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Original Research

Oxidative Stress-Mediated Cytotoxicity of Cadmium in Chicken Splenic Lymphocytes

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Abstract

Cadmium (Cd), a potent immunotoxicant, has been reported to affect immunocytes both in humans and in rodents. However, whether such an effect exists in birds is still unclear. To delineate the cytotoxicity of Cd on the bird immunocytes *in vitro*, chicken splenic lymphocytes were exposed to Cd. A significant increase in the occurrence of cell damage was observed in chicken splenic lymphocytes following Cd administration. The enhanced level of LPO and the overproduction of ROS suggested that Cd caused oxidative stress in chicken splenic lymphocytes. A pronounced inhibition of SOD and GPx was seen after being indicated with 10 μM Cd for 36h or indicated with 15 μM Cd for 24 h. In addition, DNA damage and apoptosis was observed in chicken splenic lymphocytes treated with Cd. The degree of DNA damage and the number of apoptotic cells rose in a time- and dose-dependent manner. These results clearly demonstrate that chicken splenic lymphocytes appear to be more susceptible than mammalian immunocytes to the adverse effects of Cd. The oxidative stress and subsequent DNA damage and apoptosis induced by Cd are important mechanisms of Cd cytotoxicity to bird immunocytes.

Keywords: cadmium, chicken splenic lymphocyte, oxidative stress, DNA damage, apoptosis

Introduction

Cadmium (Cd) is an abundant, nonessential element that is generating concern due to its accumulation in the environment as a result of industrial activity. Cd is widely used in electroplating and galvanizing, as a color pigment in paints, and in batteries. Cd also is a byproduct of zinc and lead mining, and smelting. Agricultural uses of phosphate fertilizers and sewage sludge and industrial uses of Cd have been identified as a major cause of widespread dispersion of the metal at trace levels into the general environment. A global perspective on Cd pollution has been increasing. Human activities have enormously aggravated Cd pollution in ecosystems. The effects of Cd compounds on birds have been a widely studied topic in ecotoxicological studies.

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Present studies indicate that Cd is highly toxic toward multiple organs and a potent immunotoxicant. Immunosuppressive properties of Cd were demonstrated both *in vivo* and *in vitro* on humans and rodents [1-8]. Based on experimental evidence mostly from *in vivo* animal models, Cd is able to cause damage both to humoral immune response and cell-mediated immunity [9, 10]. It has been reported that seabirds, black-tailed godwit (*Limosa limosa*), lesser scaup (*Aythya affinis*), and tree sparrows were susceptible to Cd that entered their food chain [11-13]. Finkelstein et al. [14] indicated that it was a positive significant relationship between multiple contaminant concentrations including Cd and immune function in endangered black-footed albatrosses. In addition, numerous studies indicated that Cd exposure reduced the cell viability of lymphocytes in redonts and humans, and also induced oxidative stress, apoptotic and necrotic death [2-8]. Our previous studies confirmed that, in chickens, Cd causes immune suppression including spleen atrophy and suppression of cell-mediated and humoral immune responses. However, it was not clear how Cd affects bird immune cells after initial cell activation. The mechanisms responsible for the toxicity of Cd on bird immunocytes are not well understood.

It has been well known that the mechanisms responsible for Cd immunotoxicity involved oxidative stress. This metal could cause intracellular reduction in the level of the main antioxidant compounds, and enhance the production of free radicals and lipid peroxides, and disrupt the antioxidant systems. Reactive oxygen species (ROS) are often implicated in Cd immunotoxicology, either in a variety of immunocytes (including thymocytes and splenic lymphocytes) [3, 4], or in the immune organs through all routes of exposure [5, 6]. Indeed, Cd inactivates enzymes and other antioxidant molecules by interaction with the thiol groups contained in these molecules (glutathione or proteins) [15]. The inactivation of cellular antioxidant molecules can result in an increase in ROS [16]. Cd-increased ROS in turn produces lipid peroxidation (LPO), and results in DNA damage and apoptosis. However, no information is available regarding oxidative stress induced by Cd in chicken splenic lymphocytes.

Therefore, we designed experiments to investigate the oxidative stress, DNA damage and apoptosis induced by Cd on chicken splenic lymphocytes. In the present study, our work was complete with the aim of better understanding the mechanisms of Cd-induced immunotoxicity in birds using the chicken splenic lymphocytes as a model.

Materials and Methods

Preparation of Chicken Splenic Lymphocytes Suspension and Treatment

Spleens were dissected from Isa brown cocks (60 days old) and were collected aseptically and placed in sterile phosphate-buffered saline (PBS, 0.1 M phosphate buffer with 0.85% NaCl, pH 7.2). Single cell suspension was prepared by gently pushing the splenic pulp through a sterile stainless steel mesh with a pore size of 100 μM. Cells were washed and resuspended in 5 mL sterile PBS and then layered over 5 mL lymphocytes separation medium (Tian Jin Hao Yang Biological Manufacture Co., LTD., China). The splenocyte preparations were enriched by centrifuge (2,000×g) for 15 minutes at 18ºC. Cells were recovered from the interface, resuspended, and washed twice in 8mL cell culture medium (RPMI 1640, Gibco, USA). The cells were suspended in complete cell culture medium [RPMI 1640 containing HEPES and 2 mM glutamine, supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% antibiotic-antimycotic solution (Sigma, USA)]. Splenic lymphocyte density was adjusted to 1.5×10^6 cells/mL and the viability of the freshly isolated cells was always above 95% (trypan blue exclusion test).

