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Morphological characterisation of BGM (*Buffalo Green Monkey*) cell line exposed to low doses of cadmium chloride

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Abstract

Morphological changes in the *Buffalo Green Monkey* (BGM) cell line after exposure to a subcytotoxic dose (0.062 mM, equivalent to EC_{10}—effective concentration 10%) of cadmium chloride have been evaluated. Cells were exposed for 24 h and the effects observed at the ultrastructural level by transmission and scanning microscopy. Using transmission electron microscopy, the most notable findings in treated cells were the presence of intranuclear inclusion bodies and thin intracytoplasmic granules associated to myelin figures and the presence of apoptotic bodies. Other morphological alterations included cell vacuolisation and a reduced cytoplasm volume, condensation of the mitochondria and a decreased number of cytoplasmic organelles, except lysosomes and autophagic vacuoles, which increased in number. Scanning electron microscopy pointed to a cell with a disrupted perinuclear region and a decrease in the number of surface microvilli. We conclude that the BGM cell line may be considered an useful tool for toxicological studies involving cadmium.

Keywords: BGM; Cytotoxicity; Cadmium; Apoptosis; Ultrastructural alterations

1. Introduction

Cell culture methods have been widely used in toxicological studies, at first in basal cytotoxicity assays, the results of which can be used to design more specific studies. As new cell lines are introduced and for improving the characterization of established cell lines (Zimmerhackl et al., 1998). Heavy metal toxicity, for example, has been assessed in vitro using renal, hepatic, nervous and other cell cultures, including tumor cells.

Cadmium is present in the environment mainly as a result of human activity and is considered one of the most toxic heavy metals. Its harmful effect on human and animal health has led to the development of many analytical and experimental procedures designed to determine the severity of such effects and its concentration in biological samples (El-Azzouzi et al., 1994; Hamada et al., 1994; García-Fernández et al., 1995; Matsuoka and Call, 1995; Bayoumi et al., 1998). LLC-PK1 an established cell line from pig kidney, are the most frequently used cells in in vitro assays for cadmium toxicity assessment (Ishido et al., 1998a, 1999; Zimmerhackl et al., 1998; Alvarez-Barrientos et al., 2001; Barrouillet et al., 2001; Olabarrieta et al., 2001). However, other renal cell lines, such as MDCK (Madin-Darby canine kidney), have been used to contrast the results obtained with the above-mentioned LLC-PK1 cells (Prozialeck and Lamar, 1997; Zimmerhackl et al., 1998; Alvarez-Barrientos et al., 2001). Several studies have used microscopic methods in both experimental animals (Nishizumi, 1972; Goyer et al., 1989; Early et al., 1992; Sudo et al., 1996) and cell cultures (Hazen-Martin et al., 1989; Hamada et al. 1994; Bucio et al., 1995; Lyons-Alcantara et al., 1998).

As a "low dose" of cadmium Bucio et al. (1995) considered a concentration 10-fold lower than the EC_{50} and as a "high dose" a concentration similar to EC_{50}. In the case of other metals, such as mercury, Lachapelle et al. (1993) consider as a "low dose" the concentration equivalent to the no-observed-effect level (NOEL),

Abbreviations: BGM, *Buffalo Green Monkey*; DSP, dysentery shellfish poisoning; EC_{10}, effective concentration 10%; FCS, foetal calf serum; MEM, minimum essential medium; NOEL, no-observed-effect level; NR, neutral red

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while Aleo et al. (1992) classified as a "subcytotoxic dose" a concentration 1.4-fold lower than the EC_{50}.

Renal cell cultures have been described as useful tools for studying heavy metal-induced cell damage and toxicity mechanisms (Cherian, 1985; Wilson, 1986), but no references to the BGM cell line appears in the literature, despite it having been widely and successfully used in microbiological studies (Dahling and Wright, 1986; Wilson, 1986). As regards toxicological matter, the BGM cell line as only been referenced in studies on dysentery shellfish poisoning (DSP) (Croci et al., 1997).

In the present study, the morphological response of the BGM cell line exposed to low doses of cadmium was assessed for its suitability for future toxicological research. The basal toxicity data were obtained using the neutral red (NR) cytotoxicity assay (Borenfreund and Puerner, 1984), and ultrastructural changes were evaluated after exposure to a subcytotoxic dose equivalent to EC_{10} (0.062 mM), a concentration 1.2-fold lower than the EC_{50}.

