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Apoptotic Effects of Cadmium on Siberian Tiger Fibroblast Cells

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ABSTRACT

Cadmium (Cd^{2+}) is a toxic heavy metal element that does severe harm to health. Since Cd^{2+} is known to induce apoptosis in a variety of cell types, this study investigated the apoptotic effects and mechanisms of Cd^{2+} on Siberian tiger fibroblast cells. This research observed morphological alterations with confocal microscopy and transmission electron microscopy, performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and detected apoptotic rates, cell cycle progression, mitochondrial transmembrane potential and intracellular calcium homeostasis. The results demonstrated that typical apoptotic morphological alterations occurred after cadmium treatment. Cadmium exerted a strong inhibitory to the proliferation of Siberian tiger fibroblast cells and induced apoptosis in a dosage and duration dependent manner. Cell cycle was arrested at G_0/G_1 phase, mitochondrial transmembrane potential dropped and calcium homeostasis was disturbed. It is concluded that cadmium induced apoptosis of Siberian tiger fibroblast cells via arresting cell cycle progression, reducing mitochondrial transmembrane potential and disturbing intracellular calcium homeostasis.

Key words: Apoptosis, cell cycle, calcium homeostasis, heavy metal, mitochondrial transmembrane potential

INTRODUCTION

Tiger inhabited in Asia, represents strengthened bravery for its unique appearance and predation characters (Song *et al*., 2007). There are four generally accepted tiger subspecies in China, Indochinese tigers (*Panthera tigris corbetti*) exist in Yunnan province, Siberian tigers (*Panthera tigris altaica*) survive in northeastern China, Amoy or South China tigers (*Panthera tigris amoyensis*) now exist in Middle and South China and Indian or Bengal tigers (*Panthera tigris tigris*) live in Tibet.

Today an estimated fewer than 400 Siberian tigers survive in eastern Russia, northeastern China and Korea (Miquelle and Pikunov, 2003; Sugimoto *et al*., 2006; Wei *et al*., 2008) and less than 20 now exist in China. The remaining wild tiger (*Panthera tigris*) populations in China continue to shrink and its genomic resource diversity loses degree aggravating gradually under ever increasing human-related pressures from 1900 sec. The deterioration of the habitat is one of the reasons for Siberian tiger to the brink of the extinction. With the deepening of the industrialization process, the natural habitat of wildlife has changed. Factors

such as heavy metals, ultraviolet light, radiation, viruses that can cause normal cells apoptosis is widespread in natural environment, to cause great harm to wildlife.

Heavy metals have been studied extensively for the pollution to environment that causes various diseases to living beings by disturbing the biological homeostasis and metabolism (Jacobson and Turner, 1980). A lot of heavy metals have long half-life so they can accumulate in the environment day by day. In living organisms, heavy metals usually accumulate to a pathogenic high level in the form of complexes. Generally, heavy metals cause damages to proteins by combining with some specific group, which would destroy or harass their functions as enzymes or transporters and so on. At last, the metabolism of cells in good order is substituted by chaos.

Cadmium is one kind of heavy metals that exists in nature mainly in the greenockite. It is widely used in the production of alloys, pigments and batteries. Along with the development of modern industry, cadmium, with a long biological half-life of 10-30 years, is polluting environment and detected in earth, water, air, even food (Dudley *et al*., 1982; Tzirogiannis *et al*., 2003; Christina *et al*., 2010).

Cadmium is also known as one of the most toxic toxicants and strong registered carcinogens (McMurray and Tainer, 2003). Long time inhaling of fumes containing cadmium would ultimately induce chronic poisoning with the clinical symptoms like chemical pneumonitis, pulmonary oedema, acute tracheobronchitis and emphysema. Studies on cadmium toxicity have shown that, cadmium is able to induce renal, hepatic and testicular injury. According to previous *in vitro* research, cadmium can induce apoptosis in low concentration and lead to necrosis in high concentration (El-Azzouzi *et al*., 1994). Internal accumulation of cadmium is dominantly in liver and kidney, besides, it also presents in blood and affects the nucleated cells, especially lymphocytes (Enger *et al*., 1983). Study on human T-cell line (CEM-C12) demonstrated that the toxic effects of cadmium is due to inducing apoptosis (El-Azzouzi *et al*., 1994), while another study on cell lines of immune system showed perturbation of the immune development with the cadmium treatment (Tsangaris and Tzortzatou-Stathopoulou, 1998). In oyster hemocytes cadmium-induced apoptosis exhibits a dose-dependent manner from 10 to 100 mM (Sokolova *et al*., 2004). And in *in vitro* cultured mammalian cells, cadmium compounds results in morphological transformations, chromosomal aberrations and gene mutations (Yang *et al*., 1996; Hwua and Yang, 1998).

Siberian tiger exposure to cadmium results from consumption of contaminated water, food and metal smoldering fumes. Exposure to elevated levels of cadmium and other heavy metals has been associated with an increased incidence of Siberian tiger (Yin and Huang, 1999). Currently, nothing is known about the apoptotic effect and mechanism of cadmium on apoptosis in Siberian tiger fibroblast cell, although cadmium-induced apoptosis has been studied in other cells of the vertebrates (Shih *et al*., 2004). This study aims to investigate the apoptotic effect and mechanism of cadmium on Siberian tiger fibroblast cell. And provide a scientific basis for the protection of the Siberian tiger.

