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Chemo-protective Effect of Monoisoamyl 2, 3-Dimercaptosuccinate (MiADMS) on Cytokines Expression in Cadmium Chloride Treated Human Lung Cells

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Abstract

Cadmium is commercially profitable element, but it causes toxicity in humans and animals leading to diseases in various organs. The main route of cadmium exposure to humans is through inhalation. Lungs respond to insult through secretion of cytokines. In this study, the chemo-protective effect of monoisoamyl 2, 3-dimercaptosuccinate (MiADMS) was evaluated on viability and cytokines expression in CdCl₂ treated human lung A549 cells by cytokine array. Cells were treated with 0, 50, 75 and 100 µM CdCl₂ alone, 300 µM MiADMS alone, and co-treated with 300 µM MiADMS and 75 µM CdCl₂ for 24 h. The viability was measured by crystal violet dye. The level of cytokines in the cells' lysate and cell culture medium was measured using Ray Biotech's Human Cytokine Array 6 in control cells, 75 µM CdCl₂ alone and MiADMS co-treated cells. Array results were validated by ELISA kit. The CdCl₂ caused a dose dependent decrease in cell viability, while MiADMS co-treatment resulted in a significant increase in viability of CdCl₂ treated cells. Morphology of the cells treated with CdCl₂ was destroyed, while MiADMS restored the lost shape in CdCl₂ treated cells. In addition, the cells co-treated with MiADMS and CdCl₂ showed modulation of cytokines expression in comparison to the CdCl₂ alone treated cells. The ELISA results showed the similar pattern of cytokine expression as Human Cytokine Array and validated the array results. These results clearly show the chemo-protective effect of MiADMS and suggest that MiADMS can be used as antidote at moderate dose against CdCl₂ toxicity.

Keywords

viability; cadmium; cytokine; monoisoamyl 2, 3-dimercaptosuccinate; lung cells

INTRODUCTION

Cadmium is a non-essential, carcinogenic and immunotoxic metal (IARC, 1993; Goering et al., 1995; Dan et al., 2000). It targets many organs in the mammalian system and mostly accumulates in the lungs, liver and kidney after absorption (Habeebu et al., 1998; Rikans and Yamano, 2000). It is a highly reactive metal affecting many human organs depending on the dose, route, and duration of exposure (Chin and Templeton, 1993; Bridges and Zalups, 2005).

Cytokines are a family of proteins that are necessary for optimal functioning of our defense (immune system) and repair systems. They are secreted by a variety of cells in response to injury, infections or toxin insults. Cytokine antibody arrays were very helpful to study many cytokines expression simultaneously in a single experiment. So far, there were no reports of cadmium toxicity studies with cytokine antibody arrays.

Many agents have been used to reverse the toxicity of metals in various organs of mammalian system. External agents such as metals (zinc and selenium); antioxidants agents, such as N-acetyl cysteine, Picroliv, ascorbic acid and internal agents such as metallothionein were studied against cadmium toxicity in various cell lines or animal models (Forrester et al., 2000; Jihen et al., 2008; Odewumi et al., 2011a). Very few studies were reported on the inflammatory action of cadmium in lungs and chemo protective effect of compounds. Hence, it is of great interest to investigate the effect of cadmium on the expression of cytokines that are produced as an inflammatory response of the lung cells to environmental insult.

The monoisoamyl 2,3-dimercapitosuccinic acid (MiADMS) is an ester of 2,3-dimercaptosuccinic acid (DMSA) containing two sulfhydryl (SH) groups. This compound is more hydrophilic and less toxic to the cells than other metal chelators (Aposhian and Aposhian, 1990). In addition, MiADMS is an efficient heavy metal mobilizer/metal chelator in soft tissues which contains more sulfhydryl groups that makes it stronger than bioligands (Andersen, 1999; Smith, 2000). In the present study, we investigated the chemo-protective mechanism of Monoisoamyl 2, 3-Dimercaptosuccinate (MiADMS) on the viability and cytokines expression in cadmium chloride treated human lung A549 cells.

MATERIALS AND METHODS

Maintenance of Cell Line

Human lung (A549, catalog number CCL-185) cell line was purchased from the American Type Culture Collection (ATCC). The supplied frozen cells were cultured according to ATCC instructions. The cells were grown in 10 mL of F12K medium containing 100 units of penicillin per mL, 100 µg of streptomycin per mL, 0.025 µg of Amphotericin B per mL, and 10% Fetal Bovine Serum (FBS) in T-75 cm² tissue culture flasks at 37°C in a 5% CO₂ incubator (Nuair Inc, Plymouth, MN, USA).

Synthesis of Monoisoamyl meso-2,3-dimercaptosuccinate (MiADMS)

Monoisoamyl meso-2,3-dimercaptosuccinate (MiADMS) was synthesized in the organic chemistry laboratory (Dr. Ikediobi's Lab) at Florida A&M University. In brief, it was prepared by the controlled esterification of Dimercaptosuccinic acid (DMSA) with the corresponding alcohol in acid medium according to Jones et al., 1992 as mentioned in previous report (Odewumi et al., 2011b).