2. Material and methods

2.1. Cell culture and test chemical

The BGM cell line was cultured in Eagle's minimum essential medium (MEM; Sigma) supplemented with 10% (v/v) foetal calf serum (FCS), 100 UI penicillin/ml and 100 μg streptomycin/ml. Cells were sown in 75-cm^2 bottles at a density of 1.5×10^4 cells/cm^2 and incubated at 37 °C in a CO_2 (5%) humidified incubator. The growth of this cell line followed three well-defined growth phases: latent, exponential and stable, allowing us to identify the best moment for cadmium chloride treatment (48 h after the beginning of subculture, just at the beginning of the exponential growth phase).

The cadmium chloride was obtained from Sigma, and prepared in purified and sterilised water (Milli-Q) at 10× for each concentration to be evaluated. These solutions were added to MEM to obtain the final concentrations. The MEM used in the control cultures was prepared in the same form but adding 10% purified water. Prior to carrying out the treatment, the osmolarity of these solutions (including the control) was measured so that any differences in cell viability after addition of the cadmium chloride salt could be evaluated.

2.2. Neutral red (NR) cytotoxicity assay

The cells were sown in 96-well microtitre plates and the cadmium chloride was added 48 h later. The initial concentration range assayed was between 20 μM and 0.2 mM, while controls consisted of medium with untreated cells and without cells. The cultures were tested using NR, which is selectively taken up by the lysosomes of living cells (Borenfreund and Puerner, 1984), thus pointing to the degree of viability. Twenty-four hours after the treatment, the NR solution was added to each well and incubated at 37 °C for 3 h. Cells were fixed with 0.5% (v/v) formaldehyde in 1% $CaCl_2$ solution and NR dye was eluted from the living cells using 100 μl acid alcohol (1%, v/v, acetic acid in 50% ethanol) (Babich and Borenfreund, 1993). Absorbance was read on a Multiskan MCC/340P plate reader at 560 nm with a 690 reference filter (Bayoumi et al., 1998). The mean absorbance for each concentration was expressed as a percentage of the value obtained for the blank wells and plotted against the cadmium chloride concentration. The effective concentrations were determined from the dose–response curve by linear regression.

2.3. Transmission electron microscopy

The EC_{10} dose of cadmium chloride (0.062 mM) was added 48 h after the cells were plated on 6-cm Petri dishes at a density of 1.5×10^4 cells/cm^2. Cells were exposed to this toxic compound for 24 h at 37 °C. Control plates were prepared without cadmium chloride. After exposure, the cells were trypsinised, collected and centrifuged (482 *g*, 10 min, +24 °C). The pellets were immediately fixed in 3% (v/v) glutaraldehyde, postfixed in 1% (v/v) osmium tetroxide and stained with 4.8% uranyl acetate. After dehydration with a graded series of alcohol concentrations, the samples were rinsed in propylene oxide and impregnated with epoxy resins. The ultrathin sections were contrasted with uranyl acetate and lead citrate for electron microscopy study. Electron micrographs were taken with a Zeiss EM 10C transmission electron microscope.

2.4. Scanning electron microscopy

The cells were seeded in Leighton tubes and exposed to 0.062 mM cadmium chloride for 24 h at 37 °C. They were then fixed in 3% (v/v) glutaraldehyde and postfixed in 1% osmium tetroxide. Samples were dehydrated with a graded series of alcohol concentrations and 100% acetone. They were dried using an acetone–CO_2 bath in a *CPDO$_2$ Balzers Union* chamber. After sputtering with gold, images were taken with the *Link-Isis* program (Oxford Instruments, UK).

3. Results

3.1. Cytotoxicity assay

The $CdCl_2$ cytotoxic concentration range, as measured by the NR assay, was between 0.03 and 0.11 mM (Fig. 1). The effective concentrations were obtained by