MATERIALS AND METHODS

Siberian tiger fibroblast cell was provided by Institute of Animal Science, Chinese Academy of Agricultural Sciences. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Propidium Iodide (PI) and Cadmium chloride ($CdCl_2$), was purchased from Sigma Inc. Annexin V-FITC Apoptosis Detection Kit I was purchased from BD corporation. Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS) was purchased from Gibco, Carlsbad, CA. This research was conducted from year 2011 to year 2012.

Cell culture and growth dynamics: The cells were cultured in DMEM supplemented with 10% FBS at 37°C under humidified with 5% CO_2 and 95% air at one atmosphere. Cells were dissociated when they reached 80-90% confluence and were subcultured into sterilized flasks at the ratio of 1:2 or 1:3.

Following the method of Kong *et al.* (2007), cells at the concentration of 2.0×10^4 cells mL^{-1} were plated into 24-well microplates. Data on cell growth and density were calculated and recorded each day until plateau phase; three wells were counted each time. The growth curve was then plotted and the Population Doubling Time (PDT) was calculated according to this curve.

Drug solution preparation and treatment: $CdCl_2$ was dissolved with DMEM medium, filtered for sterihzation, aliquoted. It should be diluted to the required concentration with DMEM medium prior to treatment. The experimental cells were logarithmic phase, after treatment with DMEM medium containing Cd^{2+}, they were cultured to the scheduled time.

Fluorescent observation using confocal microscopy: To visualize the morphological alterations of apoptotic nuclei, Acridine Orange (AO) and Ethidium Bromide (EB) fluorescent staining of cadmium chloride treated cells was performed. Cells were treated for 24 h and stained with AO, EB solution (both 2 mg mL^{-1} in ethanol). The samples were observed using confocal microscopy (Nikon TE-2000-E, Japan) immediately.

Observation by transmission electron microscopy: The cells of controls and experimental samples were collected and Fixed with 2.5% (m/v) glutaraldehyde, then washed with 0.1 mol L^{-1} phosphate buffered and subjected to serial dehydration with 30, 50, 70, 80, 90 and 100% acetone (v/v). The samples were embedded with epoxy resin (SPURR) for polymerization, then slice thin slicer with ultramicrotome (LEICAUC6i), Uranyl acetate and lead citrate for double staining. The samples were observed using transmission electron microscopy (JEM-123O).

MTT assay: The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazolium salt, is metabolized by active succinate dehydrogenase in the mitochondria of living cells into a blue Formazan product. MTT assay was used to evaluate cytotoxicity as described by (Ho *et al.*, 2005). Cell suspension of 200 μL was plated on each well of 96-well microplates at the concentration of 1.0×10^5 cells $well^{-1}$. Eight replicates were prepared for each treatment and cultured until 12, 24, 36 and 48 h. After the addition of MTT 20 μL (5 mg mL^{-1} Phosphate Buffered Saline (PBS) each well, the cells were cultured for another 4 h. The supernatant was discard. After the addition of 200 μL DMSO in each well, the samples were incubated in the dark for 30 min and then swirled for mixing. Absorbance A at 490 nm was measured using enzymatic reader.

Annexin V-FITC/PI double-labeling: The detection of apoptosis using annexin V-FITC/propidium iodide (PI) staining was performed as described previously (Cetindere *et al.*, 2010). The cells were collected and adjusted to the concentration of $1\text{-}5\times10^5$ cells mL^{-1} in binding buffer. For each sample was stained with fluorescein isothiocyanate (FITC) and PI, then detected with flow cytometer (FCM) (BD FACSCalibur, USA) within 1 h.

Cell cycle progression: The DNA and RNA intercalating fluorescent dye PI was used to quantify cellular DNA content and cell cycle distribution. The cell cycle stages were measured by FCM as previously described (Ho *et al.*, 2005). The cells were collected and resuspended in ice-cold 70% ethanol (v/v) and kept at 4°C overnight. Before analysis, cells were incubated with RNase A

(0.02 mg mL^{-1}) and stained with PI solution (PI 0.05 mg mL^{-1}, NaCl 0.585 g mL^{-1}, sodium citrate 1 mg mL^{-1}, pH 7.2-7.6) at 4°C for 10 min in the dark and then detected with FCM (BD FACSCalibur, USA) immediately. A total of 10,000 events were recorded per sample and the cell fractions in sub-G_1, G_0/G_1, S and G_2/M phases were quantified in histograms.

Mitochondrial transmembrane potential: Mitochondrial transmembrane potential was determined as described previously (Yang *et al*., 2006; Zhang *et al*., 2006). The cells were collected and add JC-1 working solution (5 μg mL^{-1}, 0.5 mL/sample), the cells were incubated at 37°C in the dark for 10 to 15 min. Centrifugation and the supernatant was discarded. Each sample was resuspended with 0.5 mL PBS and then detected with FCM (BD FACSCalibur, USA) immediately.

Intracellular calcium homeostasis: The intracellular Ca^{2+} release was assessed by staining with Ca^{2+}-sensitive dye Fluo3-AM (Adachi, 2008). Fluo-3/Am (Invitrogen, USA) was added to each sample to reach a final concentration of 5-10 μmol L^{-1}. Incubated at 37°C, 5% CO_2 in the dark for 30 to 60 min. Prepare negative controls (without Fluo-3/Am) for reference. The cells were centrifuged and washed twice with calcium-free PBS buffer, in order to remove the excessive dye. Each sample was resuspended in 0.5 mL calcium-free PBS and then detected with FCM (BD FACSCalibur, USA) immediately.

