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The Inhibitory Effect of Zinc on the Cadmium-induced Apoptosis in Human Breast Cancer Cells

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유방암세포에서 카드뮴에 의해 유도된 아폽토시스에 대한 아연의 저해 효과

오지영, 이수정, 신재호, 김태성, 문현주 강일현, 강태석, 김안근[1], 한순영[※]

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요 약

아연은 다양한 독성 물질로부터 유도된 아폽토시스를 저해하는 것으로 알려져 있으나 이 기전에 대해서는 명확히 밝혀지지 않았다. 본 연구에서는 인간 유방암 세포 MCF-7에 카드뮴을 처리하였을 때 유도되는 아폽토시스에 대한 아연의 저해효과를 살펴보았다. 아연의 아폽토시스 저해 효과는 DNA 분절현상, 핵의 쪼개짐 그리고 caspase-9의 발현을 통하여 확인하였다. 또한 아연의 아폽토시스 저해효과가 카드뮴에 의한 산화적 스트레스와 관련이 있는지 확인하기 위하여 활성산소인 peroxide의 농도를 세포내에서 측정하였다. 나아가 superoxide dismutase (SOD), catalase (CAT) 그리고 glutathion reductase (GR)같은 활성산소에 대한 인체내 방어기작으로 작용하는 항산화 효소의 활성을 측정하였다.

본 연구를 통해 아연이 카드뮴에 의해 생성된 세포내의 활성산소의 양을 감소시키고 항산화 효소를 회복시키는 기전이 카드뮴에 의한 아폽토시스를 저해하는 한 요인으로 사료되어진다.

Key words : zinc, cadmium, apoptosis, ROS, antioxidant enzyme

INTRODUCTION

Apoptosis is a positive programmed form of cell death, which eliminates genetically damaged and infected cells from the body, which is of importance for the control of development and of homeostasis in multi-cellular organisms (Li *et al.*, 2000; Owuor and Kong, 2002). The morphological and biochemical features of cells undergoing apoptosis are characterized by cell shrinkage, membrane blebbing, and nuclei breaks, and by protein expressions such as Bcl-2 family proteins and caspases (Thornberry and

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Lazebnik, 1998). Oxidative stress is defined as an imbalance between pro-oxidants or free radicals and anti-oxidizing enzymes as like SOD, GPx, CAT, and GR (Mates *et al.*, 1999). ROS, which include the free radical superoxide anion ($O_2 \cdot^-$), the hydroxyl radical (OH ·), and non-free radical hydrogen peroxide (H_2O_2) are the byproducts of the normal cellular metabolism or are formed by extra cellular environment challenges-e.g. UV radiation, ionization, cigarette smoking and metals (Kumar and Jugdutt, 2003). ROS have sufficient reactivity to cause protein oxidation and degradation, lipid peroxidation, and DNA damage. Severe oxidative stress progressively leads to cellular toxicity and ultimately induce cell death (Bonnefoy *et al.*, 2002).

Cadmium is a highly toxic metal to humans because it negatively affects various metabolic processes, e.g., membrane transport system and protein synthesis. In addition, cadmium may act on DNA and interfere with gene regulation and repair processes (Hartwig and Beyersmann, 1989; Hartwig, 1994; Waisberg *et al*, 2003). Environmentally, human are exposed to cadmium as a food contaminant, in industrial products such as paints, plastics, batteries, and by cigarette smoke. Moreover, several reports have demonstrated that cadmium can induce apoptosis in various organs and cells, e.g., in the liver (Habeebu *et al.*, 1998), kidney (Hamada *et al.*, 1996), lung epithelial cells (Hart *et al.*, 1999), and immune cells (el Azzouzi *et al.*, 1994; Tsangaris and Tzortzatou-Stathopoulou, 1998). Also, Farris (1991) suggested that cadmium toxicity may be related to ROS production and could have a negative influence on the intracellular enzymatic system (Hussein *et al.*, 1987). On the other hand, zinc is a well-known inhibitor of apoptosis, and many suggestions have been made concerning its apoptosis inhibitory mechanisms, for instance, suppression of caspase expression (Perry *et al.*, 1997), blocking of calcium/magnesium-dependent endonuclease (Barbieri *et al.*, 1992), and ROS generation (Bray and Bettger, 1990).

Despite several researches, there is no report about inhibitory effect of zinc on the cadmium-induced apoptosis in human breast cancer MCF-7 cells. So, we investigated the mechanism of the inhibitory effect of zinc in MCF-7 apoptosis by cadmium. In particular, we herein focused on the aspect of oxidative stress by cadmium.

MATERIALS AND METHODS

1. Chemicals

Cadmium chloride ($CdCl_2$) and zinc chloride ($ZnCl_2$) were obtained from Sigma (St. Louis, MO, USA). Both were dissolved in dimethylsulphoxide (DMSO), also from Sigma.