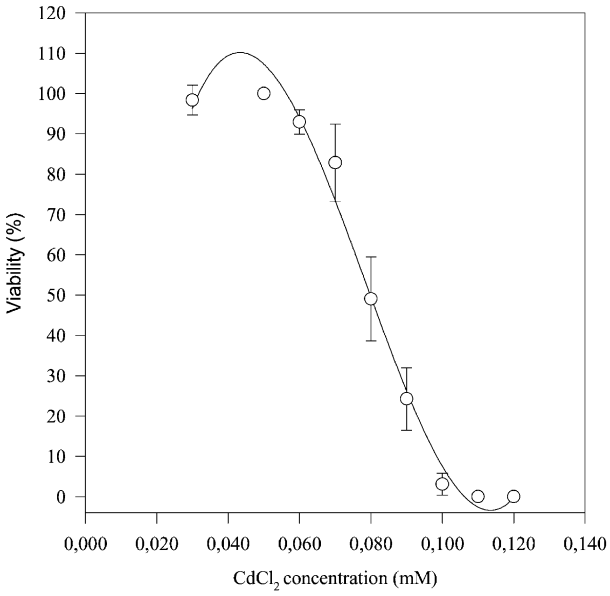

Fig. 1. Cytotoxicity curve of BGM cell line exposed to $CdCl_2$. The cultures were tested using the neutral red (NR) test 24 h after metal exposure (Babich and Borenfreund, 1993). Absorbance was read at 560 nm with a 690 reference filter (Bayoumi et al., 1998). The mean absorbance for each concentration was expressed as a percentage of the value obtained for the blank wells and plotted against the cadmium chloride concentration.

linear regression from the dose–response curve. The narrow range between the EC_0 and EC_{100} demonstrated that the BGM cell line is susceptible to the toxic activity of cadmium (Table 1).

3.2. Morphological findings

3.2.1. Untreated cells

Transmission electron microscopy showed that the BGM cells were rounded, with a well-defined outline and a variable number of microvilli. Spherical or oval mitochondria with well-defined transversal cristae, abundant smooth endoplasmic reticulum, few phagolysosomes and clearly recognisable vacuoles were characteristic of this cell line. Abundant chromatin, scarce peripheral heterochromatin and one to four nucleoli were observed in the spherical or oval nuclei, which were situated in a central position in the cell (Fig. 2). Scanning electron microscopy showed BGM cells to have an epithelial morphology with multiple nucleoli in a central nucleus. The nucleus/cytoplasm ratio was greatly in favour of the cytoplasm. Abundant short microvilli were evenly distributed over the cell surface, with the longest being observed around the nucleus. Perinuclear rupture was slight and probably associated to the method (Fig. 3).

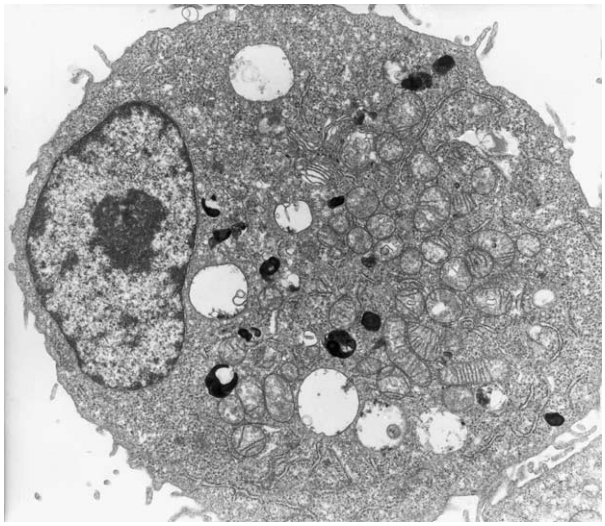

Fig. 2. Transmission electron micrograph of control (untreated) BGM cells (13,000×). BGM cells have abundant cytoplasm, a well-defined cell outline and a variable number of microvilli. Spherical or oval mitochondria with well-defined cristae and vacuoles in the cytoplasm. Abundant chromatin in nucleus.

3.2.2. Treated cells

Following exposure to 0.062 mM $CdCl_2$, visual observation confirmed that the proportion of non-viable or altered cells was close to 10%. Affected cells presented a nucleus/cytoplasm ratio close to one (1:1) or greater, and the cytoplasm was occasionally reduced to a thin fringe around the nucleus. Microvilli were few or absent. Other alterations included vacuolisation of the cytoplasm, condensed and deformed mitochondria and a decreased number of cytoplasmic organelles, while an increased number of lysosomes, autophagic vacuoles

Table 1
Percentage of viable cells of the BGM cell line as a function of $CdCl_2$ concentration and with respect to the control