Statistical analysis: Study repeated each type of experiment at least three times and confirmed that similar data were obtained. All values are presented as Means±SD. Cytotoxicity data, apoptotic rates, cell cycle, mitochondrial transmembrane potential and intracellular calcium data were analyzed using the GLM procedure in Statistical Analysis System (SAS Inc., Cary, NC, USA) and compared with a multiple comparison test (DUNCAN). A value of $p<0.05$ and $p<0.01$ was thought of as statistically significant.

RESULTS

Growth dynamics: The growth curve of Siberian tiger fibroblast cell displayed an obvious "S" shape and the PDT was proximately 30 h. The cells were in the latent phase in days 1 and 2 and then entered logarithmic phase in days 2 to 5. The concentration reached its peak on day 5 and then the cells entered the plateau phase in day 6, followed by an overall degeneration thenceforth (Fig. 1a).

Observation using confocal microscopy: Acridine Orange (AO) and Ethidium Bromide (EB) differ in permeability and fluorescence, distinguishing cells in early apoptosis and late apoptosis. Viable apoptotic cells have intact membrane which prevents EB from entering the interior of cells, possess yellow cytosol and condensed nuclei, with condensation-like or dead-like nuclear chromatin. Membrane of non-viable apoptotic cells is permeable, through which both AO and EB will enter, thus cells will display condensed nuclei and orange fluorescence. Siberian tiger fibroblast cell treated with $CdCl_2$ of 0.5, 1, 1.5, 1 and 1.5 μM $CdCl_2$ significantly increased apoptosis to 4.4 and 5.6% of total cells counted, 0.5 μM $CdCl_2$ did not induce necrosis at 24 h but 1.5 μM $CdCl_2$ caused a significant increase in necrosis (Fig. 2), similar results were obtained with Annexin V-FITC/PI double-labeling.

Observation by transmission electron microscopy: The control group cell (Fig. 3a, c, e), cell plasma is homogenous and plump, cell nucleus is large and round, nucleoli is clear and regular, chromatin is Loose. Nucleus, nuclear membrane and subcellular structures are intact. There are a large number of microvilli on cell surface.

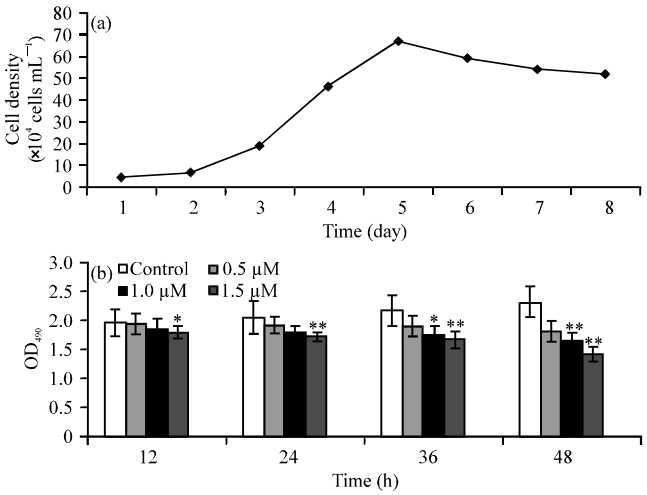

Fig. 1(a-b): Siberian tiger fibroblast cell treated with $CdCl_2$ (a) Growth curve and (b) Cytotoxicity analysis, OD values reflect viable cell population size, *,**Values are significant when compared with respective control at $p<0.05$ and $p<0.01$, respectively (n = 8)

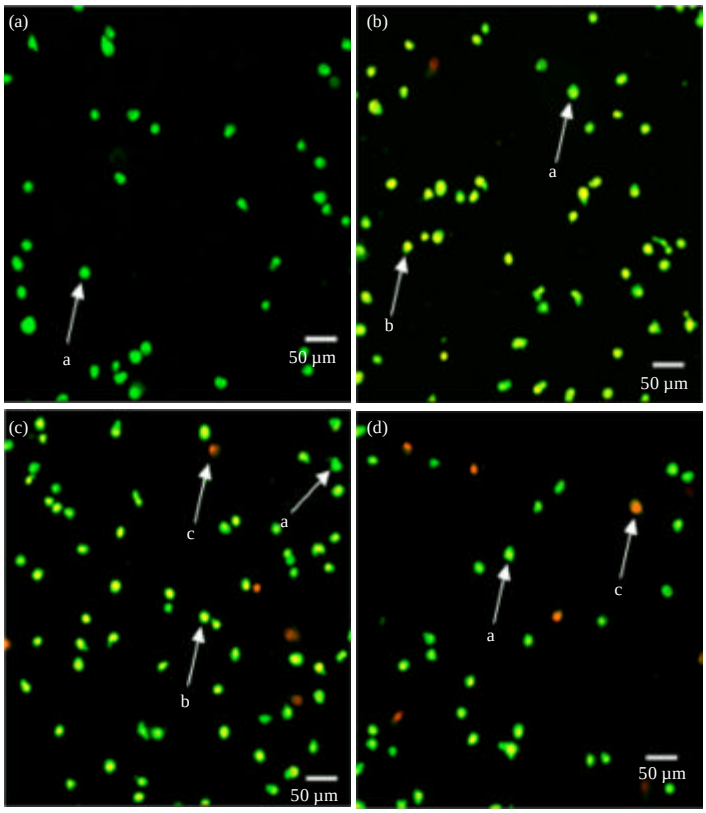

Fig. 2(a-d): Morphological observation of Siberian tiger fibroblast cell at 24 h after treatment with $CdCl_2$ (a) Control, (b) 0.5 µM, (c) 1.0 µM and (d) 1.5 µM using AO/EB double staining by confocal microscopy, Arrows; [a]Normal cells, [b]Viable apoptotic cells, [c]Late apoptotic cells and necrotic cells, Scale bars = 50 µm

