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# N-acetylcysteine prevents cadmium-induced apoptosis in human breast cancer MDA-MB468 cell line

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**Abstract**

Cadmium is a heavy metal posing severe risks and destructive effects on human health. Although cadmium was classified as a human carcinogen, it has been also shown to be a cytotoxic agent that induces cell death either by necrosis or apoptosis. In this study, we investigated the protective role of N-acetylcysteine, a free radical scavenger, on cadmium induced apoptosis in MDA-MB468 cells. The breast cancer cells were exposed to increasing concentrations of $CdCl_2$ in the presence and absence of NAC and the cell viability was assessed using MTT assay. The microscopic studies of apoptosis were carried out with fluorescent staining. To investigate the induction of apoptosis, cellular DNA was isolated using DNA kit extraction and analyzed electrophoretically. The results showed significant decrease in cellular viability upon 48 hours exposure to $CdCl_2$ in a dose-dependent manner ($p < 0.05$). Pretreatment of MDA-MB468 cells with N-acetylcysteine (1mM) reversed the cadmium cytotoxicity effects and protected cells from apoptotic death. DNA Hoechst staining showed the apoptotic bodies. The electrophoresis of extracted DNA identified a fragmentation pattern consistent with apoptosis mechanism. The results suggest that cytotoxic effects and induction of apoptosis caused by $CdCl_2$ are mediated, by oxidative stress.

***Key words:*** Cadmium, N-acetylcysteine, MDA-MB468, Human breast, Apoptosis.

## Introduction

Relationship between metals and cancer has been widely studied. Almost all metals are capable of production of reactive oxygen species (ROS). This characteristic highlights their important role in carcinogenesis as well as cancer treatment. Based on Domingo health classification, metals are divided into four categories. The highest cytotoxic heavy metals such as arsenic, cadmium, mercury, uranium and lead are widely dispersed in the environment. The ability of heavy metals in the production of ROS can be used to destroy cancer cells and its clinical applications are also considered (1,2). Cadmium is found in water, soil, sediment and air, and has been used without limitations in larger quantities since 1940. According to US Environmental Protection Agency, cadmium is in the 129 priority environmental pollutants (http://water.epa.gov/scitech/methods/cwa/pollutants.cfm).One common example of the importance of cadmium as an environmental pollutant, is Itai-Itai disease in Japan. A disease with severe pain, bone fractures, severe proteinuria and progressive osteomalacia, caused by the consumption of rice and water contaminated by cadmium mines waste waters (3). Activities such as burning of fossil resources, smoking, or nickel-cadmium/ solar batteries industry are important sources of cadmium exposure to human. Due to its long biological half-life (20-15 years), cadmium can accumulate in the human body. Cadmium is absorbed through the gastrointestinal tract, inhalation and skin into the blood and then be transported to other tissues. Cadmium binds to melatonin in blood circulation and concentrates mainly in the erythrocytes (4).

In spite of its ability to induce apoptosis and cell death, cadmium may facilitate the induction of various tumors. Epidemiological studies have shown that cadmium causes tumors in the lung, prostate, kidney and stomach. So, cadmium is placed in category I of carcinogens by the International Agency for Research on Cancer (IARC). It is thought that cadmium acts via genotoxic mechanisms such as increasing mutation rate, activation of proto-oncogenes as well as inhibition of DNA repair and apoptosis. The cadmium carcinogenicity seems to be mediated through the production of ROS and oxidative DNA damage. Likewise The genotoxicity of cadmium was significantly inhibited by scavengers of free radicals (5,6). However, the different conditions and sensitivities of the cells are the main explanations for both inhibitory and stimulatory effects of cadmium. It was shown that cadmium has dual functions on angiogenesis and induces apoptosis through VEGF pathways in HUVECs in a dose-dependent manner (7).

In this study, we used MDA-MB468 cell line as a known model for human studies, to investigate the mechanism underlying cytotoxicity of cadmium and the role of ROS-dependent apoptosis using N-acetylcysteine.

## Materials and methods

### *Chemical and reagents*

Cadmium chloride was obtained from Merck (Germany). N-acetyl-L-cysteine (NAC) was purchased from Sigma (CAS Number 616-91-1, Sigma Grade, ≥99%

(TLC), powder). RPMI medium, FBS and all other cell culture reagents and chemicals were purchased from Gibco and invitrogen. Flasks, petri dishes and multi-well plates were obtained from Nunc (Denmark). All other chemicals were of analytical grade and were purchased from Sigma Aldrich (St. Louis,MO, USA) or were of the highest purity commercially available.