Concentration (mM)	Mean percentage	Standard error	Standard deviation	Maximum	Minimum
0.03	98.35	1.65	3.69	100.00	91.76
0.05	100.00	0.00	0.00	100.00	100.00
0.06	92.92	1.36	3.04	97.88	90.00
0.07	82.83	3.91	9.57	93.68	71.15
0.08	49.08	4.24	10.38	57.69	31.59
0.09	24.27	3.16	7.74	31.64	13.46
0.10	3.11	1.12	2.74	6.62	0.00
0.11	0	0	0	0	0
0.12	0	0	0	0	0

and myelin figures were commonly seen. Thin granular intracytoplasmic and occasional intranuclear inclusion bodies were evident. In general, the nuclei and nucleoli were similar in appearance to the controls (Fig. 4). The form of some apoptotic bodies differed between cells but fragmented nuclei with areas of condensed and marginalized chromatin were characteristic of cells (Fig. 5). Some affected cells presented a decreased number of microvilli. Scanning microscopy showed that treated BGM cells were frequently smaller than untreated cells and had fewer nucleoli per nucleus. Peripheral cytoplasmic vacuolisation, cell rupture around the nucleus and the disappearance of superficial microvilli were also common alterations (Fig. 6).

4. Discussion

The mean osmolarity of the medium used for both the treated and control cultures was close to that described as optimum for in vitro cell growth (270 mOsm) (Waymouth, 1970). This strongly suggested that both basal cytotoxicity data and morphological changes observed in the treated cells were solely due to the effect of the treatment.

A better characterisation of established cell lines has become important for physiological and toxicological research (Zimmerhackl et al., 1998). Cadmium-induced toxicity assays using the renal cell line, LLC-PK1, have been developed in order to assess genetic and biochemical effects, while other cell lines (human renal, canine

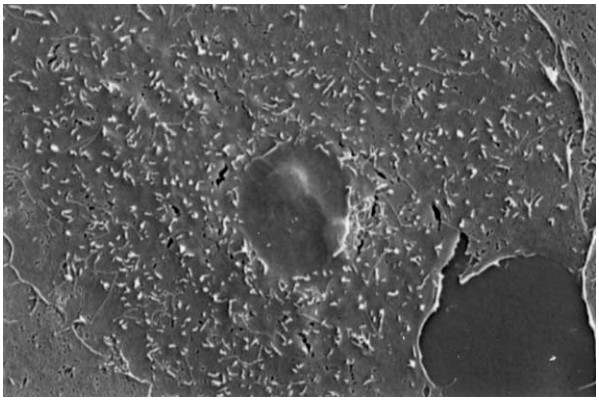

Fig. 3. Scanning electron micrograph of untreated BGM cells (1800×). Epithelial cell morphology with multiple nucleoli in a central nucleus. Minimal cell rupture around the nucleus. Abundant short microvilli homogeneously distributed around the cell surface.

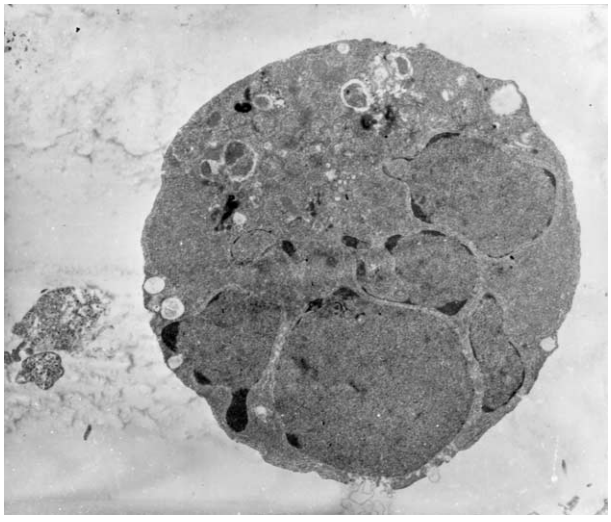

Fig. 5. Transmission electron micrograph of BGM cells exposed to 0.062 mM (EC_{10}) cadmium chloride for 24 h (8500×). Apoptotic bodies. Fragmentation of nuclei. Areas of condensed and marginalized chromatin.