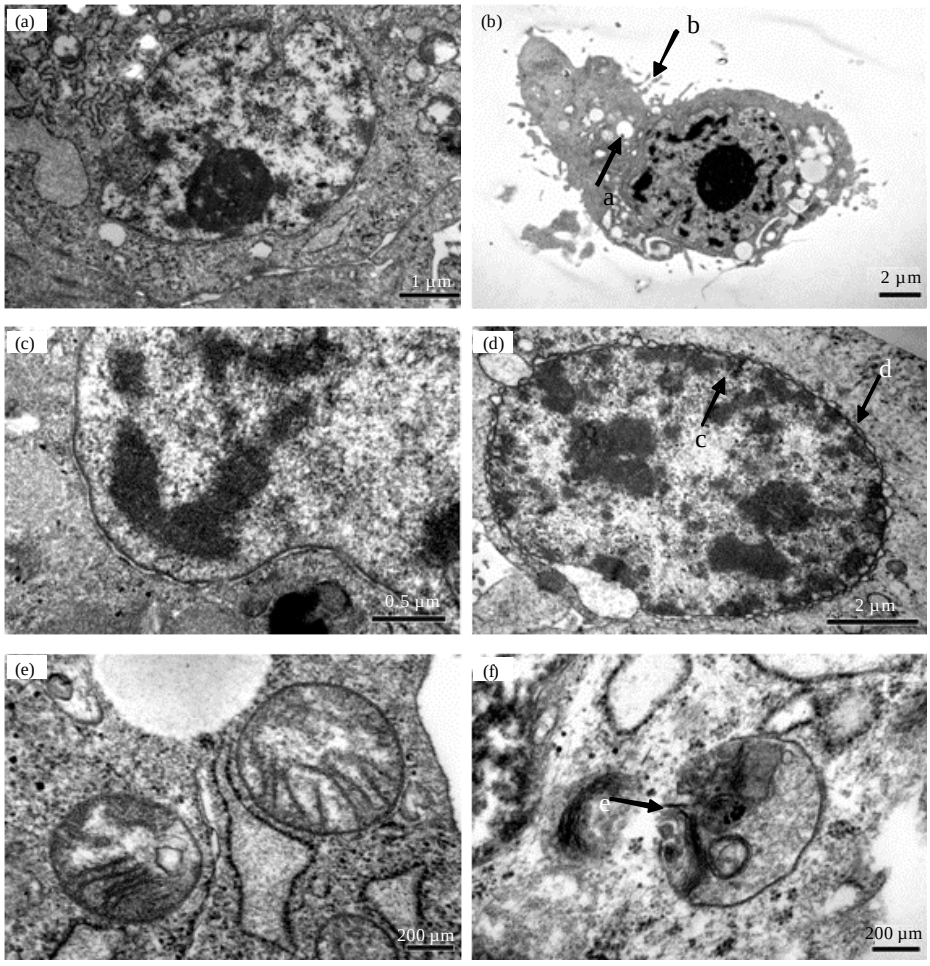

Fig. 3(a-f): Subcellular observation of Siberian tiger fibroblast cell (a, c, e) Control and (b, d, f) Treated with $CdCl_2$; 1.5 µM using transmission electron microscopy after 24 h, Scale bars (a) 1 µm, (b, d) 2 µm, (c) 0.5 µm and (e, f) 200 nm, Arrows: [a]Vacuoles, [b]Microvillus, [c]Chromatin condensation, [d]Breaked nuclear membrane, [e]Breaked mitochondrion

Cells in the experimental group (Fig. 3b, d, f), cell plasma format vacuoles (Fig. 3b), cell nucleus is pyknosis, smaller, Nucleolus is shrink, concentrate or even disappear. Chromatin is aggregate, marginalization and is lumpish in the inner nuclear membrane, heterochromatin is increase. nuclear membrane is breakdown (Fig. 3d), microvilli on cell surface is reduce (Fig. 3b). Mitochondrial disintegration (Fig. 3f), Mitochondrial are a major target in cadmium induce apoptosis, play a central role.

MTT assay: The MTT assay determines the activity of mitochondrial succinate dehydrogenase and is therefore able to detect alterations of mitochondrial function. This is used as a measurement of cell viabihty and hence an indicator of cell death. But this test does not distinguish between

apoptosis and necrosis as well as the inhibition of cell growth (Mosmann, 1983). MTT assay showed that, for cadmium chloride treated Siberian tiger fibroblast cell at 12, 24, 36 and 48 h, the viable cell population decreased significantly with elevated cadmium chloride concentration and duration of treatment, appearing dose-and duration-dependent (Fig. 1b). The same trend of results was obtained with Annexin V-FITC/PI double-labeling.

Annexin V-FITC/PI double-labeling: Quantitative analysis of apoptotic effects of $CdCl_2$ on Siberian tiger fibroblast cell. Cells by flow cytometry for Annexin V-FITC and PI staining. Annexin V-FITC and PI staining serves as a measure of phosphatidylserine externalization. Double staining was used to distinguish between viable, early apoptotic, necrotic and late apoptotic cells. Results interpretation: the first quadrant ($FITC^-/PI^-$), viable cells; the second quadrant ($FITC^-/PI^+$), cell debris, result from the mechanical factor for cell processing; the third quadrant ($FITC^+/PI^+$), late stage apoptotic cells and some necrotic cells; the fourth quadrant ($FITC^+/PI^-$), early stage apoptotic cells. These results shown that the apoptotic effect of dose-dependent and time-dependent (Fig. 4). The data of apoptotic rate at 36 and 48 h not shown. The 0.5 μM $CdCl_2$ did not induce necrosis at 24 h but 1.5 μM $CdCl_2$ caused a significant increase in necrosis, even more necrosis than apoptosis.