2. Cell culture

MCF-7 cells were obtained from the ATCC (American Type Culture Collection, USA) and grown in RPMI 1640 media (Gibco BRL, Grand Island, N.Y., USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL), 10,000 units/mL penicillin and 10 mg/mL streptomycin. Cells were incubated in a humidified 5% CO_2 atmosphere at 37°C and detached with 0.25% trypsin containing 1mM EDTA when 70% confluent.

3. Observation of apoptosis inhibitory effect

1) Quantitative DNA fragmentation assay

To detect DNA fragmentation, regarded as a hallmark of apoptosis, DNA fragmentation assay was performed. Briefly, cadmium and/or zinc treated cells were dissolved in lysis buffer [10 mM Tris/HCl, 1 M NaCl, 10 mM EDTA, 0.6% (w/v) SDS, pH 8.0] and incubated overnight. DNA was then extracted twice using phenol : chloroform : isoamyl alcohol (25 : 24 : 1) (Sigma) and isopropyl alcohol. RNA was removed by incubating the above extracted pellet with RNase (10 μg/mL). Extracted DNA was washed with 70% Et-OH and visualized by electrophoresis in a 1.5% agarose gel containing ethidium bromide. Photographs were taken under UV (312 nm) transillu-

mination (Vilber lourmat, France) to visualize DNA mobility.

2) Morphological evaluation of nuclei

Cells were rinsed twice with cold PBS and fixed with formalin solution for 3 h. Following a second washing with PBS, cells were incubated with 4′, 6-diamidino-2-phenylindole (DAPI, Sigma) solution (1 μg/mL) for 2 h and apoptotic bodies were observed by fluorescence microscopy (Olympus, Japan).

3) Cell cycle analysis

To analyze cell cycle distributions, cells were collected after chemical treatment and washed with PBS. Fixed cells (10^6/mL) with 70% Et-OH were washed PBS and incubated with RNase (1 mg/mL) and PI (400 μg/mL, Sigma). Percentage of degraded DNA was examined by flow cytometry (Beckman-Coulter, USA) using EPICS system II software (Ver.3.0).

4) Detection of caspase-9 expression

For detection of caspase-9 expression, cells were resuspended in lysis buffer (0.5% triton X-100, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, 25 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4) and cytoplasmic extracts were separated by centrifugation at 15,000 rpm for 15 min. Protein concentration was determined using the Bradford method (Bio-Rad protein assay kit, CA, USA). Same protein (20 μg/mL) was then boiled for 5 min at 92°C and loaded onto 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membrane (Bio-Rad) and blocked overnight with 5% skimmed milk in PBST [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Tween 20]. Membrane was then incubated overnight with primary anti-rabbit caspase-9 (1 : 500) (Santacruz, CA, USA) at 4°C. After incubation with goat anti-rabbit IgG-horseradish peroxidase secondary antibody (1 : 1,000) (Santacruz) for 2 h, membrane was developed by enhanced chemiluminescence (ECL, Amersham Biosciences, UK, England).

4. Determination of peroxides production

The level of peroxides was determined by measuring Fe^{2+} oxidation at an acidic pH (Jiang *et al.*, 1991). A standard curve for tert-butyl hydroperoxide was prepared, 90% methanol reagent containing 4 mM butylated hydroxytoluene (BHT) and 125 μM xylenol orange was added to cytoplasmic fractions. After approximately 30 min at room temperature, absorbance was read at 560 nm and converted to peroxide concentration. Peroxides level was determined using a PeroxiDetect kit, according to the manufacturer's instructions (Sigma).

5. Measurement of anti-oxidative enzyme activities

1) Superoxide dismutase assay

SODs (E.C. 1.15.1.1.) were measured using SOD kit (OxisResearch, Inc. USA). SODs were determined by measuring increased rate of autoxidation of 5, 6, 6a, 11b-tetrahydro-3, 9, 10-trihydroxybenxofluorene at pH 8.8 (Nebot *et al.*, 1993). A mixture of 1, 4, 6-trimethyl-2-vinylpyridinium trifluoromethanesulfonate in HCl and the individual homogenized cytoplasmic enzyme samples were incubated for 1 min at 37°C, and then 30 μL 5, 6, 6a, 11b-tetrahydro-3, 9, 10-trihydroxybenxofluorene in HCl containing diethylenetriaminepentaacetic acid (DTPA) and ethanol was added and absorbance read at 525 nm as 6 sec intervals, 10 times. One unit of SODs activity was calculated using the following formula.

$$\text{Units/mL} = A/B$$

$$A: 0.93 \times \{(V_S/V_C) - 1\} \quad B: 1.073 - 0.073 \times (V_S/V_C)$$

(V_S: slope of the linear portion of the sample absorbance curve,

V_C: slope of the linear portion of the blank absorbance curve)

2) Catalase assay

CAT (E.C. 1.11.1.6) activities were determined using the CAT kit (OxisResearch). Catalase was measured by a quenched H_2O_2 amount by 4-aminophenazone 4-aminoantipyrene (AAP), 3, 5-dichloro-2-

hydroxybenzenesulfonic acid (DHBS) and horseradish peroxidase (HRP) (Aebi, 1984; Wakimoto *et al.*, 1998). Briefly, 500 μL sodium azide was added to cytosolic fraction containing 10 mM H_2O_2. Two ml of HRP/Chromogen reagent was then added and incubated for 10 min. Catalase activity was defined as the catalase enzyme's standard curve at 520 nm.