***Cell Line and Cell Culture Conditions***

MDA-MB468, an estrogen receptor negative human breast cancer cell line was obtained from National Cell Bank of Iran (NCBI, Pasteur Institute of Iran).The cell line was grown in RPMI-1640 media supplemented with heat-inactivated 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were maintained in a humidified atmosphere at 37°C in 5% CO2/95% air. Cells were passaged two or three times weekly and examined regularly for contamination and growth assessment. The cells were cultured in 25 $cm^2$ cell culture flasks. For experimental purposes, cells were cultured in 96 multi-well plates. The optimum cell concentration as determined with the growth profile of the cell line was 50,000 cells/ml.

***Cadmium Chloride Treatment***

The cells (50,000 cells/ml) were plated and allowed to attach. After 24 hours, the medium was replaced with fresh medium supplemented with 1% FCS. Stock solution of $CdCl_2$ (10 mM) was prepared in distilled water and sterilized by filtration and then further diluted with medium to the desired concentrations. Different concentrations of cadmium chloride (1-1000 µM) were added to the plates which were incubated for 48. The concentration range and the exposure time have been selected based on our previous publications (8).

***Evaluation of cell viability using MTT assay***

After the incubation of MDA-MB468 human breast cancer cells with different concentration of $CdCl_2$, the viability of living cells were determined colorimetrically using the MTT assay. At the end of 48 hours incubation MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) solution was added to culture media (0.5 mg/ml) and incubation continued further for 3 additional hours to determine the extent of cytotoxicity caused by the test metal. MTT was reduced by live cells having functional mitochondrial succinate dehydrogenase to insoluble formazan. Then the medium was removed and DMSO was added to dissolve the formazan crystals to purple color. The optical density of samples was read on an ELISA microplate reader at 570 nM. The color formation and absorbance were directly proportion to the number of living cells. The results of cell viability are expressed as percentage of control untreated cells. To study the effect of N-acetylcysteine (NAC), MDA-MB468 cells were pre-incubated with 1 mM NAC for 2 hours before treatment with the indicated concentrations of cadmium chloride.

***Evaluation of apoptosis by DNA fragmentation***

The cells were seeded in 6-well plates at a concentration of $1x10^6$ per ml of medium and allowed to enter the logarithmic phase of cell growth. Then, the cells was treated with different concentrations of cadmium chloride (25, 50, 100 and 200 micromolar) in the presence and absence of 1 mM N-acetylcysteine for 48 hours. The DNA fragmentation (internucleosomal DNA cleavage) assay was carried out using a High Pure PCR template preparation kit (Roche Applied Science, Catalog Number: 11796828001) according to the manufacturer instruction. Briefly, the cells were harvested and lysed with lysis buffer supplemented with proteinase K. After heating and several step centrifuging, lysates were mixed with DNA loading buffer. Equal amounts of DNA from different samples were run on 1.5% agarose gels containing 1 µg/ml ethidium bromide. Separated DNA fragments (DNA ladders) were visualized and photographed on the BioRad Gel Imager and their size was determined by comparison with DNA size markers.

***Evaluation of apoptosis using fluorescence staining***

A fluorescent nuclear stain, Hoechst 33342 (Sigma-Aldrich, CAS Number 23491-52-3, ≥98%, HPLC and TLC), was used for morphological studies. Hoechst 33342 staining was performed in 6-well plates. Briefly, the cells ($1x10^6$cells/ml) were plated and allowed to attach. Then MDA-MB468 cells were exposed to the indicated concentrations of cadmium chloride for another 48 h. Untreated and treated cells were washed by PBS and fixed with paraformaldehide (4%-30 min) and stained with Hoechst 33342 (10 µM) for 60 min in the dark at 37 °C. The morphological changes were visualized with a fluorescence microscope using a 320 to 350-nm filter. Normal cells were considered to have Hoechst-stained smooth nuclei, whereas cells with brightly stained condensed chromatin, nuclear fragmentation or apoptotic bodies were considered as apoptotic.

***Statistical Analysis***

Nonparametric one-way analysis of variance (ANOVA) was performed with the Dunnett's test (when required), using SPSS software, v.11.0. Each experiment was carried out in triplicate and repeated three to four times independently. A value of $p < 0.05$ was considered significant in comparison to the respective untreated control. Differences were considered statistically significant when a $p < 0.05$ was achieved. All values in the figures and text are expressed as means ± SD of $n$ observations (with $n > 9$).