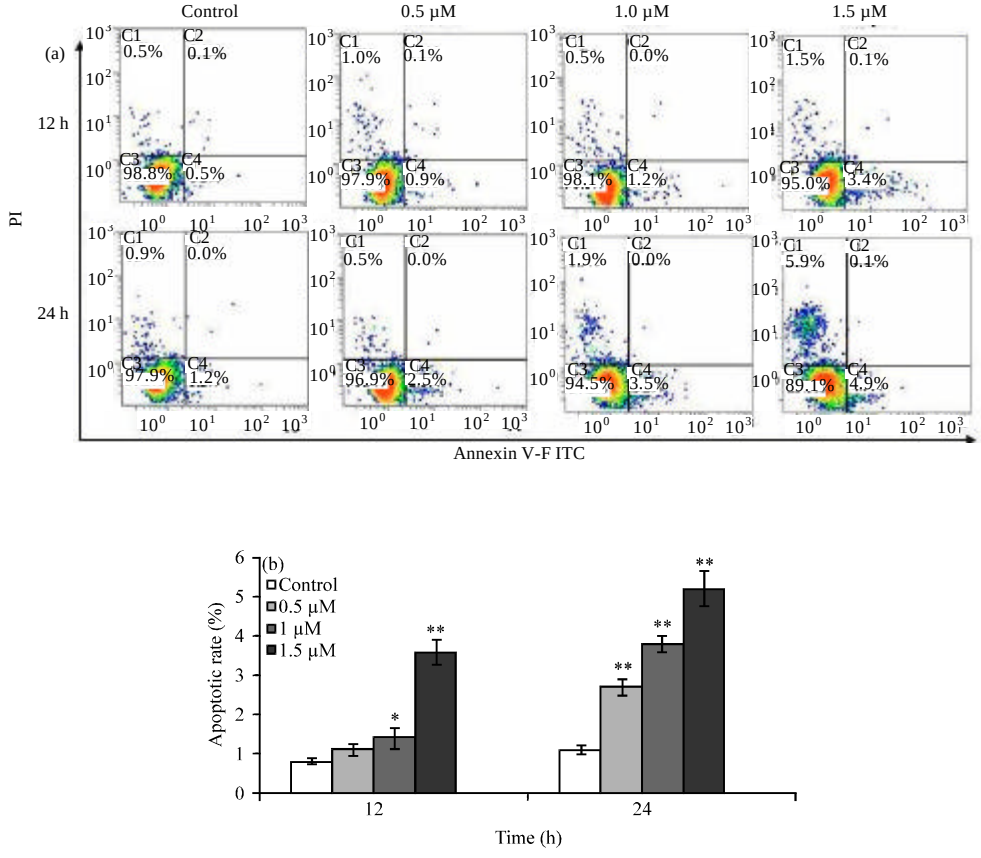

Fig. 4(a-b): FACS Analysis of apoptotic rates of Siberian tiger fibroblast cell treated with different concentration of $CdCl_2$ at 12 and 24 h (a) FACS analysis and (b) Graphical representation, *,**Values are significant when compared with respective control at $p<0.05$ and $p<0.01$, respectively (n = 3)