For the monitoring of various parameters in the present investigation, cells were incubated with 1 μM Cd for 12 h, 24 h, 36 h, 48 h, and 72 h, and also were treated for 24 h in the absence and presence of various concentrations of Cd (5 μM, 10 μM, 15 μM, 20 μM, and 30 μM).

Cell Viability Assay

Cell viability was measured by the 3-(4,5-dimethyl-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) reduction method [17]. Splenic lymphocytes at a concentration of 1.0×10^5 cells were seeded in a 96-well plate. After incubating chicken splenic lymphocytes with Cd, 10 μL MTT (5 mg/mL, Sigma-Aldrich, USA) was added to the wells 4 h prior to the completion of incubation time. The plate was centrifuged at 1200×g for 10 minutes and 100 μL dimethylsulfoxide (DMSO, AMRISCO, USA) was added after removing the supernatant to dissolve the formed formazan. Five minutes later, the absorbance was read at 570 nm in a microplate reader (Synergy HT Multimode Reader, BioTek Instruments, USA).

DNA Damage Evaluated by the Comet Assay

After the chicken splenic lymphocytes were incubated with Cd, DNA damage was detected using the alkaline of the comet assay that was carried out under the alkaline conditions as previously described [18, 19]. Briefly, the frosted slides were prepared and covered with 20-50 μL of 0.8% normal melting point agarose (NMPA, Biowest, Spain) and recoated with 100 μL of 0.8% NMPA after solidification. Cell suspension and 1% lower melting point agarose (LMPA, Cambrex, USA) were mixed at the top layer of slides. After 10 minutes of solidification on ice, slides were covered with 0.5% LMPA. Afterwards the slides were immersed in lyses buffer (2.5 M NaCl, 10 mM Tris, 1% Na Sarcosinate, NaOH to pH 10.0, and 1% Triton X-100) for 3 h at 4ºC in dark and then washed and equilibrated in the alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13) for 20 minutes in order to unwind DNA. The electrophoresis was carried out for 20 minutes at 25 V and 300 mA, and then the slides were rinsed 3 times for 10

minutes each with neutralization buffer (0.4 M Tris-HCl, PH 7.5) and stained with 25 μg/mL ethidium bromide (EB) for 40 minutes.

One hundred cells per group (five slides of 20 randomly selected nonoverlapping cells each) were analyzed at 400× using a fluorescence microscope (DM-4000B, Leica, Germany) with a blue (488 nm) excitation filter and yellow (515 nm) emission (barrier) filter. One scorer was used throughout the study and all slides were coded. Quantification of DNA breakage was achieved by visual scoring and the cells were classified into five categories representing different degrees of DNA damage, ranging from no visible migration (type 0: no DNA damage) to the maximum length comet (type 4: extensive DNA damage) [20]. The frequency of damaged DNA was obtained by adding up the number of damaged cells from classes 0 to 4, then dividing by the total number of cells analyzed in each treatment. The total score for 100 comets was obtained by multiplying the number of cells in each class by the damage class, according to Manoharan and Banerjee's formula [21].

$$\text{Total score} = (0\times n_0)+(1\times n_1)+(2\times n_2)+(3\times n_3)+(4\times n_4),$$

...where n = number of cells in each class.

Thus, the total score could range from 0 to 400.

Apoptosis Analysis

Flow Cytometric Analysis of Apoptotic DNA

The following assays were carried out on chicken splenic lymphocytes at a concentration of 1.5×10^6 cells/mL. The cells with hypodiploid DNA were determined by cell cycle studies. After treatment, the cells were harvested and washed with PBS, fixed by adding ice cold 70% ethanol dropwise and stored at 4ºC overnight. The fixed cells were washed and suspended in 1 mL PBS. Following the addition of 200 μL phosphate citrate buffer (pH 7.8) to the cells, these were incubated for 60 minutes at room temperature. After centrifugation, the cells were resuspended in 0.5 mL propidium iodide (PI) stain (10 mg PI from Sigma-Aldrich, USA, 0.1 mL Triton-X 100 and 3.7 mg EDTA in 100 mL PBS) and 0.5 mL RNase A (50 μg/mL), incubated for 30 minutes in dark. The PI fluorescence was measured through an FL-2 filter (585 nm) and 10,000 events were acquired. The flow cytometric analysis was performed on a BD-LSR flow cytometer (BD Biosciences, Canada). Cell debris characterized by low FSC/SSC was excluded from analysis. The data analyzed by Cell Quest software (BD Biosciences, Canada) and mean fluorescence intensity was obtained by histogram statistics.