3) Glutathion reductase assay

This assay was performed using a GR kit (Sigma). GR (E.C. 1.6.4.2) was determined by following the reduced absorbance caused by the oxidation of NADPH (Garcia-Alfonso *et al.*, 1993). Briefly, the enzyme reaction was conducted in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. Absorbance of the mixed solution of 2 mM EDTA, oxidized glutathion 500 μL, homogenated protein 100 μL and 2 mM NADPH 50 μL in assay buffer was read at 340 nm as 10 sec intervals, 10 times. The oxidation of NADPH was calculated using a mM extinction coefficient of 6.22 for NADPH.

6. Statistical analysis

Values are expressed as means±S.D. from at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Dunnett's test. P values of <0.05 were considered statistically significant.

Table 1. Occurrence of apoptotic bodies. MCF-7 cells were exposed to various concentrations of $CdCl_2$ (0~200 μM) for 12 h. Nuclei were stained with DAPI (1 μg/mL) and apoptotic cells were counted among 100 cells under a fluorescence microscope. % of apoptotic bodies is the mean value±S.D. of three independent experiments

Concentration (μM)	% of apoptotic bodies
0	0.67±0.29
25	3.00±0.87
50	2.67±0.58
75	6.33±0.77
100	15.00±0.50
125	17.67±0.50
150	17.00±1.04
175	22.33±0.77
200	28.33±0.77

RESULTS

1. Assessment of $CdCl_2$-induced apoptosis and cytotoxicity

To observe whether the cell death by $CdCl_2$ is due

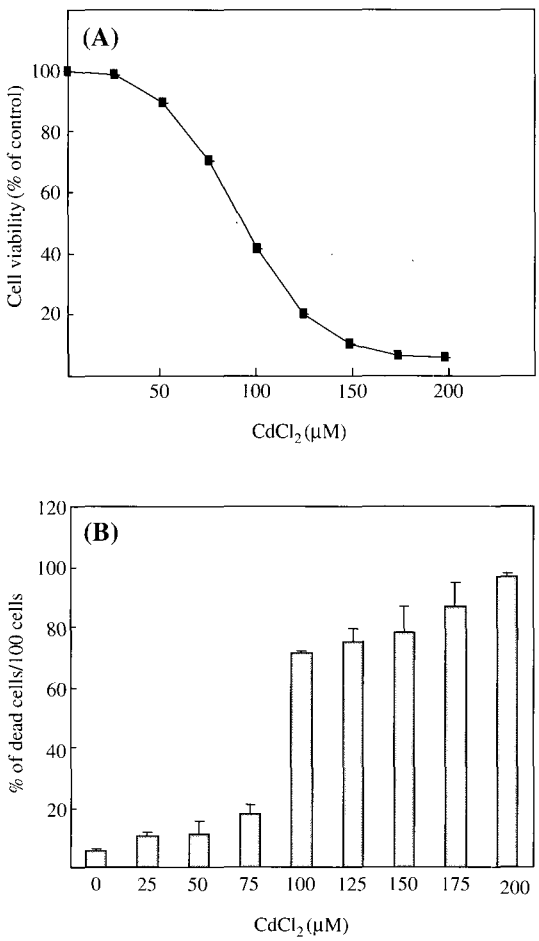


Fig. 1. Dose dependent course of MCF-7 cell viability after exposure $CdCl_2$. The cells were exposed to the various concentrations of $CdCl_2$ (0~200 μM) for 12 h. Cell viability was determined by MTT assay (panel A). And dead cells were counted by trypan blue exclusion assay (panel B). Values on panel A show the mean (n = 6), and the % of dead cells among total 100 cells in panel B were obtained by triplicate experiments.

to an apoptotic mechanism and to determine appropriate dose and time for the test, we counted apoptosis bodies after treating $CdCl_2$ (0~200 μM) for 12 h. A number of nuclei breaks, cell shrinkage and nuclei condensation were found on $CdCl_2$ dose dependently by DAPI stain (Table 1). Especially, when we exposed 100 μM $CdCl_2$ for 12 h, we observed the occurrence of strong DNA fragmentation strongly (data not shown). Moreover, we investigated cytotoxicity of cadmium by cell viability assay. Through two assays, MTT and trypan blue assay, cadmium was shown the decrease of cell viability dose responsively (0~200 μM). MCF-7 cells showed 50% cell viability at approximately 100 μM cadmium (Fig. 1).

