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Mitigative action of mono isoamyl 2, 3-dimercaptosuccinate (MiADMS) against cadmium-induced damage in cultured rat normal liver cells

Caroline O. Odewumi^{a,*}, Rebecca Buggs^a, Veera L.D. Badisa^a, Lekan M. Latinwo^a, Ramesh B. Badisa^b, Christopher O. Ikediobi^c, Selina F. Darling-Reed^b, and Marcia A. Owens^d

^aDepartment of Biology, Florida A&M University, Tallahassee 32307, USA.

^bCollege of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee 32307, USA.

^cDepartment of Chemistry, Florida A&M University, Tallahassee 32307, USA.

^dEnvironmental Sciences Institute, Florida A&M University, Tallahassee, FL, 32307, USA

Abstract

Cadmium is non-essential, carcinogenic and multitarget pollutant in the environment. Monoisoamyl 2, 3-dimercaptosuccinate (MiADMS) is an ester of dimercaptosuccinic acid that acts as an antioxidant and chelator. Therefore, the mitigative action of MiADMS on viability, morphology, antioxidative enzymes and cell cycle were studied on rat liver cells treated with cadmium chloride (CdCl₂). The cells were treated with 150 μ M CdCl₂ alone or cotreated with 300 μ M MiADMS (concurrently, 2 h or 4 h post CdCl₂ treatment) for 24 h. The viability of cells treated with CdCl₂ alone was decreased in comparison to the control cells. Cotreatment with MiADMS resulted in an increase in cell viability in comparison to the CdCl₂ alone treated cells. The CdCl₂ treatment altered the morphological shape of the cells, while cotreatment with MiADMS restored the shape. Antioxidative enzymes activities were decreased in the cells treated with CdCl₂ alone, while MiADMS cotreatment resulted in an increase in enzyme activities. The CdCl₂ arrested the cells in S phase of the cell cycle. Cotreatment with MiADMS alleviated cell cycle arrest by shifting to G1 phase. These results clearly show the mitigative action of MiADMS on CdCl₂ toxicity and may suggest that MiADMS can be used as an antidote against cadmium.

Keywords

Glutathione peroxidase (GPx); Glutathione reductase (GR); Cadmium; Cell cycle; Monoisoamyl 2, 3-dimercaptosuccinate; liver cells

1. Introduction

Cadmium is one of the naturally occurring elements found in rocks, soil and water. It is used commercially for batteries, pigments for coloring plastics, glass, and paints, stabilizers for processing of PVC polymers, and preparation of special alloys. The manufacture and use of these products give rise to high environmental concentration of and exposure to cadmium through atmospheric emissions, liquid effluents, wastewaters, sludges, and solid wastes

*Corresponding author. 1530 S Martin Luther King Dr., Jones Hall Room No. 501. caroline.odewumi@famu.edu (C.O.O).

Conflicts of interest

The authors would like to state that there are no conflicts of interest related to this work.

(Saffron, 2001). Fossil fuel combustion in classical thermal power plants is responsible for 50% of the total cadmium emitted to the atmosphere. Over 500,000 U.S. employees each year work in an environment that potentially exposes them to cadmium (Wittman R, 2002). Cadmium is an abundant, nonessential element and has been classified as one of the most toxic environmental contaminants that are carcinogenic in humans and experimental animals due to its long biological half life of 10-30 years and range of organ toxicity (IARC, 1993, Jarup et al., 1998).

Cadmium enters the human body via three main routes: inhalation, ingestion and dermal. Smoking of a cigarette results in the inhalation of 0.1-0.2 µg of cadmium (WHO, 1992). For the general population, ingestion of contaminated food products is the major route of cadmium exposure. Consumption of vegetables and grains presents the major source of cadmium exposure due to soils amended with phosphate fertilizers, and cadmium-contaminated sewage sludge and irrigation waters (ATSDR, 1999). On average, a person ingests about 30 µg of cadmium per day from food, but only absorbs 1-3 µg of cadmium in the body (ATSDR, 1999). Dermal exposure of cadmium to humans is mostly from the industrial production of cadmium containing products and their use. It is also due to the use of cadmium nanoparticles in the medical field for imaging technique (Zhang et al., 2010). Exposure of cadmium via dermal exposure caused lesions on the skin (hyperkeratosis, acanthosis and scabbing, alopecia and erythema) (Fasanya-Odewumi et al., 1998). Upon penetration of cadmium into the body, half of the absorbed cadmium highly accumulates in the liver and kidney, causing injuries of different types to the tissue cells and eventually leading to cancer (Klaassen et al., 1999; Waalkes, 2000, Santos et al., 2005).