**Results**

***The cytotoxicity effects of cadmium chloride on the growth of MDA-MB468 cell line***

The present study shows that cadmium chloride at 1 micro molar concentrations did not show significant cytotoxicity, but with increasing concentration up to 100 micro molar a significant reduction in the number of live cells can be observed (Figure 1). However, the concentrations greater than 100 µM don't affect the percentage of viable cells compared to lower effective doses of cadmium. In these studies, according to the figure 1, the incipient $EC_{50}$ value for the cytotoxic action of cadmium chloride was calculated to be 70 µM. In our previous work we used different times of incubations (24, 48 and 72 hours) and the best effect of cadmium chloride was obtained at 48 h (8). Therefore in this study, the incubation time was 48 h with cadmium chloride.

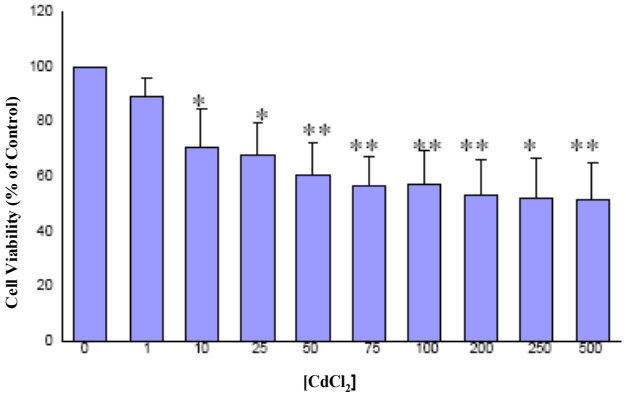


**Figure 1. The effect of cadmium chloride on the viability of MDA-MB468 breast cells.** Cells exposed to different concentrations of cadmium chloride (1-500 µM). After 48 hours, MTT viability test was performed as described in "Materials and Methods". Results (Mean ± SD) were calculated as percent of corresponding control values. Statistical analysis was performed by ANOVA followed by Dunnett's test. Each point represents 3 repeats of triplicate. Stars show that each value is significantly different when compared with control (*$P$< 0.05 and **$P$< 0.01). * P < 0.05 (significant difference from control), ** P < 0.01 (significant difference from control).

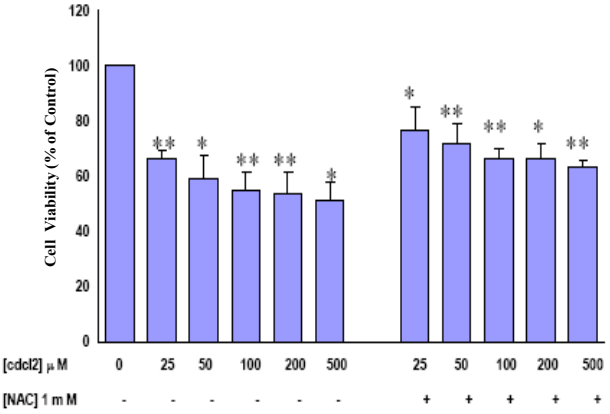


**Figure 2. The effect of cadmium chloride on the viability of MDA-MB468 breast cells, A: without N-Acetylcystein (NAC) B: in presence of NAC.** Cells were exposed to different concentrations of cadmium chloride (In the presence and absence of 1 mM NAC). After 48 h incubation, the viability was determined by MTT assay. Results (Mean ± SD) were calculated as percent of corresponding control values. Statistical analysis was performed by ANOVA followed by Dunnett's test. Each point represents 3 repeats of triplicate. Stars show that each value is significantly different when compared with control (*$P$< 0.05 and **$P$< 0.01). * P < 0.05 (significant difference from control), ** P < 0.01 (significant difference from control).

### *Effects of cadmium chloride cytotoxicity in the presence of N-acetylcysteine*

The protective role of NAC on the cytotoxicity of cadmium chloride was accessed by pretreatment of the cells with NAC (1 mM , 2 hours). According to the results showed in Figure 2, the concentrations of 25, 50, 100 , instead of and 200 and 500 µM of $CdCl_2$ were selected and considered in two groups. The first group was treated with cadmium in the absence of NAC in the culture medium. The second group contains the aforementioned concentrations of cadmium chloride in the presence of 1 mM NAC pretreatment (2 hours before exposure to cadmium). The results of this experiment show that the cytotoxic effects of cadmium chloride was reversed by N-acetylcysteine pretreatment (Figure 2). As shown in Fig 2 the reduced cell numbers by cadmium chloride at concentrations of 25, 50, 100, 200 and 500 micromolar was markedly attenuated by 1 mM NAC (16, 20, 22, 24 and 26 per cent respectively , $EC_{50}$ value >200 µM). These results suggest that NAC can exert greater protective effects in higher concentrations of cadmium