Morphological Study with Fluorescence Microscope

Apoptotic morphology was studied by staining the cells with a combination of the fluorescent DNA-binding dyes acridine orange (AO) and EB [22]. Cells were harvested and washed three times with PBS after being incubated with Cd as described above, and were then stained with 100 μg/mL AO (AMRISCO, USA) and EB (Sigma-Aldrich, USA) for 2 minutes. After 2 minutes of incubation, cells were washed twice with PBS (5 minutes each) and visualized under a fluorescence microscope (DM-4000B, Leica, Germany) at 400× magnification with excitation filter 480/30 nm. Three independent cell counts (counting a minimum of 100 total cells each for apoptosis analysis) were obtained on the basis of differential staining of the nuclei (live cells have a normal green nucleus; early apoptotic cells have bright green nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange chromatin; necrotic cells display a structurally normal orange nucleus) (Fig. 4).

DNA Ladder Analysis

The DNA fragmentation pattern (DNA ladder) was analyzed by agarose gel electrophoresis. An aliquot of 2 mL (1.5×10^6 cells/mL) was incubated with Cd as described above. At the end of incubation, cells were pelleted by centrifuge at 200×g for 10 minutes and the pellet was lysed with 0.5 mL lysis buffer (10 mM Tris-HCl, pH 7.5, 20 mM EDTA, 0.5% Triton X-100) on ice for 30 minutes. The DNA in lysed solution was extracted with phenol/chloroform and precipitated with 3 M sodium acetate (pH 5.2) and cold ethanol. After repeated washings the DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA was then loaded on 1.8% agarose gel for electrophoresis analysis. The bands were visualized by EB staining under UV light.

Determination of ROS, LPO and Antioxidant Enzymes

Intracellular ROS Measurement

The generation of ROS was detected by 2,7-dichlorofluorescein (DCF) fluorescence. After treatment, cells were harvested, washed with PBS (pH 7.2), and incubated with 10 μM 2,7-dichlorofluorescein diacetate (DCFH-DA) for 30 minutes in dark at 37ºC. And then ROS generation was measured by the fluorescence intensity (FL-1, 530 nm) of 10,000 cells using the fluorescence microplate reader (FLx800, BioTek Instruments, USA).

Preparation of the Cellular Extracts

Cells were harvested after the different treatments. The pellets obtained after centrifugation (5 minutes, 3,000×g, 4ºC) were resuspended in isotonic Tris-HCl buffer (20 mM, pH 7.4, 300 mOsm), centrifuged for 5 minutes at 3,000×g at 4ºC, and rinsed twice with the Tris-HCl buffer. Then cells were lysed in hypotonic Tris-HCl buffer by five freeze-thaw cycles. The determinations were performed on the supernatants obtained after centrifugation of the lysates at 4ºC for 10 minutes at 3,000×g. The extracts were stored at -70ºC until analyses.

Determination of Protein Content

Protein determinations were made using the dye-binding method of Bradford [23]. Bovine serum albumin (BSA) was used to construct the standard curve.

Determination of LPO

LPO was assessed by measuring malondialdehyde (MDA) formation, using thiobarbituric acid (TBA) assay [24]. The content of MDA was carried out with the MDA detection kit (A003-1, Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol. MDA content was expressed as nmol per mg of soluble cell proteins.

Determination of Antioxidant Enzymes

The activities of antioxidases, superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured in the cellular extracts. GPx activity was determined by a method of Flohe and Gunzler [25]. The GPx activity assay was performed according to the manufacturer's protocol of the GPx detection kit (A005-1, Nanjing Jiancheng Bioengineering Institute, China). GPx activity was expressed as international units per gram of soluble cell proteins. SOD activity was determined using the pyrogallol assay following the procedure described by Marklund and Marklund [26]. The SOD activity was measured with the SOD detection kit (A001-1, Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol. SOD activities were expressed as international units per mg of soluble cell proteins.

Statistical Analysis

Significance of the mean of different parameters between the treated groups was analyzed using one way analysis of variance (ANOVA) after ascertaining the homogeneity of variance between the treatments. Pair wise com-

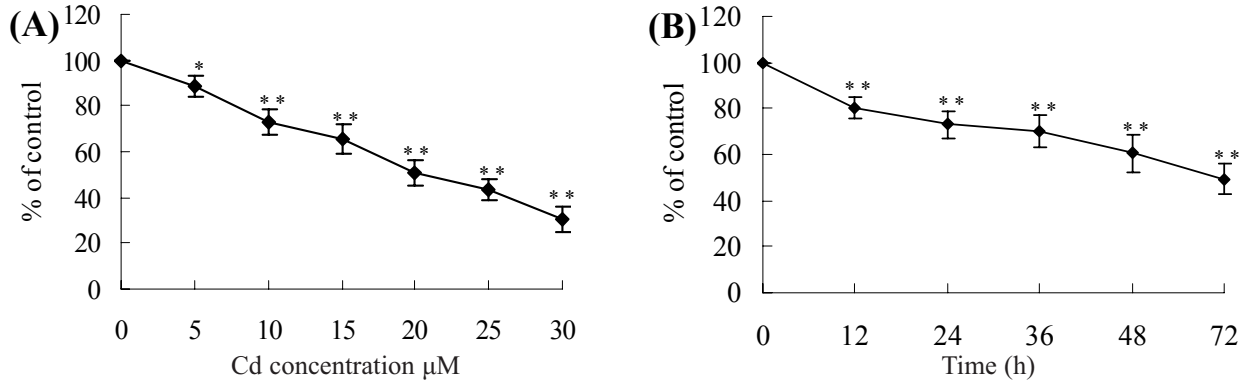

Fig. 1. Effect of Cd on the viability in chicken splenic lymphocytes. Cells were treated with the absence and presence of various concentrations of Cd (5 μM, 10 μM, 15 μM, 20 μM and 30 μM) for 24 h (A), or with 10 μM Cd for 12 h, 24 h, 36 h, 48 h and 72 h (B). Cell viability was determined using the MTT assay. Values represent mean ± SD of at least eight replicate determinations and are relative to values determined by control set as 100%. Values with non-identical superscripts are significantly different (*P<0.05, **P< 0.01 compared with control).