The mechanism of cadmium toxicity has yet to be fully understood; however, cadmium is known to induce an oxidative stress (Ikediobi et al., 2004; Santos et al., 2005; Latinwo et al., 2006). The antioxidant enzymes play an important role in protecting against reactive oxygen species or free radicals in the body. Cadmium indirectly inhibits the activities of antioxidant enzymes by depleting the SH-group containing biomolecules resulting in the production of reactive oxygen species such as superoxide ion, hydrogen peroxide, and hydroxyl radical (Wasowicz et al., 2001; Casalino et al., 2002; Eybl et al., 2004; Ikediobi et al., 2004; Latinwo et al., 2006). In addition to its action on antioxidant enzymes, cadmium has been reported to cause retardation of the cell cycle progression in Chinese hamster ovary (CHO) and human lung adenocarcinoma cells (Chao and Yang, 2001; Yang et al., 2004).

Many mitigating substances that act as chelators (N-acetylcysteine (NAC), diallyl tetrasulfide, picroliv) or antioxidants (selenium, Zinc) have been experimentally proven to provide protection against cadmium toxicity (Wang et al., 2009; Murugavel and Pari, 2007; Yadav and Khandelwal, 2006; Obianime and Roberts, 2009; Jihen et al., 2009). Monoisomly-2,3-dimercaptosuccinate (MiADMS) which contains disulfhydryl groups is a potent lipophilic metal chelator as well as an antioxidant (Tandon et al., 2003). In this study, we investigated the mitigating action of MiADMS on cadmium-induced cytotoxicity in the cultured rat normal liver CRL-1439 cells on the morphology, viability, antioxidative enzyme activities and cell cycle.

2. Materials and methods

2.1. Materials

F12 K medium(1x), penicillin-streptomycin antibiotic solution (100x), Fetal Bovine Serum (FBS), Trypsin-EDTA solution (1x), phosphate buffer saline (PBS), CdCl₂, glutaraldehyde, crystal violet, 5'5-Dithiobis(2-nitrobenzoic acid) (DTNB), NADPH, glutathione reductase, oxidized glutathione, sodium azide, Tris-HCl, Propidium Iodide (PI), RNase, *meso*-2,3-Dimercaptosuccinic acid (DMSA), isopentyl alcohol, methyl chloride, dimethyl sulfoxide

(DMSO), hexane, and cyclohexane were purchased from Sigma-Aldrich company (St. Louis, MO, USA). Potassium phosphate, EDTA, chloroform, D-glucose, SDS, sodium chloride, sodium citrate, and ethyl alcohol were purchased from Thomas Scientific Company (Swedesboro, NJ, USA).

2.2. Synthesis of monoisoamyl-2,3-dimercaptosuccinate

Monoisoamyl-2,3-dimercaptosuccinate (MiADMS) was synthesized in organic chemistry laboratory (Dr. Ikediobi's lab) at Florida A&M University. It was prepared by the controlled esterification of DMSA with isopentyl alcohol in acidic medium according to Jones et al (1992). The purified product was characterized and confirmed using spectral and analytical methods like HPLC, Infra-red and GC-MS before using for treatment studies on the liver cells. MiADMS was stored in the refrigerator (desiccator) to avoid oxidation and thermal decomposition.

2.3. Maintenance of cell line

Rat normal liver CRL-1439 epithelial cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured as per the guidelines supplied. The cells were maintained in F12K medium containing 100 units of penicillin/ml, 100 µg of streptomycin/ml, 2 mM L-glutamine and 10% FBS in T-75 cm² flasks at 37 °C in a 5% CO₂ incubator.