2. Apoptosis inhibitory effect of $ZnCl_2$ on $CdCl_2$ -induced apoptosis

To observe apoptosis inhibitory effect of zinc, we evaluated DNA fragmentation, nuclei breaks, flow cytometry analysis, and caspase-9 expression were measured as apoptotic features to observe apoptosis inhibitory effect of zinc. After co-treating $ZnCl_2$ and $CdCl_2$ for 12 h, we observed that $ZnCl_2$ (10, 50, 100, and 150 μM) dose-dependently inhibited $CdCl_2$-induced DNA fragmentation. In particular, fragmented DNA mobility of cells co-treated with same concen-

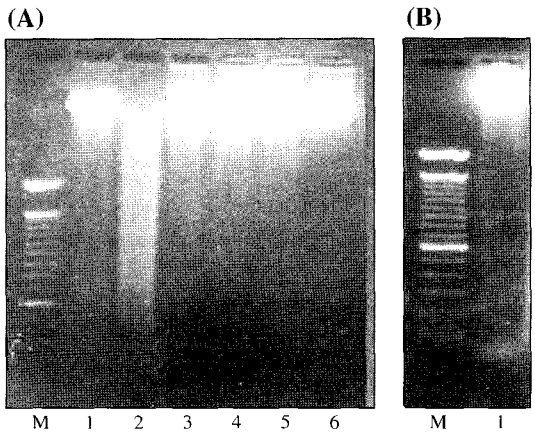


Fig. 2. Inhibition of DNA fragmentation by $ZnCl_2$. MCF-7 cells were treated with 100 μM $CdCl_2$ and/or the various concentration of $ZnCl_2$ for 12 h. Total genomic DNA (10 μg) extracted from the cells was electrophoresed. Results are representative of two independent experiments [(A) Lane1: Control, Lane2: $CdCl_2$ 100 μM, Lane3: $CdCl_2$ 100 μM+$ZnCl_2$ 10 μM, Lane4: $CdCl_2$ 100 μM+$ZnCl_2$ 50 μM, Lane5: $CdCl_2$ 100 μM+$ZnCl_2$ 100 μM, Lane6: $CdCl_2$ 100 μM+$ZnCl_2$ 150 μM, (B) Lane1: $ZnCl_2$ 100 μM, M: DNA 100 bp size marker].

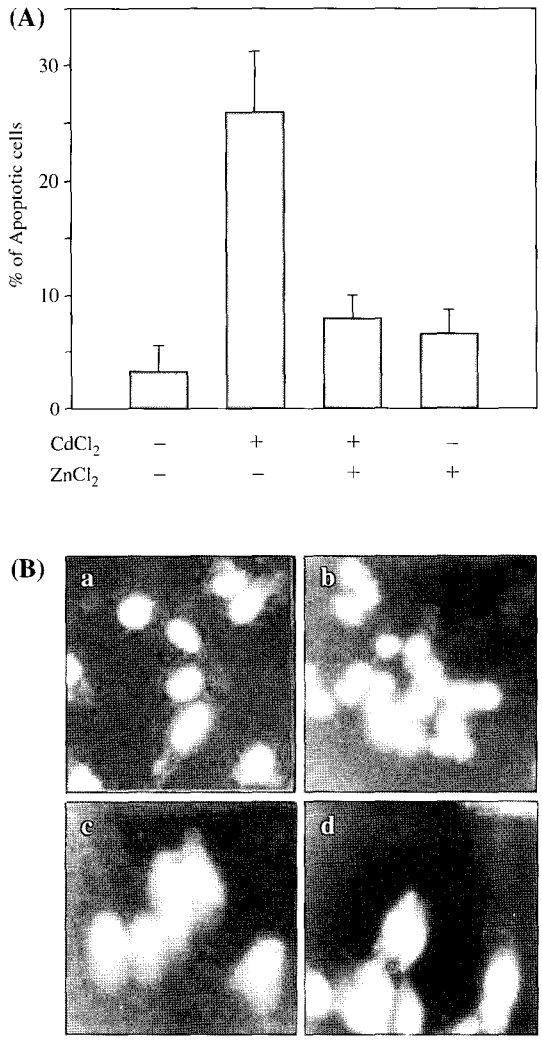
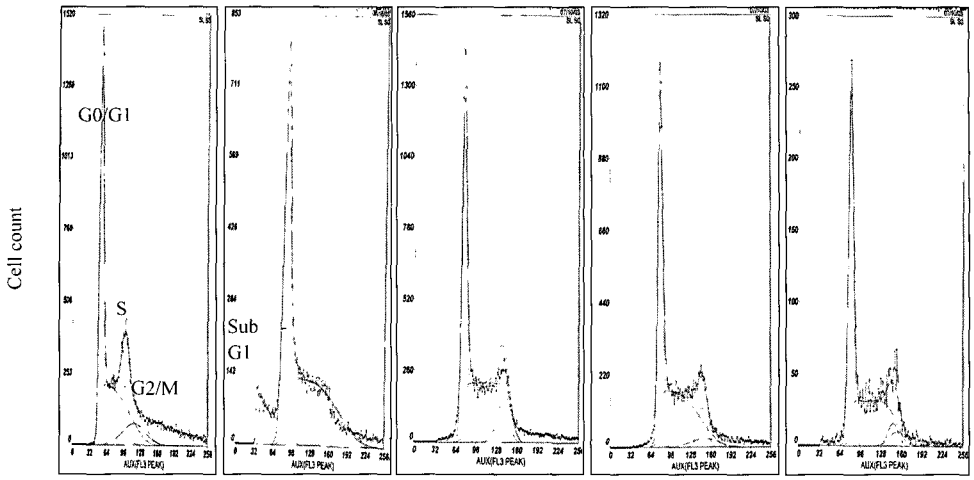