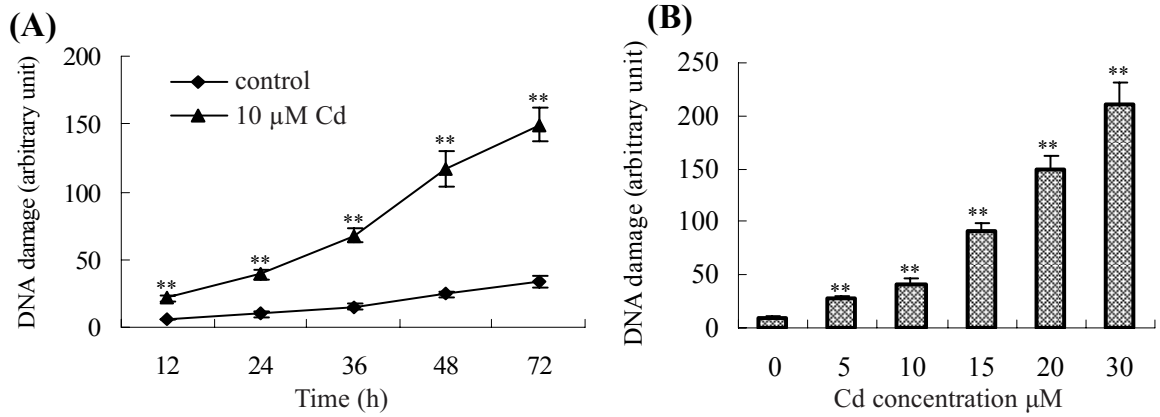

Fig. 2. Cd-induced DNA damage in chicken splenic lymphocytes. Untreated cells or cells treated with the various concentrations of Cd (5 μM, 10 μM, 15 μM, 20 μM and 30 μM) for 24 h (A), or with 10 μM Cd for 12 h, 24 h, 36 h, 48 h and 72 h (B) were processed by the comet assay. An arbitrary unit was used to score the level of DNA damage in cells(see Materials and Methods). Each value is the mean ± SD of at least five separate cultures. Values with non-identical superscripts are significantly different (**P< 0.01 compared with control).