2.4. Treatment of cells with CdCl₂

The cells at a density of 10×10^4 cells per well were plated in polystyrene, flat bottom 24-well microtiter plates (Corning Costar, Rochester, NY) in F12K medium containing 10% FBS and allowed to stabilize overnight in a CO₂ incubator at 37 °C. Following this, the cells were treated with 0, 50 and 150 µM CdCl₂ alone or cotreated with 150 µM CdCl₂ and 300 µM MiADMS concurrently, 2, 4 and 6 h post CdCl₂ treatment in a final volume of 1 ml per well in triplicate wells for 24 h at 37 °C in a 5% CO₂ incubator. In post treatments, cells were first treated with CdCl₂. All treatments were repeated at least twice. Cells were also treated with DMSO (0.1% final concentration) since MiADMS was dissolved in DMSO.

2.5. Evaluation of cell viability

At the end of the incubation period, the viability of the cells was evaluated by dye uptake assay according to Badisa et al (2003). After 24 h of treatment, glutaraldehyde (400 µl of 0.25%) was added to each well to give 0.07% final concentration and incubated for 30 min at room temperature to fix the viable cells. Following this, plates were rinsed with water to wash off the dead cells and dried under airflow inside of the laminar hood for 5 min. Crystal violet (400 µl of 0.1%) was added to each well, incubated for 15 min, washed and dried. To solubilize the dye, 1 ml of 0.05 M sodium phosphate solution (monobasic) in 50% ethyl alcohol was added to each well and the plates were read at 540 nm in a plate reader (Bio-Tek EL800 Plate Reader).

2.6. Preparation of enzyme extracts

Crude enzyme extracts were prepared as per the method of Ikediobi et al., (2004). Approximately 3.9×10^6 cells per T-75 flask were plated and stabilized overnight. Following this, the cells were treated with 0, 50 and 150 µM CdCl₂ alone or cotreated with 150 µM CdCl₂ and 300 µM MiADMS concurrently, 2 and 4 h post CdCl₂ treatment in a final volume of 10 ml per flask in triplicate for 24 h at 37 °C in a 5% CO₂ incubator. All treatments were repeated at least twice. At the end of the incubation, the cells were trypsinized and pelleted by centrifuging at 2,500 rpm for 5 min. The cell pellets were suspended in 1 ml of 50 mM phosphate buffer (pH 7.0) and homogenized with polytron

homogenizer in a glass vial on ice for 1 min at intervals of 15 s. The homogenates were then transferred to eppendorff tubes and centrifuged at 3,000 rpm for 10 min at 4 °C to remove the lysed cell membrane debris. The supernatants were transferred to new tubes and stored at 4 °C for enzyme assay studies.

2.7. Catalase Enzyme assay

Catalase activity was assayed according to the method of Aebi (1984). The assay total volume (450 µl) contained 50 µl of cell extract and 250 µl of 50 mM phosphate buffer (pH 7.0). The reaction at 37 °C was started by the addition of 150 µl of 30 mM H₂O₂. The decrease in absorbance at 240 nm was monitored for 1 min in a Beckman DU 7500 spectrophotometer. The enzyme activity was calculated using the extinction coefficient of 0.00394 l mmol⁻¹ mm⁻¹ and the unit of enzyme activity was expressed as mmol H₂O₂ decomposed per minute.

2.8. Glutathione peroxidase (GPx) Enzyme assay

Glutathione peroxidase activity was assayed according to the method of Yang et al., (2001). The reaction mixture (500 µl) contained 3.2 mM GSH, 0.32 mM NADPH, 1 unit glutathione reductase (GR), 1 mM sodium azide and 0.82 mM EDTA in 0.16 M Tris-HCl (pH 7.0). The sodium azide was added to the reaction mixture to inhibit endogenous catalase activity. The reaction mixture was incubated with 50 µl of sample at 37 °C for 5 min, and the reaction was started by addition of H₂O₂ at a final concentration of 100 µM. The rate of NADPH consumption was monitored at 340 nm for 3 min. One unit of GPx activity was defined as the amount of enzyme required to consume 1 µmol of NADPH/min in the coupled assay.