Treatment of Cells

To investigate the chemo-protective effect of MiADMS on human lung cells treated with cadmium chloride, 1×10^5 cells per well were seeded into 24-well culture plate in 900 μL of medium and incubated overnight in a 5% CO_2 incubator at 37°C for stabilization. Following the stabilization, the cells were treated with different concentrations of cadmium chloride (0, 50, 75 and 100 μM CdCl_2) or 300 μM MiADMS and 75 μM CdCl_2 (co-treatment) in a final volume of 1 mL in triplicate wells and incubated for 24 h at 37°C in a 5% CO_2 incubator. Cells incubated with only culture medium without CdCl_2 or MiADMS served as the control cells.

Morphology

At the end of the treatment, morphology of untreated (control), cadmium chloride alone treated, and cadmium chloride and MiADMS co-treated lung cells were observed under a phase-contrast microscope and the images were taken with a Kodak digital camera under the Nikon Diaphot phase contrast microscope with 10x objective.

Cell Viability Test

The viability was evaluated by dye uptake assay according to Badisa et al., 2008. Glutaraldehyde (400 μL of 0.25% to make 0.07% final concentration in the well) was added to each well and incubated for 30 min at room temperature to fix the viable cells. Following this, the plates were rinsed with water to wash off the dead cells and dried under airflow inside the laminar hood for 5-10 min. Crystal violet solution (400 μL of 0.1%) was added to each well and incubated for 15 min, followed by several washes and air dried. Crystal violet dye was solubilized in each well with addition of 1 mL of 0.05 M sodium phosphate solution (monobasic) in 50% ethyl alcohol. The culture plates were read at 540 nm in a plate reader (Bio-Tek EL800). The mean O.D. value of the control was taken as 100% and the rest of the other groups were calculated as a percent of the control.

Preparation of Samples for Cytokine Array

To study the various cytokines expression of the cadmium chloride treated cells and the chemo protective effect of MiADMS on cytokines expression in these cells, approximately 3.9×10^6 cells were plated in T-75 cm^2 flasks in complete F12K medium and allowed to stabilize overnight. The cells were then treated with 0, 75 μM CdCl_2 alone or co-treated with 300 μM MiADMS and 75 μM CdCl_2 in triplicate T-75 cm^2 flasks. The flasks were incubated for 24 h at 37°C in a 5% CO_2 incubator. After 24 h of incubation, the culture medium was transferred to 15 mL falcon tube and stored at -80°C till it was used for cytokine array analysis. The cells were trypsinized, pooled together and pelleted by

centrifuging at 2,500 rpm for 5 min. The cell pellet was suspended in 1 mL of 1x cell lysis buffer (from the Cytokine Array Kit) and lysed by homogenization in a vial under ice for 15 s (3x) using a Polytron homogenizer. The homogenate was transferred to an eppendorff tube and centrifuged at 10,000 rpm for 10 min at 4°C to remove the lysed cell membrane debris. The supernatant was transferred to fresh tube and cell lysate was stored at -80°C for use in the cytokine array analysis.

Protein Estimation

The protein concentration was determined by the BCA method (Pierce company) using a BSA standard. Diluted albumin (BSA) standards and working reagent were prepared according to the kit instructions. Different concentrations of each standard and each unknown sample (25 µL) was pipetted in triplicate into appropriately labeled eppendorff tubes with 500 µL of working reagent and mixed well. The tubes were incubated at 37°C for 30 min and then read at 562 nm in a Beckman spectrophotometer. From the standard curve, the protein concentrations of cell lysate were determined.

Cytokine Array Analysis

The cytokines expression was determined in cell lysate and cell culture medium using Ray Biotech's Human Cytokine Antibody Array 6 kit (catalog # AAH-CYT-6). The array procedure was carried out according to manual instructions with minor changes. The membranes were blocked for 30 min and then hybridized with 400 µg of cell lysate protein or 1 mL of cell culture medium for 2 h at room temperature. The membranes were washed with buffer I (3x) and buffer II (2x) for 5 min. Following this, the membranes were incubated with biotin-conjugated primary antibodies for 2 h at room temperature and then washed. Later, membranes were incubated with the HRP-conjugated streptavidin secondary antibodies at room temperature for 2 h, washed again and developed by incubation with detection buffer for 5 min. The chemiluminescence of the arrays was detected using Alpha Innotech's FluorChem FC2 machine and density of spots was analyzed by AlphaEaseFC software.

