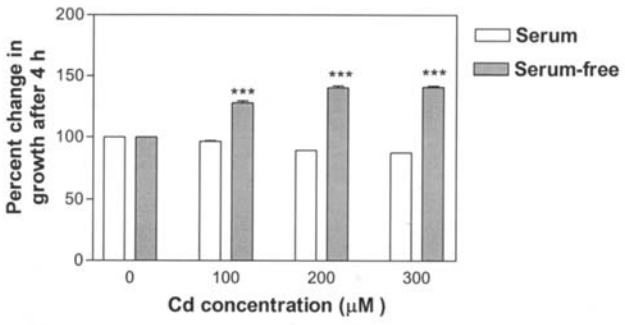

Figure 1. Comparison of cell growth of rat normal liver cells after 4 h of incubation with 0-300 μM $CdCl_2$ in serum-containing or serum-free media as measured by the crystal violet dye intensity incorporated by the live cells. The results were expressed as means ± SD (n=6). ***Significant difference from the control ($p<0.01$).

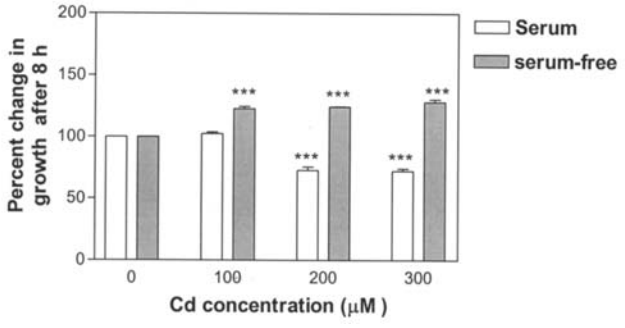

Figure 2. Comparison of cell growth of rat normal liver cells after 8 h of incubation with 0-300 μM $CdCl_2$ in serum-containing or serum-free media as measured by the crystal violet dye intensity incorporated by the live cells. The results were expressed as means ± SD (n=6). ***Significant difference from the control ($p<0.01$).

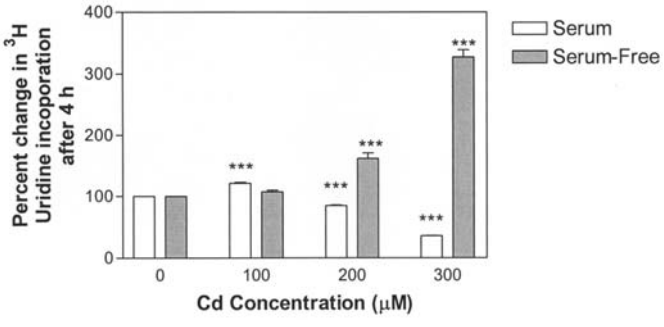

Figure 3. Comparison of total RNA levels of rat normal liver cells after 4 h of incubation with 0-300 μM $CdCl_2$ in serum-containing or serum-free media as measured by ^{3}H uridine incorporation. The results were expressed as means ± SD (n=6). ***Significant difference from the control ($p<0.01$).

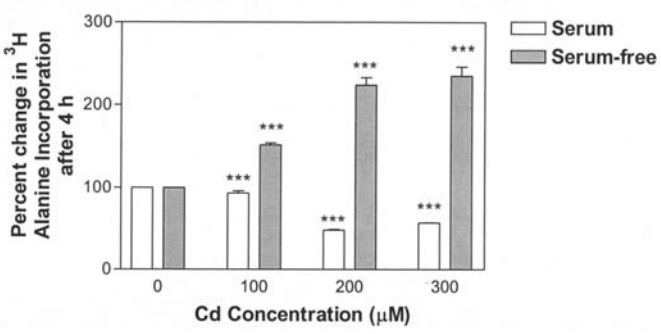

Figure 4. Comparison of total protein levels of rat normal liver cells after 4 h of incubation with 0-300 μM $CdCl_2$ in serum-containing or serum-free media as measured by ^{3}H alanine incorporation. The results were expressed as means ± SD (n=6). ***Significant difference from the control ($p<0.01$).