2.9. Glutathione reductase Enzyme assay

Glutathione reductase activity was assayed according to Smith et al (1988). This assay is based on the following reactions:

Change in absorbance at 412 nm due to the formation of TNB was measured. The assay volume (2 ml) contained 1 ml of 0.2 M potassium phosphate with 1 mM EDTA buffer (pH 7.5), 500 µl of DTNB in 0.01 M phosphate buffer (pH 7.0), 250 µl water, 100 µl of 2 mM NADPH in water, 50 µl cell extract, and 100 µl of 20 mM oxidized glutathione (GSSG). The increase in absorbance at 412 nm was monitored for 3 min in a Beckman DU 7500 Spectrophotometer at 24 °C. The enzyme activity was calculated using the extinction coefficient of TNB ($E_{412} = 13.6 \text{ L mmol}^{-1} \text{ cm}^{-1}$). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmole NADPH per min.

2.10. Cell cycle analysis by flow Cytometer

Cell proliferation of CdCl₂ treated cells and the mitigative activity of MiADMS were examined in rat normal liver cell line, CRL-1439 using FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) according to Badisa et al (2009). In T-25 flask, 1.3×10^6 cells were plated and incubated overnight. The following day, the cells were treated with 0, 25, 50 and 75 µM CdCl₂ alone or cotreated with 300 µM MiADMS concurrently in triplicate flasks for 24 h in a 5% CO₂ incubator at 37 °C. At the end of incubation, the cells were trypsinized

and centrifuged at 2,500 rpm for 10 min at room temperature. Each pellet was re-suspended in 100 μ l PBS and made singlet cells by passing three times through 25 G needle. The cells were fixed in pre-cooled 95% ethanol (5 ml added in a drop-wise manner to each tube while vortexing) and incubated at 4 °C at least for 24 h. The cells were then harvested and re-suspended in ethanol (100 μ l of 95%). The cell suspensions were transferred into BD falcon tubes and were shielded from light. Staining solution (1 ml) containing final concentrations of 1.25 mg/ml ribonuclease A, 1 mg/ml D-glucose and 50 μ g/ml PI was added to each tube in the dark. The tubes were incubated at room temperature for 1 h in the dark with occasional stirring. The distribution of cells in each phase was analyzed within 2 h with the FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). In each sample, a total of 10,000 events from the gated subpopulation were analyzed separately. CELLQuest software was used for acquisition and analysis of the data, and the percentage of cells in each phase was determined with ModFit 3.0 software.

2.11. Statistical Analysis

The viability and enzyme assay results were presented as mean $\pm$ standard deviation (SD (n=3)). All cadmium treated cells data were presented in percentage value in comparison to the untreated control cells (100%). The data were analyzed for significance by one-way ANOVA, and then compared by Tukey's multiple comparison tests, using GraphPad Prism Software, version 3.00 (San Diego, CA, USA). The test values $p < 0.05$ and $p < 0.01$ were considered significant and highly significant in comparison to the respective untreated control or CdCl₂ alone.