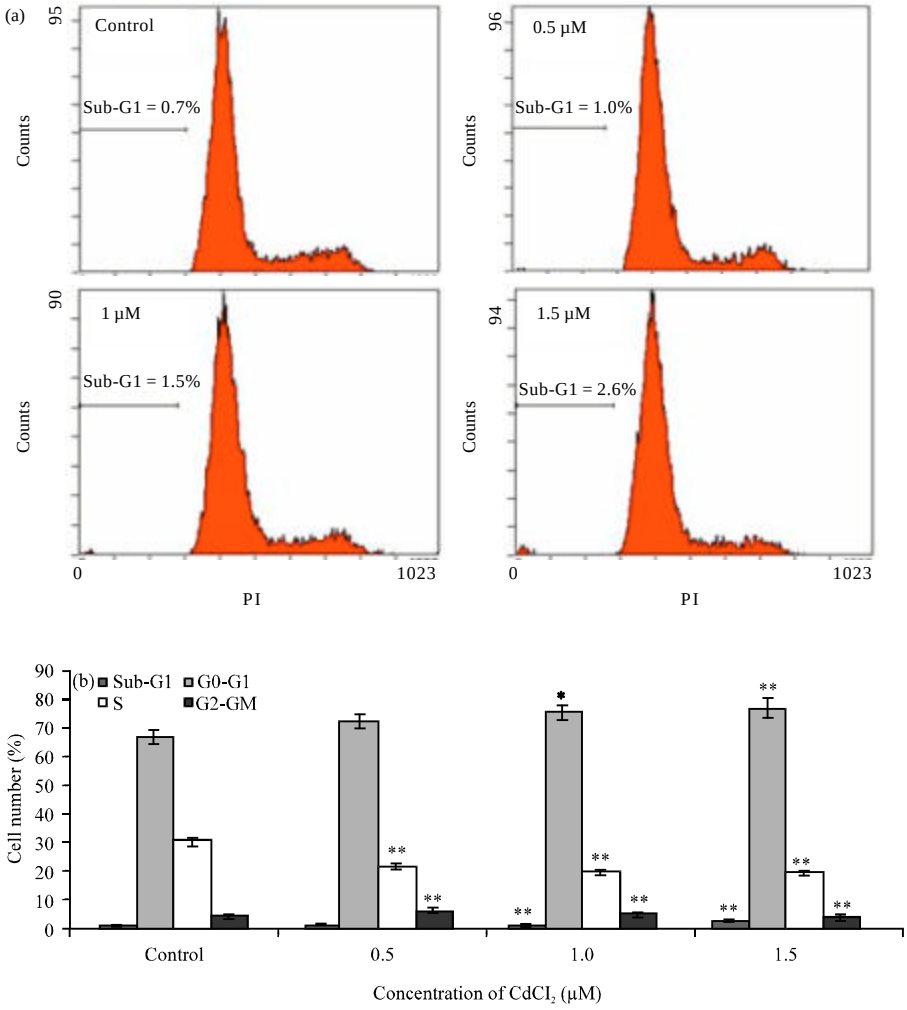

Fig. 5(a-b): FACS analysis of cell cycle progression and sub-G_1 content of Siberian tiger fibroblast cell at 24 h after treatment with $CdCl_2$ (a) Cell cycle distribution histograms, The percentage of apoptotic cells in hypodiploid DNA peak (sub-G_1 population) is given in each plot and (b) Distribution of cells in the Sub-G_1, G_0/G_1, S and G_2/M phases of the cell cycle,*,**Values are significant when compared with respective control at $p<0.05$ and $p<0.01$, respectively (n = 3)

Cell cycle progression: To test the mechanisms of $CdCl_2$ on Siberian tiger fibroblast cell apoptosis, cell cycle progression was analyzed by FCM. With the increasing concentration of $CdCl_2$, the percentage of cells increased in G_0/G_1 phase and decreased in S phase, indicating an arrest in G_0/G_1 phase and that DNA synthesis was inhibited. The effects on cell cycle were even more significantly with elevated $CdCl_2$ dose, 0.5 µM $CdCl_2$ did not increased the percentage of G_0/G_1 phase significantly but 1 µM and 1.5 µM $CdCl_2$ caused a significant increase at G_0/G_1 phase, reflecting a dose dependent correlation (Fig. 5). The percentage of apoptotic cells in hypodiploid DNA peak (sub-G_1 population) was calculated by sub-G_1 population/total cell cycle populations and indicated by numbers shown in each histogram, 1 µM and 1.5 µM $CdCl_2$ caused a significant increase in hypodiploid DNA peak, the same trend of results was obtained with Annexin V-FITC/PI double-labeling and MTT assay, sub-G_1 population also showed dose-dependent.

Mitochondrial transmembrane potential: JC-1 is a lipophilic, cationic dye that can selectively enter mitochondria and reversibly change color from green to red as the membrane potential increases. JC-1 dye accumulates as aggregates in the mitochondria in normal cells, which results in red fluorescence, whereas, in apoptotic or necrotic cells, JC-1 exists in monomeric form and stains the cytosol green. The cells number of C gate reflects the change of mitochondrial transmembrane potential, The increase of cells number of C gate means decrease of mitochondrial transmembrane potential (Fig. 6a). Mitochondrial transmembrane potential significantly dropped after treatment