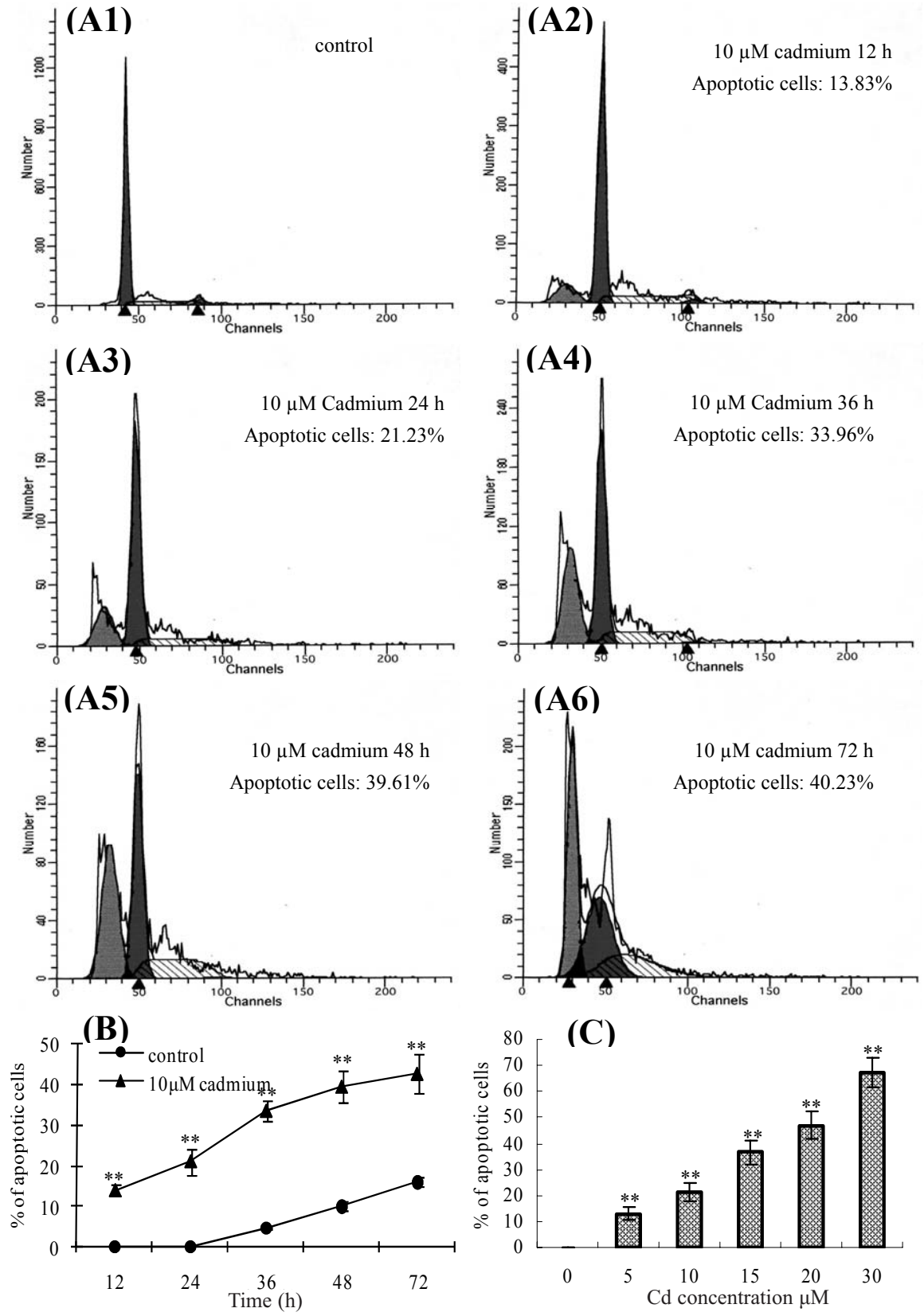

Fig. 3. Effect of Cd on the apoptotic rates of cells. Freshly isolated chicken splenic lymphocytes (1.5×10^6) treated with 10 μM Cd for 12 h, 24 h, 36 h, 48 h, and 72 h (B), or with the absence and presence of various concentrations of Cd (5 μM, 10 μM, 15 μM, 20 μM and 30 μM) for 24 h (C). The propidium iodide fluorescence measured using flow cytometer with FL-2 filter. Results expressed as histogram representing the % of apoptosis population (A, 24 h) and the % of apoptotic cells obtained from the histogram statistics (B, C). Each value represents mean ± SD of at least four separate cultures. (**P<0.01 compared with control).

Table 1. Effects of increasing Cd concentrations on oxidative stress marker levels and antioxidant enzyme activities of chicken splenic lymphocytes.

parameters	Cd concentration (μM)					
	0	5	10	15	20	30
GPx activity (U/mg.Pr)	3.92±0.28	3.58±0.36	3.07±0.29*	2.42±0.35**	2.12±0.24**	1.64±0.21**
SOD activity (NU/mg.Pr)	54.36±5.29	49.98±4.99*	47.5±2.29*	40.20±3.16**	38.51±5.03**	32.62±5.84**
MDA content (nmol/mg.Pr)	2.17±0.22	2.39±0.25	2.479±0.364*	3.18±0.32**	3.49±0.38**	4.14±0.57**
ROS level (DCF-fluorescence)	119.6±10.4	153.8±16.9*	201.6±23.8**	295.7±30.4**	357.7±37.8**	528.8±66.6**

Values are expressed as mean ± SD, for six separate cultures.
*Values differs significantly from control group ($P<0.05$).
**Values differs significantly from control group ($P<0.01$).

parisons were done by calculating the least significant difference. Results were expressed as mean ± SD from at least six independent experiments. Significant difference was taken as $P<0.05$ or $P<0.01$.

Results

Cytotoxicity of Cd

The effects of Cd on chicken splenic lymphocyte viability were assessed by MTT. The results obtained were expressed as a percentage (%) of survival, using the untreated cells as reference (Fig. 1 A and B). The decrease of cell viability was evident when chicken splenic lymphocytes were exposed to Cd. The results showed that chicken splenic lymphocytes exposed to Cd displayed a time- and dose-dependent decrease in cell viability.

DNA Damage Induced by Cd

Comet assay was performed to evaluate the DNA damage caused by Cd. The DNA damage degree of chicken splenic lymphocytes, exposed to Cd, enhanced along with the increase of incubation time and Cd concentration, which showed a dose- and time-dependent pattern (Fig. 2 A and B).

Induction of Apoptosis by Cd

Apoptotic chicken splenic lymphocytes treated with Cd were detected with flow cytometry, DNA Ladder analysis and staining with AO/EB. The number of apoptotic cells rose in a time- and dose-dependent manner as shown in Figs. 3, 4, and 5. The earliest significant increase in hypodiploid DNA was at 12 h and 5 μM Cd in chicken splenic lymphocytes. At 72 h the hypodiploid DNA increased from 14.37% (control) to 40.23% (10 μM Cd) (Fig. 3 A2-A5). The fluorescence intensity of cell staining with AO/EB began to increase as early as 12 h after exposure to either 5 or 30 μM Cd, resulting in Cd-induced condensation of nuclear chromatin (Fig. 4 A2). The highest fluorescence intensity was observed in cells exposed to 30 μM for 24 h. In comparison, untreated cells exhibited low levels of fluorescence with unaltered chromatin, which was uniformly spread throughout the nuclei. The biochemical hallmark of apoptosis, typical formation of 200-bp DNA fragments shown as ladders on agarose gels, was also observed in Cd-treated chicken splenic lymphocytes in a time- and dose-dependent manner (Fig. 5). Kinetic analysis of DNA fragmentation revealed that the formation of 200-bp DNA fragments in chicken splenic lymphocytes became apparent at 24 h under the Cd exposure conditions between 5 μM and 30 μM Cd, respectively. Under these experimental conditions, a good relationship between Cd-induced chromatin condensation and DNA fragmentation was observed. Taken together, all these results revealed that Cd-induced apoptosis in chicken splenic lymphocytes was time- and concentration-dependent.