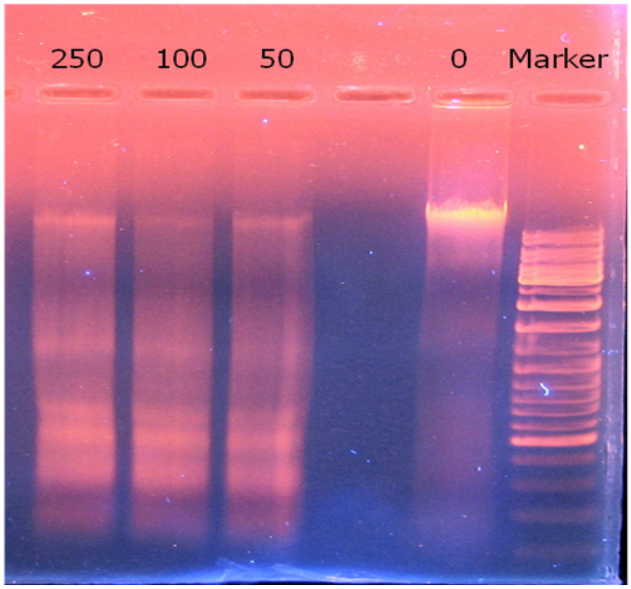


**Figure 3. Agarose gel electrophoresis of DNA extracted from MDA-MB468 cells treated with cadmium chloride for 48 h shows ladder pattern.** Lane 1: 100bp DNA marker, Lane 2: zero concentration of cadmium, lanes 3: treatment with 50 µM, lane4: treatment with 100 µM and lane5: treatment with 250 µM cadmium chloride, indicating that this pattern ladder are confirmed because the apoptosis is caused by cadmium chloride.

### *The protective role of N-acetylcysteine on the apoptotic DNA fragmentation induced by cadmium chloride*

During apoptosis, the cellular DNA is broken into several pieces. This DNA fragmentation leads to the formation of DNA ladder pattern on agarose gel electrophoresis (9). Our results demonstrate that the cytotoxic effect of cadmium chloride leads to the apoptotic cell death in the MDA-MB468 human breast cancer cell line. As shown in figure 3 the cells was incubated for 48 hours with different concentration of cadmium ( 50, 100 and 250 µM) and then genomic DNA was extracted and transferred to agarose gel to prove the ladder pattern (Figure 3). To assess the protective role of N-acetylcysteine, NAC (1mM) was added 2 hours prior to incubation with cadmium chloride. Figure 4 shows that DNA ladder fragmentation pattern was not observed in the presence of NAC, which indicates that NAC inhibits ROS production and subsequent apoptosis induced by cadmium.

### *The results represent staining with Hoechst (33342)*

One of the features of cells that undergo apoptosis is nuclear chromatin condensation and DNA fragmentation that form apoptotic bodies (10). The cells were incubated for 48 hours with the 100 µM concentration of cadmium chloride. Then the cells were stained with a DNA-binding bisbenzimide fluorochrome (Hoechst 33342) and examined with the fluorescence microscope. Apoptotic bodies that are characteristic of apopto-