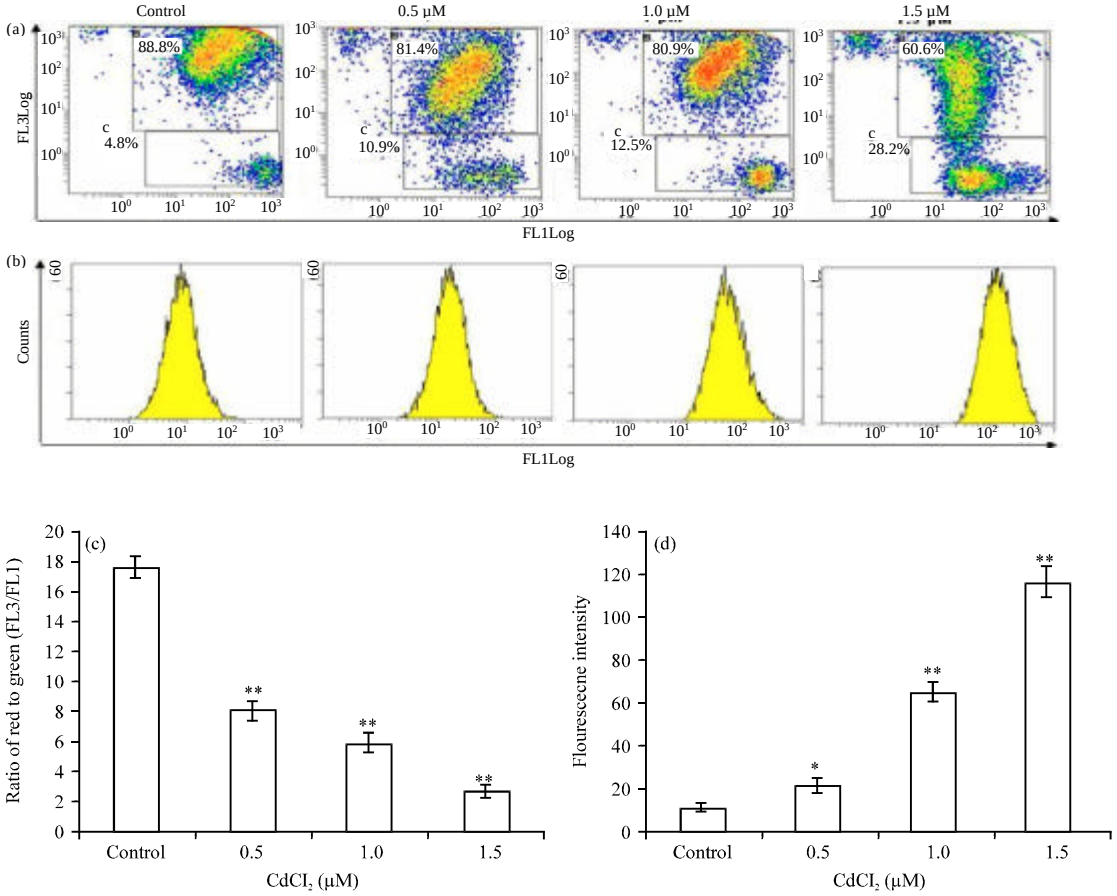

Fig. 6(a-d): (a) FACS analysis of the mitochondrial membrane potential of Siberian tiger fibroblast cell at 24 h after treatment with $CdCl_2$, Active mitochondria with high transmembrane potential form JC-1 aggregates, which are red (FL3, 620 nm), whereas in mitochondria with low transmembrane potential, JC-1 remains in a monomeric, green form (FL1, 527 nm). The ratio of red to green (FL3/FL1) reflects the change in mitochondrial membrane potential, (b) FACS analysis of intracellular calcium homeostasis of Siberian tiger fibroblast cell at 24 h after treatment with $CdCl_2$, Peak moving to the right means a increase of intracellular Ca^{2+} concentration, (c) Analysis of the mitochondrial membrane potential,*,**Values are significant when compared with respective control at $p<0.05$ and $p<0.01$, respectively ($n = 3$), and (d) Analysis of intracellular calcium homeostasis,*,**Values are significant when compared with respective control at $p<0.05$ and $p<0.01$, respectively ($n = 3$)

with $CdCl_2$, displayed significant differences compared with the control (Fig. 6c). Decreased mitochondrial transmembrane potential has been linked to apoptotic cell.

Intracellular calcium homeostasis: Siberian tiger fibroblast cell at 24 h after treatment with $CdCl_2$ and were subsequently labelled with the molecular probe Fluo-3/AM. The results show that peak position in the histograms reflects the intracellular Ca^{2+} concentration (Fig. 6b). The Ca^{2+} concentration of experimental samples treated with cadmium chloride of 0.5, 1, 1.5 μM displayed significant differences compared with the controls. It was revealed that there is a positive correlation between Ca^{2+} release and cadmium chloride concentration (Fig. 6d).

DISCUSSION

Heavy metals, being extremely toxic, pose an environmental threat. When air, earth or water is contaminated by some heavy metals, they may accumulate in the organisms over a long period and finally cause disease. During that accumulation, the metal can directly influence various processes, including enzyme and signal transduction pathways, in addition to indirect effects such as gene expression and formation of free radicals, peroxides or cytokines (Kasprzak, 1997).

Cadmium is not essential element of human beings. In higher eukaryotic cells, cadmium manifests its toxicity via impairing mitochondrial function, inducing generation of free radicals and DNA damages. Toxicology of metals recapitulates the cadmium mediated cytotoxic and metabolic effects at the cell level, including altering the activities of various enzymes, interfering with the normal protective actions of essential metals (Ca^{2+}, Zn^{2+}, Fe^{2+}, Se^{2+}), inducing oxidative stress, inhibiting mitochondrial ATP production and altering gene expression that may all culminate in the triggering of cell death by either apoptosis or necrosis (Zalpus and Koropatnick, 2000).

Our study showed that exposure to Cd^{2+} of Siberian tiger fibroblast cells resulted in cell morphology changes, subcellular structure destruction, decreased cell viability and apoptotic effects in a dose-and time-dependent manner.

It is widely known that apoptosis stimulated physiologically or pathologically is associated with cell cycle progression (Siegers *et al*., 1999). Unscheduled proliferation constitutes a key step in canceration and an altered death to division speed would eventually lead to malignant transformation and neoplastic growth (Frantz *et al*., 2000). In general, cell cycle arrest will lead to change of cell proliferation and is very possible to induce apoptosis simultaneously. Many apoptotic signals affect apoptotic machineries as well as cell cycle progression at the same time. Some processes of cell cycle are closely interrelated with apoptotic signal pathway, for example, mitochondrial cytochrome c release acts as key control point for caspase activation and apoptosis. Accordingly, cell cycle analysis is widely used in researches of apoptosis.

Cell cycle alterations and their impact on death and survival decisions present in p53-deficient Cd^{2+}-exposed cells, particularly of organs in which Cd^{2+} carcinogenesis occurs. Cd^{2+} induces p53-dependent G_1/S and/or G_2/M cell cycle arrest in some cell lines expressing p53 (Bjerregaard, 2007; Cao *et al*., 2007; Kim *et al*., 2005; Xie and Shaikh, 2006).

Our study showed that Cd^{2+} treatment of Siberian tiger fibroblasts resulted in cell cycle arrest in the G_0/G_1 phase and inhibition of cell proliferation with decreased proportion of S phase. With elevated Cd^{2+} concentration, the proportion of cells in G_1 phase, hypodiploid DNA (sub-G_1 population) increased and that of cells in S phase decreased (Fig. 5). Therefore, we inferred that Cd^{2+} induced apoptosis of Siberian tiger fibroblast cells via preventing the synthesis of DNA and

affecting cell cycle progression. This result provides an important experimental basis for future mechanism study of Cd^{2+} induced apoptosis in Siberian tiger fibroblast cell and Siberian tiger protection.