Fig. 3. Morphological change in nuclei. Cells were exposed to 100 μM $CdCl_2$ and /or $ZnCl_2$ for 12 h and stained with DAPI (1 μg/mL). Apoptotic bodies were observed under a fluorescence microscope (×400). Panel A shows the % of apoptotic bodies versus control cells. Pictures of panel B are representative of three independent tests (a: control, b: $CdCl_2$ 100 μM, c: $CdCl_2$ 100 μM+$ZnCl_2$ 100 μM, d: $ZnCl_2$ 100 μM).



$CdCl_2$	−	+	+	+	+
Zinc	−	−	50	100	100

Fig. 4. The apoptosis inhibitory effect of $ZnCl_2$ through cell cycle analysis. After treating $CdCl_2$ and/or $ZnCl_2$ (50, 100, and 150 μM) for 12 h, viable MCF-7 cell numbers were measured by flow cytometry. The above pictures are the representative of three independent experiments.

tration (100 μM) of $ZnCl_2$ and $CdCl_2$ was similar to that of control cells (Fig. 2A). In addition, we found that apoptotic bodies were significantly reduced in the $ZnCl_2$ (100 μM) co-treated group versus the $CdCl_2$-treated group. We counted apoptotic bodies (Fig. 3A) and observed them under microscope (Fig. 3B). Through cell cycle analysis by flow cytometry, we found that the sub-G1 cell populations were reduced by $ZnCl_2$ (Fig. 4). Additionally, when $ZnCl_2$ (100 μM, 12 h) was treated to MCF-7 cells, the sub-G1 peak was not shown (data not shown). The caspases, a family of cysteine proteases, are major participants in apoptotic signal transduction (Elinos-Baez *et al.*, 2003). We supposed that $CdCl_2$-induced apoptosis is partially induced through a mitochondrial dependent pathway. Another apoptosis marker caspase -9 that is mitochondria dependent caspase was suppressed by $ZnCl_2$ co-treatment (Fig. 5), but was not expressed in the 100 μM $ZnCl_2$-treated group (data not shown).

3. Generation of ROS by $CdCl_2$ and its reduction by $ZnCl_2$

When the cells were exposed to 100 μM $CdCl_2$ for 12 h, peroxides were induced, but, cells co-treated with 100 μM $ZnCl_2$ and $CdCl_2$ showed significantly lower peroxides level. Also, 100 μM $ZnCl_2$-treated cells more generated peroxides than $ZnCl_2$ and $CdCl_2$ co-treated cells, but this was less than in control cells (Fig. 6).

4. Measurement of anti-oxidative enzyme activities

After exposing $CdCl_2$ (100 μM, 12 h), we found SOD, CAT, and GR activities were reduced significantly. Whereas, cells co-treated with 100 μM $ZnCl_2$ and $CdCl_2$ showed higher SOD activity than the control group (Fig. 7A). Also, the CAT activity was recovered to the same level as the control (Fig. 7B), but the cellular activity of GR was not elevated after co-treating $ZnCl_2$ and $CdCl_2$ (Fig. 7C). Meanwhile,

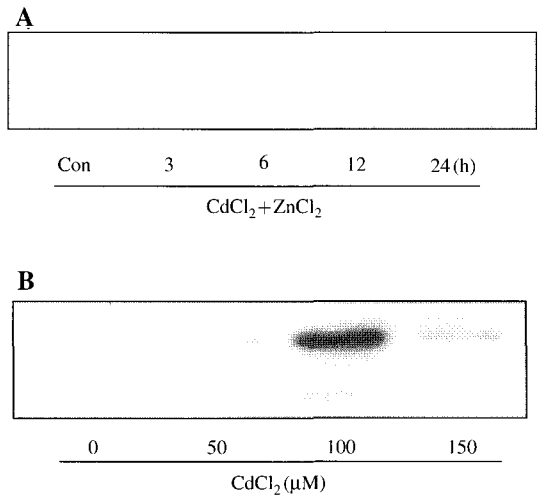


Fig. 5. Capase-9 expression. Proteins extracted from the cells were analyzed by western immuno-blotting. Results are representative of two independent experiments. [Panel A: Cells were incubated with $CdCl_2$ (100 μM) and $ZnCl_2$ (100 μM) for various times (3, 6, 12, and 24 h), Panel B: Cells were exposed to $CdCl_2$ (50, 100, and 150 μM) for 12 h]