Generation of Intracellular ROS by Cd

In order to measure the generation of ROS by Cd, we used the fluorescent probe DCF. As shown in Tables 1 and 2, there was significant increase in DCF-formation on the chicken splenic lymphocytes after being incubated with Cd. The ROS levels of Cd-treated cells were significantly higher than those of the untreated cells ($P<0.05$ or $P<0.01$), which showed a dose- and time-dependent pattern.

Lipid Peroxidation Induced by Cd

As shown in Tables 1 and 2, the MDA contents of Cd-treated cells were significantly higher than those of the untreated cells ($P<0.05$ or $P<0.01$). The MDA contents enhanced along with the increase of incubation time and Cd concentration, which showed a dose- and time-dependent pattern.

Effect of Antioxidant Enzymes by Cd

The activities of antioxidases, SOD and GPx in cells, have been shown in Tables 1 and 2. Current findings indi-

Table 2. Effects of 10 μM Cd on oxidative stress marker levels and antioxidant enzyme activities of chicken splenic lymphocytes.

Group	time	GPx activity (U/mg.Pr)	SOD activity (NU/mg.Pr)	MDA content (nmol/mg.Pr)	ROS content (DCF-fluorescence)
control	12 h	3.66±0.15	51.83±3.92	2.28±0.13	123.4±13.7
	24 h	3.22±0.12	52.23±2.36	2.18±0.12	130.6±15.8
	36 h	3.76±0.06	50.46±1.42	2.45±0.07	138.9±17.7
	48 h	3.40±0.22	54.52±3.69	2.65±0.17	149.7±20.6
	72 h	3.27±0.16	58.30±2.53	2.56±0.16	163.8±21.1
Cd treatment group	12 h	3.27±0.53	48.18±3.26	2.39±0.24	142.3±13.9*
	24 h	3.07±0.30*	47.50±2.29*	2.48±0.36*	173.8±16.4**
	36 h	2.64±0.25**	45.01±3.99*	2.75±0.15*	201.6±23.8**
	48 h	2.51±0.30**	42.40±2.10**	2.96±0.34**	259.5±20.8**
	72 h	2.32±0.41**	38.51±5.03**	3.18±0.30**	321.9±26.6**

Values are expressed as mean ± SD, for six separate cultures.
*Values differ significantly from control group at the same time point ($P<0.05$).
**Values differ significantly from control group at the same time point ($P<0.01$).

cate that Cd administration significantly attenuated the activities of SOD and GPx in comparison to the control after incubating with 10 μM Cd for 36 h ($P<0.01$) or with 15 μM Cd for 24 h ($P<0.01$).

Discussion

Cd exposure has been reported to alter a variety of immune cell functions, particularly in humans [27-29] and rodents [4, 10, 30, 31]. The immunotoxicity of Cd in a variety of mammalian and non mammalian animal species is well documented [9]. However, no literature is available regarding the toxic effects of Cd on the immune system of birds. Indeed, the cytotoxicity of Cd on the immunocytes in a variety of animals is different. In rodents, splenocytes and thymocytes treated with Cd (1-100 μM) for 24 h exhibited a dose-dependent pattern in cell viability and 25 μM Cd caused ~50% and ~45% loss in cell viability, respectively [3, 4]. Cd at 20 μM induced both apoptotic and necrotic death in murine macrophage (J774A.1) cultures at 24 h [2]. In humans, cell vitality was significantly reduced (~50% loss) after peripheral blood mononuclear cells (PBMC) were exposed to 44.48 μM Cd for 24h [29]. Tsangaris et al. [27] reported that the 50% lethal dose (LD50) for the T-cell line CCRF-CEM was 25±2.0 μM, and for the lymphoblastoid cell line Molt-3 was 22.5±2.4 μM after 18 h incubation with Cd. In the present study, the cytotoxicity effect was evident when chicken splenic lymphocytes were exposed to Cd. Both the 20 μM Cd concentration for 24 h and the 10 μM Cd concentration for 72 h caused ~50% loss in cell viability, indicating that lymphocytes of birds were relatively more sensitive to Cd than that of mammalian animals, particularly rodents and humans.