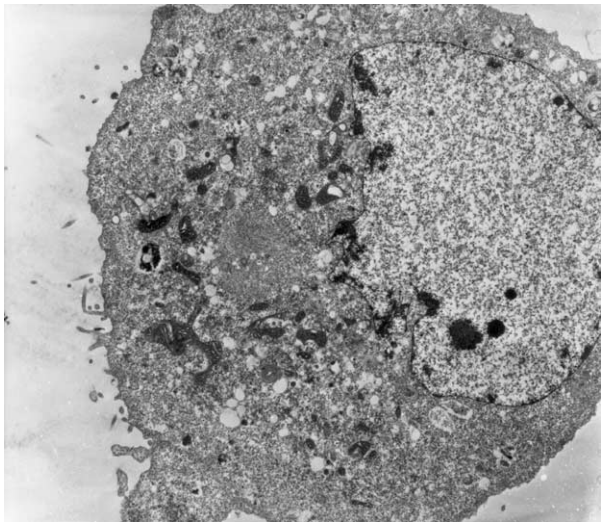

Fig. 4. Transmission electron micrograph of BGM cells exposed to 0.062 mM (EC_{10}) cadmium chloride for 24 h (8250×). Microvilli almost absent and condensed and deformed mitochondria. Intracytoplasmic and intranuclear inclusion bodies.

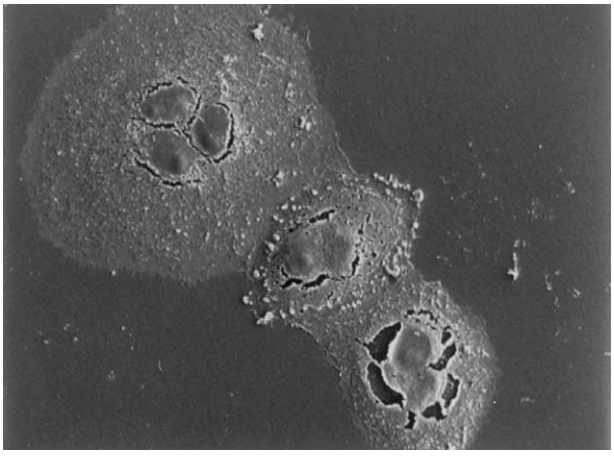

Fig. 6. Scanning electron micrograph of BGM cells exposed to 0.062 mM (EC_{10}) cadmium chloride for 24 h (650×). Severe cell rupture around nucleus. Microvilli are absent or detached. Nucleus/cytoplasm ratios close to 1.

renal, human hepatic fetal, dermis of trout) have been used for microscopic studies (Hazen-Martin et al., 1989; Hamada et al., 1994; Bucio et al., 1995; Lyons-Alcantara et al., 1998).

Few studies on cadmium toxicity have not used renal cell lines (Babich et al., 1986; Keogh et al., 1994; Lin et al., 1994, 1995; Bucio et al., 1995; Bayoumi et al., 1998) and a variable response to the EC_{50} of cadmium chloride was observed (Table 2). In this study, we have used the BGM cell line because of its renal origin and a similar response was observed to that described by Bayoumi et al. (1999) in the BF-2 cell line, both being in the middle of the range of concentrations described for other cell lines (Table 2).

The morphological changes observed in the treated cells were similar to those described in the literature in other cell lines. The fine intracytoplasmic granules and intranuclear inclusion bodies in the treated cells probably represented cadmium. It is not usual to find references to the presence of this type of cadmium inclusion in in vitro studies. In an X-ray microanalysis of renal proximal tubules in cadmium-treated rats, Marshall et al. (1994) found cadmium in the nuclei, and also in the cytoplasm and lysosomes. In a similar study, Matsuura et al. (1991) found 80–90% of added cadmium in the cytoplasm and associated to several ultrastructural changes that included nucleoli containing compact dense granules. Using a cytochemical method in a study of primary-cultured renal tubular cells from beagle kidneys, Hamada et al. (1994) observed that intranuclear cadmium was stained in the nucleolar regions. These findings demonstrated that cadmium, which is absorbed rapidly, reaches the nucleus and affects both nuclear and cytoplasmatic metabolism. The alterations in mitochondria of treated cells were similar to those described by other authors (Rehm and Waalkes, 1990; Matsuura et al., 1991). Since the energy of a cell depends on mitochondria activity and integrity, morphological alterations of this organelle might adversely affect cell functioning although not sufficiently to produce cell death.