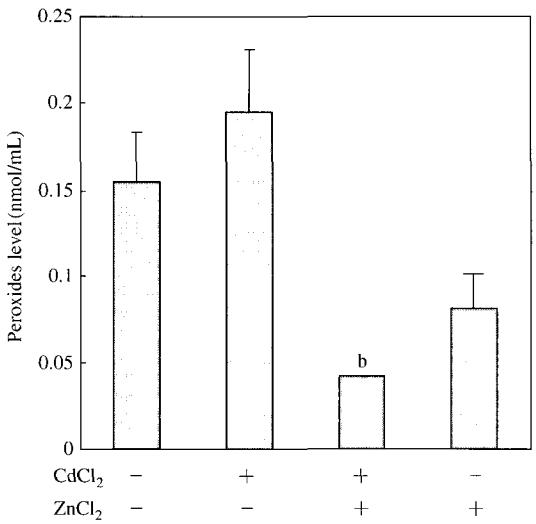


Fig. 6. Peroxides level measurement. MCF-7 cells were treated with $CdCl_2$ (100 μM) and/or $ZnCl_2$ (100 μM) for 12 h. Peroxide levels were measured by oxidizing Fe^{2+}. Each column shows the mean value of three experiments±S.D. (b) significantly different from the group exposed to 100 μM $CdCl_2$ ($P<0.05$, one-way ANOVA).

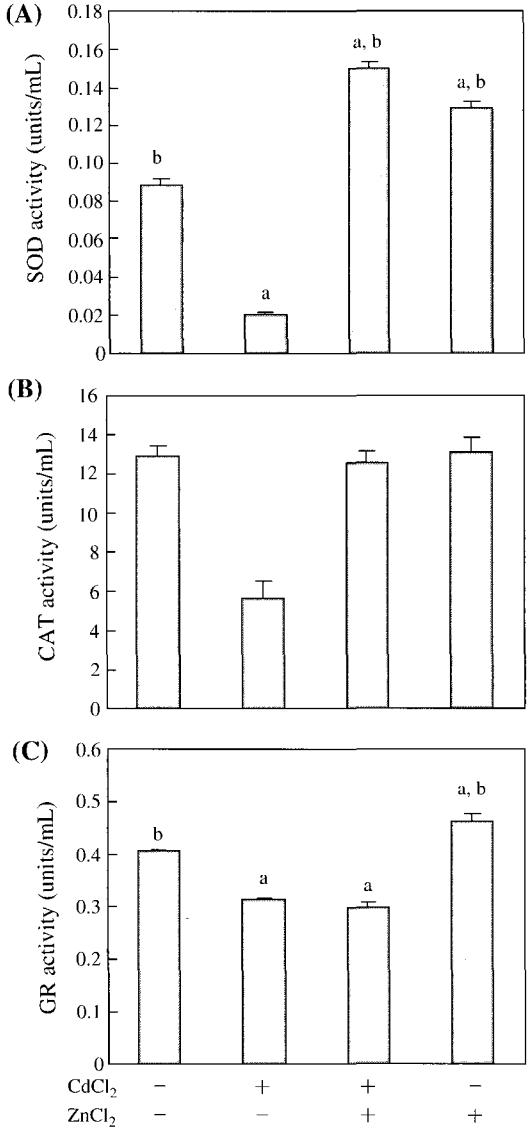


Fig. 7. Cellular anti-oxidative enzyme activities. Chemically treated MCF-7 cells (100 μM $CdCl_2$ and/or $ZnCl_2$ for 12 h) were harvested, and their protein was extracted. (A) SODs activity was determined by measuring the increase of autoxidation of 5, 6, 6a, 11b-tetrahydro-3, 9, 10-trihydroxybenxofluorene at 525 nm. (B) The CAT activity was measured by determining the quenched H_2O_2 amount. (C) GR activity was detected by the oxidation of NADPH. Each column represents the mean value of three experiments±S.D. (a) significantly different from the control ($P<0.05$, one-way ANOVA); (b) significantly different from the group exposed to 100 μM $CdCl_2$ ($P<0.05$, one-way ANOVA).

the activities of the above three enzymes in the 100 μM $ZnCl_2$-treated group were almost the same or more compared to control level.