3. Results

3.1. Mitigative action of MiADMS on CdCl₂-induced cytotoxicity

The toxic effects of CdCl₂ at different concentrations on various cell lines have been evaluated earlier (Latinwo et al., 2006; Kaplan et al., 2008). However, little is known about the mitigative action of MiADMS on CdCl₂-induced toxicity. Therefore, we studied the mitigative action of 300 μ M MiADMS (in 1:2 ratio) on 150 μ M CdCl₂ induced toxicity after 24 h. In this study, we observed that CdCl₂ toxicity was dose dependent. As the concentration of the CdCl₂ (50, 100 and 150 μ M CdCl₂) increased, the cell viability decreased to 86.3 ± 3.32 , 56.2 ± 2.55 and 38.1 ± 2.97 % respectively ($p < 0.001$, Fig. 1) in comparison to the control (100%). Cells were treated with DMSO (0.1%) alone or with MiADMS alone to examine their independent toxicity on cells. The viability of DMSO (solvent used to dissolve MiADMS) treated cells was 102.9 ± 2.26 %, while the viability of 300 μ M MiADMS treated cells was 99.6 ± 4.88 %. Both compounds showed no toxicity on the cells' viability; therefore the toxicity seen on treated cells was mainly from CdCl₂. In order to observe a significant mitigative effect role of MiADMS, the cells were cotreated with 300 μ M MiADMS at various time intervals (concurrently, 2 h, 4 h, and 6 h post treatment) and 150 μ M CdCl₂. The mitigative action of MiADMS on cell viability after acute CdCl₂ exposure was time dependent. Administration of MiADMS in cotreated cells mitigates the toxicity produced from CdCl₂ by increasing cell viability in comparison to cells treated with 150 μ M CdCl₂ alone ($38.1 \pm 2.97\%$). However, the viability within the MiADMS cotreatment groups was significantly ($p < 0.001$) decreased as the time of cotreatment increased { 83.4 ± 0.57 (concurrent), 80.1 ± 4.8 (2 h), $64.35 \pm 3.32\%$ (4 h), and $44.3 \pm 0\%$ (6 h)} respectively. MiADMS after 6 h of CdCl₂ exposure did not significantly ($p > 0.05$) increase cell viability. These results clearly demonstrated the mitigative action of MiADMS on CdCl₂ induced cell death.

3.2. Mitigative Action of MiADMS on morphology

The figure 2 shows the mitigating action of MiADMS (concurrent treatment) on the morphology of the normal rat liver cells treated with 150 μM CdCl_2 for 24 h. The untreated control cells exhibited triangular shape with extensions (Fig. 2a). The cells treated with MiADMS alone also exhibited similar morphology as the control cells (Fig. 2b) indicating MiADMS did not contribute any morphological alteration to the cells. Cells treated with 150 μM CdCl_2 alone lost extensions resulting in forming round shape; an indication that CdCl_2 caused morphological alteration to the cells (Fig. 2c). Cotreatment of 300 μM MiADMS with 150 μM CdCl_2 treated cells restored the extensions and cell morphology (Fig. 2d). These results clearly demonstrated the mitigating action of MiADMS on the morphology of CdCl_2 treated cells.

3.3. Mitigating action of MiADMS on the status of catalase enzyme

The catalase enzyme activity in CdCl_2 treated cells after 24 h and the mitigating action of 300 μM MiADMS is shown in Figure 3. In the 50 μM CdCl_2 treated cells, the catalase enzyme activity was increased to $108.2 \pm 3.54\%$ in comparison to the control cells (100%). Beyond 50 μM CdCl_2 , the catalase enzyme activity was decreased in a dose dependent manner. Cells treated with 100 and 150 μM CdCl_2 had a significant decrease in catalase activity (52.4 ± 0.42 , $17.9 \pm 5.37\%$, $P < 0.001$) respectively. The cotreatment of MiADMS (concurrent treatment, 2 h or 4 h post treatment) with the 150 μM CdCl_2 treated cells significantly increased ($p < 0.001$) the activity of catalase to 71.75 ± 4.6 , 100.45 ± 14.92 and $89.45 \pm 15.9\%$ respectively in comparison to CdCl_2 alone treated cells ($17.9 \pm 5.37\%$). Within the cotreatment groups, the activity of catalase was highest in 2 h post treatment. These results clearly demonstrated the mitigating action of MiADMS and the effect of various time intervals on CdCl_2 toxicity of treated cells.