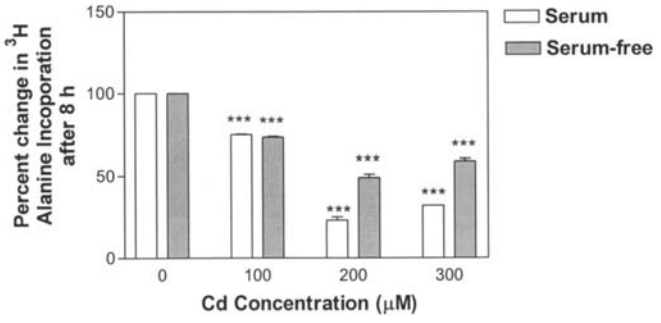

Figure 5. Comparison of total protein levels of rat normal liver cells after 8 h of incubation with 0-300 μM $CdCl_2$ in serum-containing or serum-free media as measured by ^{3}H alanine incorporation. The results were expressed as means ± SD (n=6). ***Significant difference from the control ($p<0.01$).

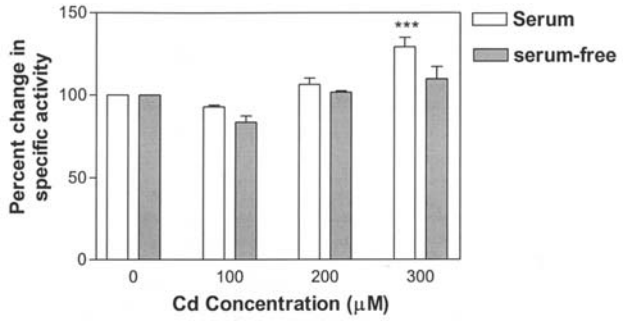

Figure 6. Comparison of glutathione peroxidase enzyme activity of rat normal liver cells after 4 h of incubation with 0-300 μM $CdCl_2$ in serum-containing or serum-free media. The results were expressed as means ± SD (n=6). ***Significant difference from the control ($p<0.01$).

to control. These observations suggest that the response of RNA levels in cells with Cd treatment in both types of media was dose dependent in comparison to control cells. These results may imply that RNA expression levels in cells grown in serum-containing medium were lower than those grown in serum-free medium due to Cd treatments. These observations are consistent with the results of Figs. 1 and 2, where reduced cell growth was observed in serum-containing medium as a result of Cd treatment.

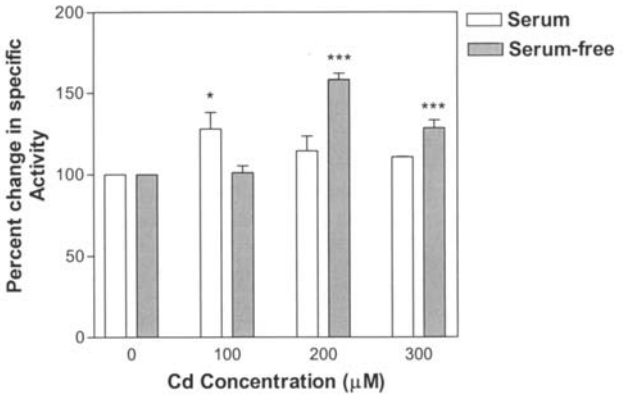

Figure 7. Comparison of glutathione reductase enzyme activity of rat normal liver cells after 4 h of incubation with 0-300 μM $CdCl_2$ in serum-containing or serum-free media. The results were expressed as means ± SD (n=6). $^{*}p<0.05$ and $^{***}p<0.05$ were considered significant and highly significant, respectively.

Comparison of total protein levels in serum-containing and serum-free media. Figs. 4 and 5 show the difference in ^{3}H alanine incorporation in proteins when the cells were exposed to 0, 100, 200 and 300 μM Cd in serum-containing or serum-free media for 4 and 8 h respectively. It was observed that in serum-containing medium, Cd at 100, 200 and 300 μM decreased total cellular protein levels by 6.5±3.75, 51.9±1.34 and 42.8±0.21%, while in serum-free medium, these levels increased by 51.4±3.54, 124±12.73 and 134.8±15.56% in comparison with their respective controls (Fig. 4). After an 8-h exposure to Cd, the total protein levels in serum-containing and serum-free media decreased with the increasing concentration of Cd (Fig. 5). However, the percentage of decrease was more pronounced in serum-containing medium. These results clearly suggest that some components in serum-free medium protect the cells from Cd-induced toxicity.

Comparison of antioxidant enzyme levels in serum-containing and serum-free media. Cd treatment generates high levels of ROS (13,14), causing severe oxidative stress to cells. Under such circumstances, the cells respond by increasing the activities of antioxidant enzymes such as glutathione reductase and glutathione peroxidase. In the present study, we investigated the effect of Cd at different concentrations on the activities of these two enzymes prepared from cells grown in serum-containing or serum-free medium after a 4-h exposure. The results are presented in Figs. 6 and 7. It was observed that glutathione peroxidase activity in both types of media was decreased by 7.3±1.4 and 16.1±5.2 when the cells were exposed to 100 μM Cd for 4 h in serum-containing and serum-free medium, respectively (Fig. 6). Notably, beyond 100 μM, the activity was increased by 6.3±5.4 and 29±5.8 when the cells were exposed to 200 and 300 μM Cd in serum-containing medium and by 1.5±1.4 and 9.8±2.7 in serum-free medium (Fig. 6).