In the apoptotic process, many important events are closely related with mitochondrion (Bouchier-Hayes *et al*., 2005), including the drop in mitochondrial transmembrane potential (Amstrong, 2006), increase of membrane permeability, opening of mitochondrial permeability transition pores (Disa and Bailly, 2005; Lucken-Ardjomande *et al*., 2005) and then release to cytoplasm of mitochondria of cytochrome C (cyt c) (Mohamad *et al*., 2005) and Apoptosis Inducing Factor (AIF) (Liu *et al*., 1997), etc. Cyt c interacts with (apoptosis protein-activating factor-1) apaf-1 and procaspase-9 to form a complex known as the apoptosome, which in turn activates effector caspases such as caspase-3. Activation of caspases eventually leads to degradation of the cell's DNA and to the cascade of other intracellular reactions that culminate in cell death by apoptosis.

In response to stimulation of cadmium, mitochondria play a central role, the mitochondrial inner membrane undergoes a permeability transition, resulting in a dramatic increase in permeability caused by an apparent opening of a channel known as the mitochondrial Permeability Transition Pore (PTP) (Green and Reed, 1998; Huttenbrenner *et al*., 2003). Previous study showed that the substrate oxidation and proton leak subsystems are the main targets for Cd toxicity in oyster mitochondria. Exposure to 12.5 μM Cd strongly inhibited the substrate oxidation subsystem and stimulated the proton conductance across the inner mitochondrial membrane (Kurochkin *et al*., 2011). In animal and plant cells, Cd promotes generation of Reactive Oxygen Species (ROS) with suppression of respiration and partial uncoupling (Cannino *et al*., 2009). Several mechanisms have been proposed to explain effects of Cd on mitochondrial bioenergetics, including inhibition of electron transport chain, oxidative damage to mitochondrial enzymes and opening of the mitochondrial permeability pore (Valko *et al*., 2005).

In this study, mitochondrial transmembrane potential decreased in Siberian tiger fibroblast cells upon treatment with cadmium chloride (Fig. 6a, c). Mitochondria are supposed to be a key intracellular target for cadmium cytotoxicity, as indicated by the inhibition of the phosphorylation, subsequently by the possible suppression of ATP production even ATP depletion and breakdown of mitochondria, eventually activating downstream apoptotic pathways. Therefore, it was concluded that cadmium chloride Induced apoptosis of Siberian tiger fibroblast cells was related to mitochondrial pathway.

The release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm has been implicated as a key-signalling event in many models of apoptosis and it may sensitize mitochondria to trigger apoptotic cell death. Furthermore, more and more evidence to prove that endoplasmic reticulum proteins affect apoptosis by either interacting with Bcl-2 family members or altering endoplasmic reticulum Ca^{2+} responses. Several endoplasmic reticulum proteins are caspase substrates that may regulate the execution phase of apoptosis (Breckenridge *et al*., 2003). In general, in response to endoplasmic reticulum stress, Bax and Bak occurs changes in the conformational structure and to cause the release of Ca^{2+} from endoplasmic reticulum lumen into cytoplasm, followed by the activation of calpain. Upon exposure to Ca^{2+}, calpain subunits undergo auto-cleavage, which facilitates activation and ultimately leads to calpain degradation (Goll *et al*., 2003). In addition to participation in apoptosis, according to the nature of the stimuli and cell types to promote apoptosis and anti-apoptotic protein cleavage, calpain has been reported to cleave caspase-4 that is supposed to function in endoplasmic reticulum stress-mediated apoptosis (Hitomi, 2004).

Mitochondrion is an intracellular calcium store. The Ca^{2+} uptake depends on mitochondrial transmembrane potential. Mitochondrial Ca^{2+} elevation mechanisms include non-specific leakage

and pore formation. Appropriate stimulation, Ca^{2+} was released from mitochondrion and endoplasmic reticulum. Mitochondrial calcium overload leads to mitochondrial damage, release of cytochrome C and caspase activation and subsequent apoptosis.

Earlier studies in mammalian and plant systems showed that Cd^{2+} might interfere with Ca^{2+} homeostasis stimulating Ca^{2+} efflux from intracellular storage sites (Shah and Pant, 1991; Verbost *et al.*, 1988). Short exposure (3-6 h) to Cd^{2+} induces apoptotic cell death mediated by the Ca^{2+}-dependent proteases calpains (Lee *et al.*, 2006). Our data demonstrated when Cd^{2+} induced apoptosis of Siberian tiger fibroblast cells, cytosolic free Ca^{2+} concentration increased, so that Ca^{2+} homeostasis was disturbed (Fig. 6b, d). It was indicated appropriate concentration of Cd^{2+} had significant effects in inducing apoptosis on Siberian tiger fibroblast cells. The result of disturbed calcium homeostasis, taken together with mitochondrial transmembrane potential decrease, presumably linked with endoplasmic reticulum calcium release.

In conclusion, cadmium exposure of Siberian tiger fibroblast cells resulted in significant cytotoxicity and considerably elevated levels of apoptosis and necrosis. The mechanism was illustrated to be that cadmium induced apoptosis by inhibiting DNA synthesis, reducing mitochondrial membrane potential and interfering calcium homeostasis. High rates of apoptosis due to cadmium exposure may have important implications for the immune defense of the Siberian tiger fibroblast cells. The results of our study offer some information for the conservancy of Siberian tiger.

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