DISCUSSION

Cadmium induced-apoptosis has been observed in cells or organs e.g. mouse liver and T cell. (El Azzouzi *et al.*, 1994; Habeebu *et al.*, 1998) We also confirmed apoptosis phenomenon triggered by cadmium in MCF-7 cells by using several parameters such as DNA fragmentation, nuclei breaks or condensation and special protein expression. A number of theories about cadmium-induced apoptosis have been suggested. In genotoxicity respects, cadmium ions cause conformational DNA changes, chromosomal aberrations, and DNA strand breaks (Hamada *et al.*, 1996; Habeebu *et al.*, 1998). When cells are exposed to cadmium, several genes with an important role in apoptosis are induced e.g. c-jun, c-fos, c-myc, tumor suppressor gene p53 (Matsuoka and Call, 1995; Zheng *et al.*, 1996). From the view of oxidative stress, the main point of this study is that cadmium can negatively affect mitochondria (Koizumi *et al.*, 1994). Our data show that oxidative stress could be related with cell death. When we treated cadmium (12 h, 100 μM) in MCF-7cells, we observed that the peroxide level was elevated. ROS induced by cadmium increase lipid peroxidation, thiobarbituric acid reactive substance formation, malondialdehyde in the liver, brain, and lung (Manca *et al.*, 1991). Also, ROS directly affect protein and DNA (Mates *et al.*, 1999) and leads to apoptosis (Farris, 1991). Caspase plays a central role in apoptosis. These proteins exist in the cytosol as inactive pro-enzymes that become activated during apoptosis. After we treated $CdCl_2$ (50, 100, and 150 μM) for 12 h, we observed caspase-9 expression at all doses (Fig. 5B). Also, cadmium has a negative effect on anti-oxidative enzymes that are the major scavengers of intracellular ROS (Hussein *et al.*, 1987). Many detoxifying and cytoprotective proteins, i.e. metallothioneins, glutation, heat hock proteins (Hsp), heme oxygenase, and zinc transporter proteins respond to cadmium exposure (Beysersmann, 2002). We investigated three anti-oxidative enzyme activities, that is-GR, CAT, and SOD, and we confirmed that these enzyme activities were depleted in the presence of cadmium.

Zinc can play a biological important role in the control of apoptosis. After co-treating zinc and cadmium, we observed a reduction in the cellular peroxide level in MCF-7 cells. Also, zinc co-treatment recovered SODs and CAT enzyme activities to the control intracellular peroxides. But, depleted cellular GR by cadmium was not recovered by zinc co-treatment. Anti-oxidative enzyme level is known to be a modulator of the apoptotic process (Stohs and Bagchi, 1995). If anti-oxidative enzyme level is low, harmful hydroxyl radicals or superoxide anions remain in the body (Pryor, 1986; Fleury *et al.*, 2002). So, we think that the increase of anti-oxidative enzyme activity in response to cadmium is a protective factor of oxidative stress-induced apoptosis. When cells are supplemented with antioxidants such as zinc, cells can better resist oxidative stress. In addition, zinc is associated with cell membrane stabilization, metallothionein transcription, and a ZnSOD structure and ROS scavenging (Wellinghausen *et al.*, 1997). Finally, we confirmed the protective effect of zinc against cadmium-induced apoptosis by observing its inhibition of DNA fragmentation, nuclei breaks, and caspase-9 expression. In particular, the suppression of caspase-9 expression means that zinc could prevent damage of mitochondria. Through our study, we confirmed that zinc could reduce ROS induced by cadmium though elevating the activities of anti-oxidative enzyme, and the mode of its action may be associated with the mitochondria dependent apoptosis pathway, as evidenced by peroxides level and caspase-9 expression. Our results suggest that oxidative stress is an inducing factor of cadmium-induced apoptosis. Also zinc has an anti-oxidative capacity by reducing free radicals and recovering anti-oxidative enzyme activities.

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REFERENCES

Aebi H. Catalase in vitro, Methods Enzymol 1984; 105: 121-126.

Barbieri D, Troiano L, Grassilli E, Agnesini C, Cristofalo EA, Monti D, Capri M, Cossrizza A and Franceschi C. Inhibition of apoptosis by zinc: a reappraisal, Biochem Biophys Res Commun 1992; 187: 1256-1261.

Beysersmann D. Effects of carcinogenic metals on gene expression, Toxicology letters 2002; 127: 63-68.

Bonnefoy M, Drai J and Kostka T. Antioxidants to slow aging, facts and perspectives, Presse Med 2002; 31(25): 1174-1184.

Bray TM and Bettger WJ. The physiological role of zinc as an antioxidant, Free Radic Biol Med 1990; 8(3): 281-291.

El Azzouzi B, Tsangaris GT, Pellegrini O, Manuel Y, Benveniste J and Thomas Y. Cadmium induces apoptosis in a human T cell line, Toxicology 1994; 88: 127-139.

Elinos-Baez CM, Maldonado V and Melendez-Zajgla J. Caspases: apoptosis inducing molecules, Gac Med Mex 2003; 139(5): 493-499.

Farris MW. Cadmium toxicity: unique cytoprotective properties of alpha tocopheryl succinate in hepatocytes, Toxicology 1991; 69: 63-77.

Fleury C, Mignotte B and Vayssiere JL. Mitochondrial reactive oxygen species in the cell death signaling, Biochimie 2002; 84(2-3): 131-141.

Garcia-Alfonso C, Martinez-Galisteo E, Llobell A, Barcena JA and Lopez-Barea J. Horse-liver glutathione reductase. purification and characterization, Int J Biochem 1993; 25(1): 61-68.