It was observed that glutathione reductase activity in serum-containing medium was increased by 27.8±17.6, 14.7±12.9 and 10.9±0.42% when the cells were exposed to 100, 200 and 300 μM Cd, respectively for 4 h, but increased

by 1.3±5.86, 58.6±5.59 and 28.9±6.79% in serum-free medium (Fig. 7).

Discussion

Previously, most *in vitro* metal toxicity studies were conducted on rat, mouse or human cell lines grown in media containing various amounts of fetal calf or bovine serum (13,16,27). The serum provides growth and adhesion factors necessary for the growth of these cultures. It was reported that fetal bovine serum contains 36 g/l total proteins, of which albumin represents a major portion (23 g/l) (certificate of serum analysis from Cellgro). Furthermore, serum also contains other proteins including growth factors such as insulin and transferrin. While several cell culture studies on cadmium toxicity have been performed with 10% fetal calf serum in medium (4,13-16,28,29), there have also been studies where reduced-serum or serum-deprived media were utilized (21,22,30). The primary reason given for the usage of reduced serum in the toxicity studies is to minimize the interaction of divalent cadmium cations with anionic groups on proteins in serum (31). However, the drastic reduction in serum proteins during toxicity studies decreases the availability of optimum levels of factors needed for cell growth. This in turn may be simply a reflection of cells becoming more sensitive towards cadmium due to increased oxidative stress, as shown earlier (23), and thus the resulting toxicity may not indicate the true toxic nature of cadmium. Thus, in this study, commercial serum-free medium where the cells were under non-stress conditions was used to expose the effect of serum. The cadmium toxicity in the medium containing 10% FCS or commercial serum-free medium were compared for cell growth, total RNA, total proteins and the antioxidant enzymes GPx and GR. The commercial serum-free medium (Cellgro Complete™) contains a 50/50 mix of DMEM/F12 and a mixture of selected trace elements and high molecular weight carbohydrates, extra vitamins, a non-animal protein source and a small amount of high quality bovine serum albumin (1 g/l i.e. 15.1 μM) without insulin, transferrins, hormones or other growth factors.

It was observed that cells grew better in commercial serum-free medium than serum-containing medium when the cells were treated with 100-300 μM cadmium chloride for 4 or 8 h (Figs. 1 and 2). This may have been due to the binding of cadmium to factors other than albumin (the concentration of albumin is only 15.1 μM), present in the serum-free medium, and contradicts earlier studies which showed that the cells demonstrated more toxicity in serum-depleted medium (21,22). The reasons for this could be that, unlike commercially available medium, the medium employed in cytotoxic studies by these authors did not contain a number of factors such as high molecular weight carbohydrates, extra vitamins, and a non-animal protein source. It has been reported that serum proteins protect the cells from toxicity by chelation and thus reduce toxicity (21). If serum proteins offer protection against cadmium toxicity, then cadmium should induce more toxicity in cells in commercial serum-free medium. However, contrary to this idea, we found a greater protection for cells in serum-free medium than in serum medium where higher RNA and protein expression levels were observed (Figs. 2-6). Furthermore, we also observed low levels of anti-oxidative enzymes in serum-free medium which indicates less oxidative stress due to cadmium. The protection in our studies may be due to the chelation of cadmium by factors present in serum-free medium. The *in vitro* conditions are entirely different from an *in vivo* situation. Since the long term goal of *in vitro* observations is to extrapolate those results to *in vivo* situations, it is more meaningful to employ 10% FBS in culture medium for the cytotoxic evaluation of cadmium. This procedure may represent a step closer to an *in vivo* situation, where blood contains serum proteins (physiological concentration of albumin, 600 μM). Under *in vivo* conditions inside the human body, the metals enter through the blood stream before being deposited in various organs like the liver, causing the observed toxicity. Thus, the *in vitro* toxicity studies carried out in the presence of serum in the medium correlates with the *in vivo* toxicity of the metal under normal physiological conditions.

In conclusion, the medium containing FBS showed higher cadmium toxicity than the commercial serum-free medium. Hence, the medium containing FBS may be an ideal system to use in testing the toxicity of heavy metals on the cultured animal cell lines which may be correlatable with an *in vivo* toxicity situation.

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