Oxidative stress may be defined as an alteration in the steady-state balance between oxidant and antioxidant agent in the cells. However, the balance between oxidant and antioxidant in immune cells is an important determinant of their functions [32, 33]. Several studies have reported that Cd-induced cytotoxicity of immune cells is related to oxidative stress. Pathak and Khandelwal [3, 4, 7] have stated that Cd exposure to murine splenocytes and thymocytes could cause oxidative stress, i.e. ROS generation and alterations in glutathione (GSH). Oxidative stress of the erythrocytes in Cd-treated rats showed the decrease in the activities of catalase (CAT), GPx and the content of GSH, and the increase of the activity of SOD [34]. In this study, we reported that Cd exposure potentiated oxidative stress in chicken splenic lymphocytes. The generation of ROS and the level of lipid peroxidation measured as MDA in chicken splenic lymphocytes enhanced with the increase of Cd concentration, indicating oxidative stress occurred. The overproduction of ROS was one of the possible mechanisms of the oxidative stress induced by Cd treatment. These results suggest that the cytotoxicity of Cd on bird lymphocytes is consistent with mammalian results [3-8, 27-31]. Cells survive the oxidative stress induced by heavy metals by the operation of intricating antioxidative systems, comprising both enzymatic and nonenzymic systems. The enzymatic systems play an important role in eliminating ROS by some enzymes such as SOD and GPx. SOD defense against oxidative stress by converting $O_2^{\bullet-}$ into O_2 and less-reactive species H_2O_2. GPx competes with catalase for H_2O_2 as a substrate and is the major source of protection against low levels of oxidative stress [35]. Hussain et al. [36] indicated the activity of SOD was inhibited and the level of lipid peroxides was increased in the liver and kidney of rats exposed to Cd *in vitro* and *vivo*. In the present study, the activities of SOD and GPx in chicken splenic

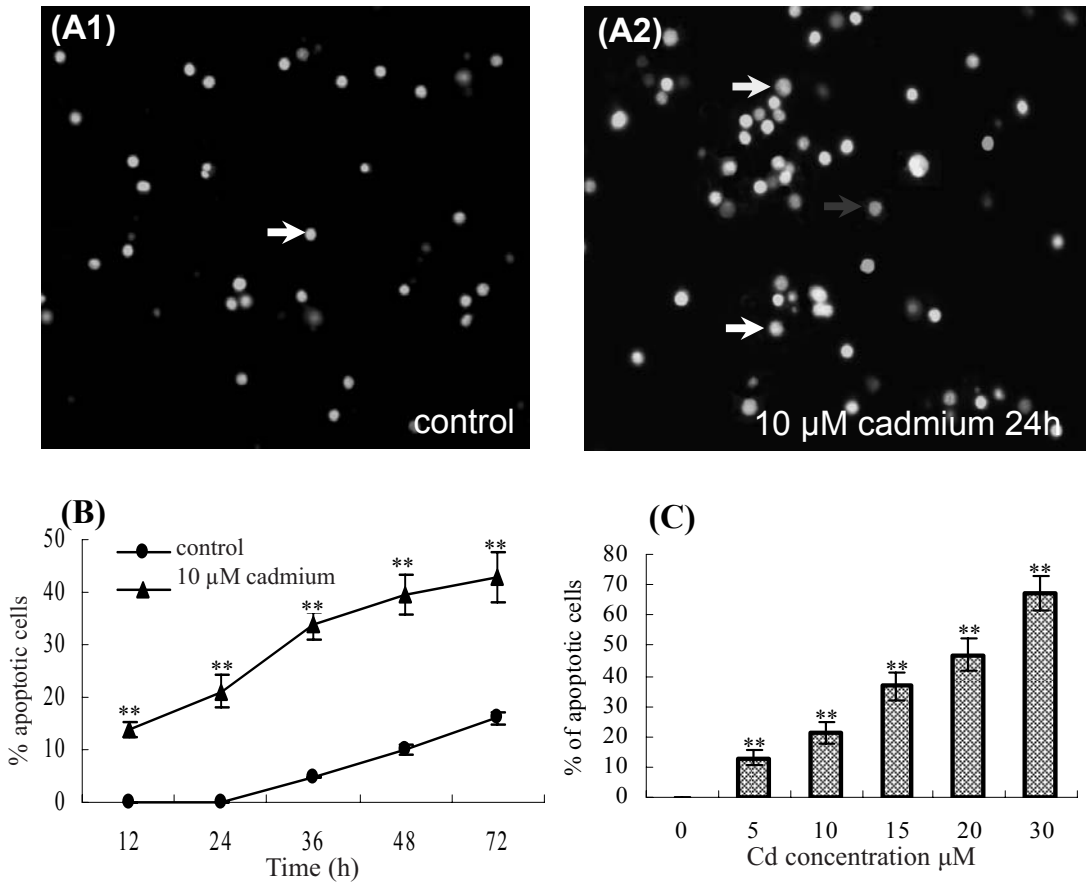

Fig. 4. Cd-induced apoptotic morphological changes on chicken splenic lymphocytes with AO/EB stained (400×). Green live cells (white arrows) were shown in control (A1), green early apoptotic cells (white arrows), orange later apoptotic cells (yellow arrows) and red necrotic cells (red arrows) were shown in Cd group (A2). Cells were treated with 10 μM Cd for 12 h, 24 h, 36 h, 48 h, and 72 h (B), or with the absence and presence of various concentrations of Cd (5 μM, 10 μM, 15 μM, 20 μM, and 30 μM) for 24 h (C). Each value represents mean ± SD of at least five separate cultures. (**P<0.01 compared with control).