3.4. Mitigating action of MiADMS on the status of GPx enzyme

The GPx enzyme activity in CdCl_2 treated and cotreated cells with 300 μM MiADMS and CdCl_2 after 24 h treatment is shown in Figure 4. In the 50 and 100 μM CdCl_2 treated cells, the GPx enzyme activity significantly increased to 121.2 ± 12.8 and $116.8 \pm 3.82\%$ respectively ($P < 0.01$) in comparison to the untreated control cells (100%). However, in the 150 μM CdCl_2 treated cells, the GPx enzyme activity was decreased to $88.1 \pm 3.54\%$ ($P < 0.001$). Cotreatment of MiADMS (concurrent treatment, and 2 h post treatment) with CdCl_2 treated cells resulted in a significant increase of GPx activity ($p < 0.001$) to 112.4 ± 1.41 and $105.8 \pm 7.91\%$ respectively compared to cells treated with 150 μM CdCl_2 alone ($88.1 \pm 3.54\%$). The 4 h post treatment of MiADMS with 150 μM CdCl_2 treated cells resulted in significant decrease of GPx enzyme ($13.75 \pm 4.31\%$) compared to the 150 μM CdCl_2 treated cells (88.1%). These results clearly demonstrated the mitigating action and the effect of various time intervals of MiADMS on cadmium-induced alteration in GPx enzyme activity of CdCl_2 treated cells.

3.5. Mitigating action of MiADMS on the status of GR enzyme

Figure 5 shows the mitigating action of 300 μM MiADMS on the GR enzyme activity in CdCl_2 treated cells after 24 h treatment. The effect of CdCl_2 on the GR enzyme activity was dose dependent; as the CdCl_2 concentration increased the GR enzyme activity decreased. In the 50 μM CdCl_2 treated cells, the GR enzyme was significantly increased to $185.6 \pm 3.18\%$ ($P < 0.01$), while it was significantly decreased to 65.2 ± 6.08 and $12.4 \pm 0.28\%$ ($P < 0.01$) respectively in 100 and 150 μM CdCl_2 treated cells in comparison to the untreated control cells (100%). In the cotreated cells with MiADMS (concurrent, 2 h and 4 h post treatment) and 150 μM CdCl_2 , the GR activity was significantly ($p < 0.001$) increased to 122.8 ± 9.62 , 157.1 ± 9.62 and $78.6 \pm 5.16\%$ respectively, compared to cells treated with 150 μM CdCl_2 .

alone ($12.4 \pm 0.28\%$). Within the cotreatment groups, the GR activity was significantly increased in 2 h post treatment compared to 4 h post treatment. These results clearly demonstrated the mitigating action of MiADMS and the effect of dosing time on CdCl₂-induced alteration in GR enzyme activity of CdCl₂ treated cells.

3.6. Mitigating action of MiADMS on cell cycle arrest

The mitigating action of MiADMS on cells treated with CdCl₂ was examined in rat normal liver cells. Liver cells were either treated without CdCl₂ (0 μ M CdCl₂) which served as the control, or treated with various CdCl₂ concentrations (25, 50 and 75 μ M CdCl₂), and/or cotreated with all CdCl₂ concentrations and 300 μ M MiADMS for 24 h followed by flow cytometer analysis. The figure 5 shows the flow analysis data of 10,000 cells (events). In cells treated with cadmium chloride alone, the population of cells in G1 phase decreased compared to all other treatments. However, in cells cotreated with MiADMS at all CdCl₂ concentrations, the cell population in G1 phase was similar to control. Significant ($p < 0.001$) cell arrest was observed in S phase of cells treated with CdCl₂ alone at all concentrations compared to control and MiADMS cotreated cells. These results indicated that MiADMS mitigated CdCl₂ toxicity by inhibiting the cell cycle arrest at all CdCl₂ concentrations, shifting it to G1 phase.