Habeebu SS, Liu J and Klaassen CD. Cadmium-induced apoptosis in mouse liver, Toxicol Appl Pharmacol 1998; 149: 203-209.

Hamada T, Sasaguri T, Tanimoto A, Arima N, Shimajiri S, Abe T and Sasaguri Y. Apoptosis of human kidney 293 cells is promoted by polymerized cadmium-Metallothionein, Biochem Biophys Res Commun 1996; 219: 829-834.

Hart BA, Lee CH, Shukla GG, Shukla A, Osier M, Eneman JD and Chiu JF. Characterization of cadmium-induced apoptosis in rat lung epithelial cells: evidence for the participation of oxidant stress, Toxicology 1999; 133: 43-58.

Hartwig A. Role of DNA repair inhibition in lead and cadmium-induced genotoxicity: a review, Environ Health Persp 1994; 102: 45-50.

Hartwig A and Beyersmann D. Comutagenicity and inhibition of DNA repair by metal ions in mammalian cells, Biol Trace Elements Res 1989; 21: 359-365.

Hussein T, Shulka GS and Chandra SF. Effects of cadmium on superoxide dismutase and lipid peroxidation in liver and kidney of growing rats: in vivo and in vitro studies, Pharmacol. Toxicol 1987; 60: 355-358.

Jiang ZY, Woollard AC and Wolff SP. Lipid hydroperoxide measurement by oxidation of Fe^{2+} in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method, Lipids 1991: 26(10): 853-856.

Koizumi T, Yokota T, Shirakura H, Tatsumoto H and Suzuki KT. Potential mechanism of cadmium-induced cytotoxicity in rat hepatocytes: Inhibitory action of cadmium on mitochondrial respiratory activity, Toxicology 1994; 92: 115-125.

Kumar D and Jugdutt BI. Apoptosis and oxidants in the heart, J Lab Clin Med 2003; 142(5): 288-297.

Li M, Kondo T, Zhao L, Li FJ, Tanabe K, Arai Y, Zhou ZC and Kasuya M. Apoptosis induced by cadmium in human lymphoma U937 cells through Ca^{2+}-calpain and caspase-mitochondria-dependent pathways, J Biol Chem 2000; 275: 39702-39709.

Manca D, Richard AC, Trottier B and Chevalier G. Studies on lipid peroxidation in rat tissues following administration of low and moderate doses of cadmium chloride, Toxicology 1991; 67: 303-323.

Mates JM, Perez-Gomez C and Nunez de Castro I. Antioxidant enzymes and human diseases, Clin Biochem 1999; 32(8): 595-603.

Matsuoka M and Call KM. Cadmium-induced expression of immediate early genes in LLC-PK1 cells, Kidney Int 1995; 48: 383-389.

Nebot C, Moutet M, Huet P, Xu JZ, Yadan JC and Chaudiere J. Spectrophotometric assay of superoxide dismutase activity based on the activated autoxidation of a tetracyclic catechol, Anal Biochem 1993; 214(2): 442-451.

Owuor ED and Kong AN. Antioxidants and oxidants regulated signal transduction pathways, Biochem Phar-

macol 2002; 64(5-6): 765-770.

Perry DK, Smyth MJ, Stennicke HR, Salvesen GS, Duriez P, Poirier GG and Hannun YA. Zinc is a potent inhibitor of the apoptotic protease, caspase-3. A novel target for zinc in the inhibition of apoptosis, J Biol Chem 1997; 272(30): 18530-18533.

Pryor WA. Oxy-radicals and related species: their formation, lifetimes and reactions, Annu Rev Physiol 1986; 48: 657-667.

Stohs S and Bagchi D. Oxidative mechanisms in the toxicity of metal ions, Free Radical Bol Med 18; 1995: 321-336.

Thornberry NA and Lazebnik Y. Caspase: enemies within, Science 1998; 281(5381): 1312-1316.

Tsangaris GT and Tzortzatou-Stathopoulou F. Cadmium induces apoptosis differentially on immune system cell lines, Toxicology 1998; 128: 143-150.

Waisberg M, Joseph P, Hale B and Beyersmann D. Molecular and cellular mechanisms of cadmium carcinogenesis, Toxicology 2003; 192(2-3): 95-117.

Wakimoto M, Masuoka N, Nakano T and Ubuka T. Determination of glutathione peroxidase activity and its contribution to hydrogen peroxide removal in erythrocytes, Acta Med Okayama 1998; 52(5): 233-237.

Wellinghausen N, Kirchner H and Rink L. The immunobiology of zinc, Immunol Today 1997; 18(11): 519-521.

Zheng H, Liu J, Choo K, Michalska AE and Klaassen CD. Metallothionein-I and -II knock-out mice are sensitive to cadmium-induced liver mRNA expression of c-*jun* and p53, Toxicol Appl Pharmacol 1996; 136: 229-235.