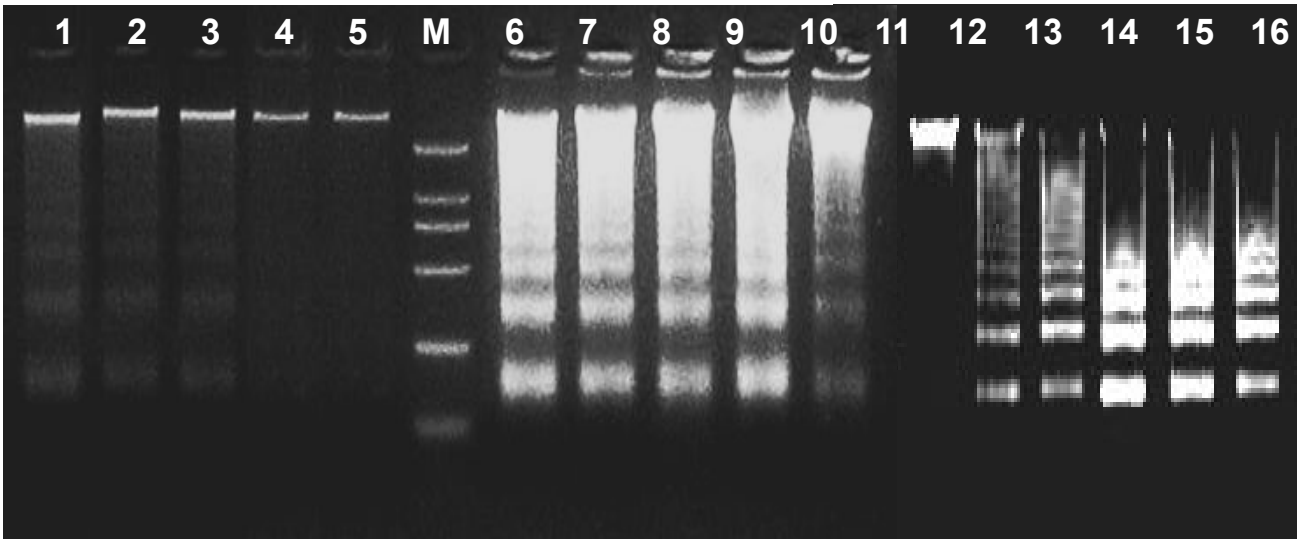

Fig. 5. Effect of Cd on DNA fragmentation by (1.8%) agarose gel electrophoresis. Lane M: DNA Marker DL2000; Lane 1-5: DNA from control lymphocytes cultured for 12 h, 24 h, 36 h, 48 h, and 72 h; Lane 6-10: DNA ladder patterns of lymphocytes treated with 10 μM Cd for 12 h, 24 h, 36 h, 48 h, and 72 h; Lane 11-16: DNA ladder patterns of lymphocytes treated with 0 μM, 5 μM, 10 μM, 15 μM, 20 μM, and 30 μM Cd for 24 h.

lymphocytes exposed to Cd was significantly attenuated compared to the control and displayed a time- and dose-dependent pattern. The MDA contents of Cd-treated cells enhanced along with the increase of incubated time and Cd concentration, which also showed a dose- and time-dependent pattern. These results were similar to the previous study [36].

The oxidative stress could induce many kinds of negative effects, including membrane peroxidation, DNA damage and apoptosis [3, 4, 7]. In the present study, DNA damage in chicken splenic lymphocytes treated with Cd was detected by the comet assay, the DNA damage degree of cells enhanced along with the increase of incubation time and Cd concentration. The enhancement in DNA damage was correlated to oxidative stress induced by Cd in chicken splenic lymphocytes, indicating that the overproduction of ROS in cells could cause single- or double-stranded DNA breaks, and MDA could react with DNA bases G, A, and C to form adducts M_1G, M_1A, and M_1C, respectively [37]. Moreover, in this study, the microscopic examination of chicken splenic lymphocytes exposed to Cd revealed morphological changes typical of apoptosis such as membrane blebs and overall shrinkage of cells (Fig. 4 A2). In accordance with these morphological changes, apoptosis was further confirmed by the observation of Cd inducing both hypodiploid DNA (Fig. 2 A2-6) and DNA fragmentation (Fig. 5). The number of apoptotic chicken splenic lymphocytes induced by Cd rose in a dose- and time-dependent manner. Cd-induced oxidative stress is known to play a major role in its potential to cause DNA damage and to induce apoptosis [38]. However, Lag et al. [39] indicated that apoptosis was not related with oxidative stress in their study on Cd-induced apoptosis of primary epithelial lung cells. Thus, the accurate mechanism of Cd-induced apoptosis needs to be elucidated in further study.

In summary, Cd is a toxic heavy metal to chicken splenic lymphocyte and inhibits the viability of immune cells in birds. The oxidative stress and subsequent DNA damage and apoptosis induced by Cd are important mechanisms of the Cd cytotoxicity to bird immune cells. Therefore, birds' immunotoxic potential of Cd will now be undertaken for better understanding of the toxicity of Cd in bird immune cells.

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