4. Discussion

Cadmium induced cell damage has been demonstrated earlier in our laboratory by the increased DNA damage, lipid peroxidation and inhibition of antioxidative enzymes in rat liver cells (Fasanya-Odewumi et al., 1998; Ikediobi et al., 2004; Latinwo et al., 2006). Many compounds that act as chelators [N-acetylcysteine (NAC), diallyl tetrasulfide, picroliv] or antioxidants (selenium, Zinc) have been experimentally proven to provide protection against cadmium toxicity (Wang et al., 2009; Murugavel and Pari, 2007; Yadav and Khandelwal, 2006; Obianime and Roberts, 2009; Jihen et al., 2009). In this present study, we used monoisoamyl-2, 3-dimercaptosuccinate (MiADMS) which is a potent lipophilic metal chelator to investigate the mitigating action against CdCl₂-induced cytotoxicity in rat liver cells. The cells were either treated with CdCl₂ alone or cotreated with MiADMS (concurrently, 2, 4 and 6 h post-treatment of cadmium) in order to study the mitigative action and time of treatment of MiADMS against CdCl₂-induced cytotoxicity. The cytotoxicity was evaluated by a simple and reproducible crystal violet dye staining assay (Badisa et al., 2003). From our previous results, it was shown that the LD₅₀ of cadmium chloride in CRL 1439 rat liver cells was 125 μ M, but in the present study, we used 150 μ M CdCl₂ for better visualization of the MiADMS mitigative action. The CdCl₂ at 50, 100 and 150 μ M treatments had decreased the viability to 86.3 ± 3.32 , 56.2 ± 2.55 and $38.1 \pm 2.97\%$ respectively ($p < 0.001$, Fig. 1) in comparison to control (100%). The MiADMS provided similar protection in concurrent cotreated and 2 h post-treated cells (83.4 ± 0.57 , $80.1 \pm 4.8\%$ Fig 1) compared to the 4 or 6 h post-treated with less protection against cadmium chloride toxicity on viability study (64.35 ± 3.32 and $44.3 \pm 0\%$ Fig. 1). However, the 4 or 6 h post-treated with MiADMS increased the cell viability to 64.35 ± 3.32 , and $44.3 \pm 0\%$ respectively when compared to the 150 μ M CdCl₂ treated cells ($38.1 \pm 2.97\%$). The time of MiADMS treatment on cadmium chloride treated cells was shown to affect its mitigative action on the cell viability in this study. As the delay in time of post-treatment with MiADMS increased, the cell viability was decreased. This may be due to the toxicity caused by the cadmium prior to the MiADMS treatment. In this case, MiADMS may be acting as a chelator by immobilizing cadmium and also as an antioxidant by removing the free radicals induced by cadmium from the site of deleterious oxidation reactions.

The change in cell morphology observed in 150 μ M CdCl₂ treated cells resulted in the loss of membrane integrity that eventually led to cell death (Fig. 2c). This result was consistent

with our previous work (Ikediobi et al., 2004) and others (Newairy et al., 2007; Murugavel and Pari 2007; Yadav and Khandelwal 2006) which revealed that cadmium induced lipid peroxidation. Addition of MiADMS to the treatment, restored cell morphology (Fig. 2d), and this observation was consistent with earlier studies which indicated that MiADMS prevented cadmium or lead-induced toxicity in liver, renal, and brain in rats (Saxena and Flora, 2004; Tandon et al, 2002 and 2003).

Cadmium exposure in vitro or in vivo altered several stress response genes involved in the production of antioxidant enzymes which play an important role in the cellular defense mechanism against oxidative stress. Catalase and GR enzymes activity were decreased drastically in the cells treated with 150 μM CdCl_2 alone, while the enzymes activity were increased in cotreatment with MiADMS (concurrent, 2 or 4 h post treatment) groups (Figs. 3 and 5). The result shown here signifies the time response of MiADMS among the cotreated cells. Cotreatment beyond 2 h resulted in the decrease of these enzymes activity. A similar pattern of result was observed in viability and enzymes data. More cells observed in 2 h MiADMS post treatment resulted in more enzymes activity and the fewer cells observed in 4 h MiADMS post-treatment resulted in the less enzymes activity. This result is consistent with earlier reports where MiADMS reversed lead or cadmium induced oxidative stress in rats (Tandon et al., 2002, 2003).

Glutathione peroxidase is another enzyme involved in protection of cells during oxidative stress. In this study, GPx activity was increased in cells treated with 50 and 100 μM CdCl_2 alone compared to the control cells (Fig. 4) which indicates the cells' response. In cells treated with 150 μM CdCl_2 alone, the GPx activity level decreased compared to other CdCl_2 alone treated and control cells which indicate an intensive ROS generation by CdCl_2 . Cotreatment of 150 μM CdCl_2 with 300 μM MiADMS restored the GPx activity in concurrent and 2 h post treatment but not in 4 h post treatment. Based on these results the mitigative action of MiADMS might be due to the reaction of sulfhydryl groups of MiADMS with metals or free oxygen radicals or with highly reactive byproducts of lipid peroxidation produced by CdCl_2 . These results clearly show that the time of dosing plays an important role in mitigating action of MiADMS on the status of GPx activity.