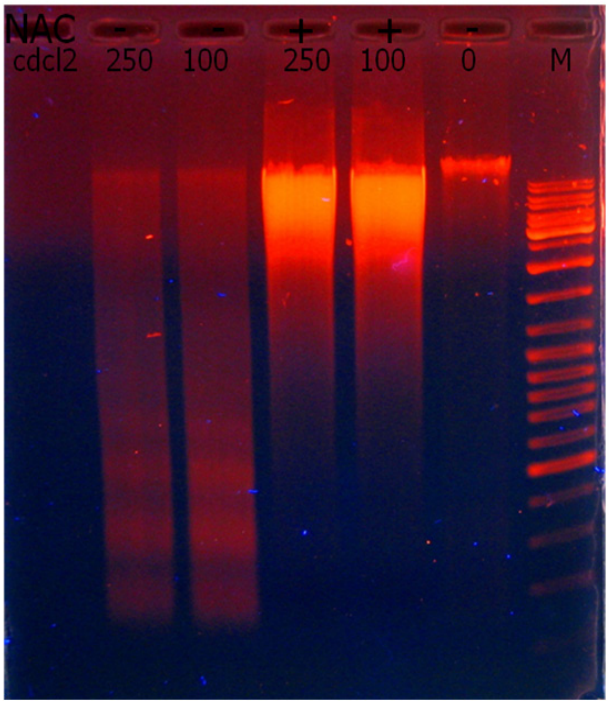


**Figure 4. Agarose gel electrophoresis of DNA extracted from MDA-MB468 cells incubated with cadmium chloride for 48 h in the presence and absence pretreatment with N-Acetyl cystein.** Lane 1: 100bp DNA marker, Lane 2: zero concentration of cadmium, lanes 3: treatment with 100 µM in the presence of 1 mM NAC, lane4: treatment with 250 µM in the presence of 1 mM NAC, lane5: treatment with 100 µM in the absence of 1 mM NAC and lane6: treatment with 250 µM in the absence of 1 mM NAC

tic index was observed while negative control cells (zero concentration of cadmium) stained with Hoechst33342 didn't show any fluorescence (Data not shown). Figure 5 show the morphological changes associated with apoptosis of the cells after exposure to cadmium chloride using light (Figure 5B) and fluorescence microscopy (Figure 5A). So the results indicate that cadmium induced cell death via apoptosis.

## Discussion

During recent years the number of articles related to apoptosis has been grown considerably. This special attention is due to defects in apoptosis that leads to diseases such as cancer or autoimmune diseases (11). Moreover many anticancer drugs and radiation destroy the cancer cells through induction of apoptosis (12, 13). The present study was conducted to study the mechanism of apoptosis induced by cadmium in the human breast cancer cell line MD-MB-468, as well as clarifying the protective role of N-acetylcysteine, a free radical scavenger, on cadmium induced apoptosis. The results clearly indicated significant decrease in cellular viability upon exposure to $CdCl_2$ in a dose-dependent manner. Moreover the preincubation with 1 mM NAC almost completely suppressed cadmium-induced cytotoxicity. Hoechst 33342 staining showed the apoptotic bodies and extracted DNA electrophoresis showed a fragmentation pattern consistent with apoptosis mechanism. So the cytotoxic effects and induction of apoptosis by $CdCl_2$ are mediated, by oxidative stress.

For a long time it was regarded that cell death induced by cadmium was a form of necrosis. But in 1991 the apoptotic nature of cadmium-induced cell death was shown in atrophic kidneys excised from beagle dogs (14). Subsequent studies showed that invitro and invivo apoptosis induced by cadmium is the consequences of production of reactive oxygen species that leads to more severe depletion of glutathione and protein-bound sulfhydryl groups (15,16).

Antioxidants such as N-acetylcysteine, vitamin E, catalase and superoxide dismutase effectively are able to confirm apoptosis induced by cadmium (16).We demonstrate that NAC as an antioxidant largely reverse cadmium chloride induced apoptosis in MDA-MB468 cells. This is because that oxidative stress through ROS production is the main source of cell death caused by cadmium chloride. (17).

The results from the present study are in agreement with previous studies. Lawal et al. showed significant increases in lactate dehydrogenase leakage, DNA damage, malondialdeyde and antioxidant enzymes activities in three cell lines (human hepatoma cell line HepG2, a human astrocytoma cell line 1321N1, and a human embryonic kidney cell HEK 293) upon exposure to cadmium. ROS production and glutathione (GSH) depletion were attenuated by the presence of N-acetylcysteine (NAC) (18). Exposure to cadmium chloride enhanced intracellular ROS generation that play a pivotal role in the reduction of extracellular SOD in COS7