Matsuura et al. (1991) observed an increase in lysosomes, myelin bodies and vesiculation. Similar changes were found in our study. We suggest that the close association of myelin figures and the increase in autophagic vacuoles and lysosomes near or around the intracytoplasmic granules (Fig. 4) represents an active but unsuccessful attempt to isolate cadmium or to expel it from the cell. Marigómez et al. (1989) defined cadmium as a metal capable of destabilising lysosomes. The degree of destabilisation might differ from cell to cell since the metabolic activity (including the cell's absorption capacity) during the first hours of exponential growth in culture might not be of equal intensity in all cells. This might also explain the different degrees to which cell morphology is altered.

Several authors have described cell apoptosis as consequence of exposure to low doses of heavy metals (Hamada et al., 1994; Rajaram et al., 1995; Duncan-Achanzar et al., 1996; Oberto et al., 1996; Kitamura et al., 1997; Shenker et al., 1997; Ishido et al., 1998a,b). The apoptotic process begins with interaction between the apoptotic agent and a membrane receptor, followed by DNA fragmentation and, finally, the appearance of apoptotic bodies. Besides the high percentage of viable cells, some necrotic cells were observed as were others in differing stages of apoptosis. Falcieri et al. (1994) suggested that apoptosis does not normally co-exist with necrotic death as it represents a completely different phenomenon, while Habeebu et al. (1998) suggested that cadmium-induced apoptosis in vivo precedes necrosis. While this may be true in vivo, we observed that exposure to low doses of cadmium in vitro induces a greater or similar degree of apoptosis as necrosis. El-Azzouzi et al. (1994) reported that human CEM-C12 cells exposed to low cadmium levels died through apoptosis, while high cadmium levels produced necrosis. Hamada et al. (1996) showed that high cadmium concentrations were less effective in inducing apoptosis than low concentrations. Quantification of cell death was not possible with the microscopy methods used in this study

Table 2
Effective concentration (EC_{50}) (in μM) after exposure to cadmium in different in vitro cell cultures

Chemical form	Cells	EC_{50}	T	ET (h)	Authors/reference
Cd acetate	REF (rat embryo fibroblasts)	1.5	CA	24	Lin et al., 1995
Cd chloride	WRL-68 (human foetal liver)	4.7	TB	24	Bucio et al., 1995
Cd chloride	CHO (hamster ovary cells)	8.3	NR	24	Bayoumi et al., 1998
Cd nitrate	CHO (hamster ovary cells)	15	TB	16	Lin et al., 1994
Cd acetate	HFW (human skin fibroblasts)	25	CA	24	Lin et al., 1995
Cd chloride	I407 (human intestinal epithelium)	53	NR	48	Keogh et al., 1994
Cd chloride	BF2 (perch dorsal fin)	88	NR	24	Bayoumi et al., 1998
Cd chloride	RTG2 (rainbow trout gonads)	32	NR	24	Bayoumi et al., 1998
Cd chloride	LLC-PK1 (porcine renal epithelium)	40	NR	24	Olabarrieta et al., 2001
Cd^{+2}	BF2 (perch dorsal fin)	80	NR		Babich et al., 1986
Cd^{+2}	RTG2 (rainbow trout gonads)	180	NR		Babich et al., 1986

T = Technique, ET = Exposure time, CA = Clonogenic assay, TB = Trypan Blue, NR = Neutral Red.

and so it would be interesting to evaluate the apoptotic process in this cell line using other methods.

Cell damage at the scanning microscopy level has not been described previously in cell cultures exposed to cadmium. It is probable that the rupture of both treated (Fig. 6) and untreated (Fig. 3) cells in our case was the result of the technique used, since such an alteration could not be seen by transmission electron microscopy. However, the greater susceptibilty of the treated cells to rupture suggests the greater fragility of cells exposed to cadmium. Indeed, Ord et al. (1998) reported that the first sign of exposure to cadmium was damage to the cell membrane. It has also been shown that exposure to cadmium results in lipid peroxidation of the cell membranes (Dudley et al., 1984; Müller, 1986; Bano and Hasan, 1989; Koizumi et al., 1996; Yang et al. 1997). However, it is also possible that cell fragility is the consequence of alterations in the cytoskeleton caused by changes in the nucleus during apoptosis.

In conclusion, we can affirm that the BGM (*Buffalo Green Monkey*) cell line may be used similarly to other cell lines of renal origin (LLC-PK1, MDCK) in studies on cadmium-induced toxicity and apoptosis.

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