Cell cycle analysis showed interesting results when mitigative action of MiADMS was examined in rat normal liver cells treated with different CdCl_2 concentrations. Compared to the control cells, cells treated with CdCl_2 alone had an increase in cell population in S phase which indicates the cell arrest with CdCl_2 treatment. The cell arrest was shifted to G1/G0 phase when cotreated with MiADMS which indicates a mitigative action of MiADMS by inhibition of cell arrest (Fig. 6). This result was supported by previous report (Gaofeng et al., 2008) which showed the cell arrest in S phase of lung embryo fibroblast cells treated with a low dose of cadmium (0.9 – 1.5 μM). In another study with CdCl_2 , the cell arrest was observed in G1 in kidney distal epithelial cells (Bjerregaard, 2007). Hence, cadmium's effect on cell cycle depends on the type of cells, cadmium concentration and duration of the treatment. Inhibition of cells treated with CdCl_2 in S phase may be as a result of its interference with genes associated with cell growth as reported in earlier studies (Joseph et al., 2001; Spruill et al., 2002). The shifting of the arrest to G0/G1 from S phase by MiADMS may be as a result of MiADMS inhibiting the signal-regulated kinase (ERK) activation responsible for cell proliferation as seen in study with NAC (Kim et al., 2005).

In conclusion, this study clearly showed that cotreatment with MiADMS in CdCl_2 treated rat normal liver cells (CRL-1439) mitigates the cadmium toxicity by increased cell viability, restored cells morphology, inhibition of cell arrest and increased antioxidative enzymes.

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Fig. 1. Mitigative action of MiADMS on CdCl₂ induced cytotoxicity of rat normal rat liver cells. All values are mean $\pm$ standard deviation [S.D. (n = 6)]. Statistically (Tukey's Multiple Comparison Test) different from the control (** $p < 0.001$) and from the 150 μ M cadmium alone (## $p < 0.001$) were marked.

Fig. 2. Mitigative action of 150 μM CdCl₂ and 300 μM MiADMS on the morphology of the normal rat liver cells. The cells were treated with 150 μM CdCl₂ alone or 300 μM MiADMS alone or cotreated with 300 μM MiADMS (concurrent) and 150 μM CdCl₂ for 24 h.

Fig. 3. Mitigative action of MiADMS on cadmium-induced alteration in catalase activity of rat normal rat liver cells. All values are mean $\pm$ S.D. (n = 6). Statistically (Tukey's Multiple Comparison Test) different from the control (** $p < 0.001$) and from the 150 μ M cadmium alone (## $p < 0.001$) were marked.

Fig. 4. Mitigative action of MiADMS on cadmium-induced alteration in GPx activity of rat normal rat liver cells. All values are mean $\pm$ S.D. (n = 6). Statistically (Tukey's Multiple Comparison Test) different from the control (* $p < 0.05$) and from the 150 μ M cadmium alone (# $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$) were marked.

Fig. 5. Mitigative action of MiADMS on cadmium-induced alteration in GR activity of rat normal rat liver cells. All values are mean $\pm$ S.D. (n = 6). Statistically (Tukey's Multiple Comparison Test) different from the control (** $p < 0.001$) and from the 150 μ M cadmium alone (## $p < 0.001$) were marked.

Fig. 6.

Mitigative action of MiADMS on cadmium-induced alteration in cell cycle phases of rat normal rat liver cells. The cells were treated with 0, 25, 50, 75 μ M CdCl₂ alone or cotreated with 300 μ M MiADMS (concurrent) for 24 h. All values are mean $\pm$ S.D. (n = 6). Statistically (Tukey's Multiple Comparison Test) different from the control (** p < 0.01) and from the respective cadmium alone (# p < 0.05 and ## p < 0.01) were marked.