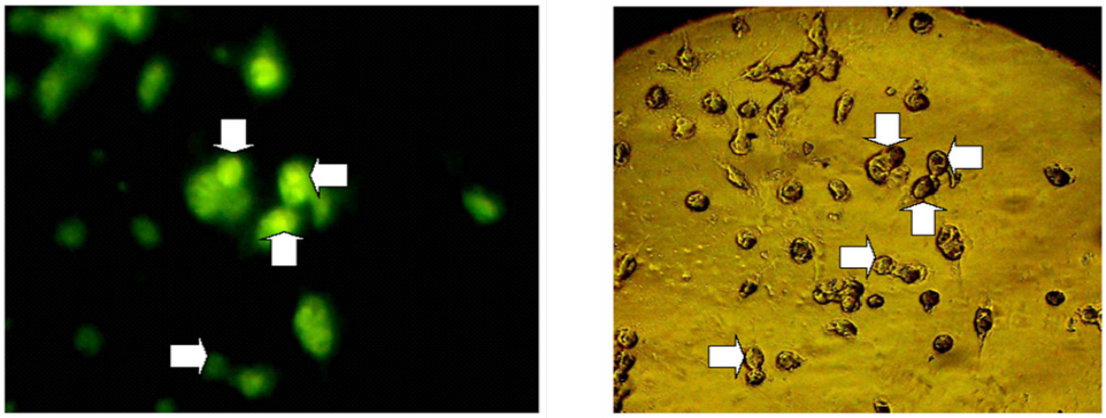

**Figure 5. Fluorescence microscopy photomicrograph of MDA-MB468.** Treated cells with Cadmium chloride stained with Hoechst 33342 in which the apoptotic cells (arrows) showed morphological changes including shrunken and condensed nuclei with a bright fluorescence appearance under a fluorescence microscope(A). Apoptotic bodies (arrows) also were clearly observed under the light microscope (B).

cells (19) or significantly decrease of superoxide dismutase and glutathione peroxidase activities in Jurkat T cells (20). Although antioxidative agents did not protect against cadmium-induced apoptosis in rat alveolar cells (21), cadmium caused cell cycle arrest in rat liver cells and co-treatment with NAC shifted the cells to the G1 phase (22).On the other hand, At low and moderate concentrations in cell culture systems cadmium primarily causes apoptosis, and at higher concentrations necrosis becomes evident in the kidney (23). There are also evidences of cadmium-induced autophagy in MES-13 mesangial cells, through the ROSGSK-3b signaling pathway (24).

Induction of apoptotic signals in cancer cells lead to the accumulation of P53 protein in the cell that increases the release of ROS, cytochrome C and several other regulatory factors from mitochondria. These factors are causing the activation cascade of caspases, which trigger the proteolysis of nuclear proteins and DNA degradation (13). So cancer cells with mutated P53 gene would be resistant to apoptosis (25). It was suggested that P53 mutation in human prostate epithelial cells is a possible contributing factor for the acquisition of apoptotic resistance in cadmium prostatic carcinogenesis (26). Cadmium mediates apoptosis in a human non-small cell lung carcinoma cell line (CL3) through activation of mitogen-activated protein kinases (JNK, p38 and ERK) (27). Yan liu and his colleagues shown that N-acetylcysteine at concentration of 500 micromolar significantly inhibits ROS production, subsequent JNK phosphorylation and conversion of procaspase-3 to activate Caspase-3, thus prevents apoptosis in Pig Kidney Epithelial LLC-PK1 cells (28).

Concerning breast cancer, very limited studies have been produced so far on the role played by cadmium on human breast cancer or non tumoral breast epithelial cells. Sirchia et al.(29) showed that cadmium regulated the expression levels of genes coding for stress response factors (e.g. heat shock proteins and metallothioneins), and for apoptosis-related factors and enzymes in MDA-MB231 estrogen receptor-negative human breast cancer cells and or HB2 non tumoral breast epithelial cells. Whilst HB2 cells appear to activate defense mechanisms against metal stress via metallothionein up-regulation, MDA-MB231 cells direct cells to a kind of death with down-regulation of Bcl-2, over-expression of Dap kinase and several caspases (29), as well as downregulation of astrocyte-elevated gene-1 (AEG-1) followed by the decrease of p65, NF-kB, c-fos and c-jun (30). In disagreement with our finding Cannino et al (31) showed that cadmium is able to exert a cytotoxic effect on tumor MDA-MB231 cells, which shows signs of a non-apoptotic type of cell death and characterized by significant increase in the production of reactive oxygen species and induced drastic changes in gene expression pattern (32). Moreover, it was shown that cadmium inactivated the p53 protein in human breast cancer MCF-7 cells. There are some reports that support a possible relationship between cadmium and breast cancer (33). It was reported that cadmium significantly higher in breast tumor tissues, blood and urine of cancer patients than in controls (34). Moreover cadmium malignantly transforms normal human breast epithelial cells through a mechanism independent of estrogen receptor into a basal-like cancer phenotype (35).

In conclusion our results suggest that cytotoxic effects and induction of apoptosis (morphological studies as well as DNA ladder fragmentation) caused by $CdCl_2$ are mediated, by oxidative stress.

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