ELISA

Ray Bio ELISA kit employs a specific cytokine (IL1- α) antibody coated on a 96-well plate. Each plate well was incubated with 40 µg of cell lysate protein at 4°C for overnight. The wells were washed with wash buffer (4x) and incubated with biotinylated antibody at room temperature for 2 h. Following this, the unbound biotinylated antibodies were washed away from the plate using wash buffer and the wells were incubated with HRP-conjugated streptavidin. Lastly, the wells were washed again and were developed with soluble HRP substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) in buffered solution. The stop solution was added and the color intensity was measured at 450 nm in a plate reader (Bio-Tek, Winooski, VT, USA). Cytokine expression level in treatment groups was compared with control cells cytokine expression (100%).

RESULTS

Chemo- Protective Effect of MiADMS on Morphological Changes in CdCl₂ Treated A549 Cells

Fig. 1, shows the morphology of A549 human lung alveolar cells treated with 0, 50, 75, and 100 μ M cadmium chloride alone, or 300 μ M MiADMS alone or co-treated with 75 μ M cadmium chloride and 300 μ M MiADMS for 24 h. The morphological changes observed in the CdCl₂ treated lung A549 cells were dose dependent. The higher the concentration of CdCl₂ in the treatment, the more morphological changes were observed. The control cells (Fig. 1A) exhibited triangular epithelium-like extensions. In the cells treated with 50 μ M CdCl₂, minor change in morphology was observed with few cells showing shortened extension and display of round circular shape (Fig. 1B). In the cells treated with 75 μ M CdCl₂, the morphology of many cells changed from rod-like to round with either short to no extensions (Fig. 1C). In the cells treated with 100 μ M CdCl₂, almost all of the cells lost their triangular shape and extensions becoming very small and circular (Fig. 1D). Treatment with 300 μ M MiADMS alone did not alter the morphology of the cells (Fig. 1E). The cotreatment of 75 μ M CdCl₂ treated cells with 300 μ M MiADMS (Fig. 1F) restored the cell extensions that were destroyed by cadmium chloride to the normal (control cells' shape, Fig. 1A). These results clearly show the chemo protective effect of MiADMS on the morphological changes in CdCl₂ treated human lung A549 cells.

Chemo Protective Effect of MiADMS on the Viability of CdCl₂ Treated A549 Cells

The viability of the cells treated with CdCl₂ (0, 50, 75, 100 μ M) or 300 μ M MiADMS alone, or co-treated with 75 μ M CdCl₂ and 300 μ M MiADMS for 24 h was shown in Fig. 2. The viability of the cells treated with CdCl₂ alone was dose dependent and was significantly decreased to $65.8 \pm 3.4\%$, $43.3 \pm 1.5\%$ and $29.2 \pm 1.4\%$ respectively in comparison to control cells (100%, Fig. 2). The cell viability of MiADMS alone was measured to see if it may have any toxic effect on the cells. MiADMS didn't show significant toxic effect on cell viability. When the viability of the cells treated with 75 μ M CdCl₂ alone ($43.3 \pm 1.5\%$) was compared to the co-treated cells (CdCl₂ and MiADMS), the viability was increased to $64.8 \pm 1.7\%$. This result clearly shows the chemo-protective effect of MiADMS on the viability of A549 human lung cells treated with CdCl₂.

Differential Expression of Cytokines in the Cells' Lysate and Cell Culture Medium of Cells Treated with CdCl₂ alone and Co-treated Cells with MiADMS

The various cytokines level in the lysate of the control, 75 μ M CdCl₂ alone, and co-treated cells with 300 μ M MiADMS and 75 μ M CdCl₂ were measured using Ray Biotech Arrays. In the lysate of CdCl₂ alone treated cells, 17 cytokines were significantly up regulated (up regulation is considered as the expression level 30% above control) and 15 cytokines were significantly down regulated (down regulation is considered as the expression level 30% below control) in comparison to control (100%). The up regulated cytokines in the lysate of CdCl₂ alone treated cells were eotaxin-2, FGF-6, FGF-7, Fit-3 ligand, fractalkine, GCP-2, GDNF, GM-CSF, IFN- γ , IGF-1, IGFBP-1, IL-1 α , IL-1 β , IL-1ra, IL-3, IL-4, IL-5, IL-10, IL-13, IL-15, IL-16, and light (Figs. 3A, B). Contrasting results were observed when the level of the cytokines expressed in the lysate of CdCl₂ alone treated cells was compared to

co-treated cells with MiADMS and CdCl₂. All up regulated cytokines in the lysate of CdCl₂ alone treated cells were down regulated in the lysate of co-treated cells. The cytokines that were down regulated in the lysate of CdCl₂ alone cells were CNTF, EGF, eotaxin, IGFBP-2, IGFBP-4, MCP-3, MCP-4, MDC, MIG, MIP-1 δ , MIP-1 α , NT-3, PARC, PDGFBB, and TGF- β 3 (Fig. 4). Similar to the up regulated cytokines level result, when the level of the cytokines in the lysate of co-treated cells was compared to the lysate of the CdCl₂ alone treated cells, all the down regulated cytokines in the lysate of CdCl₂ alone treated cells were increased in the lysate of the co-treated cells (Fig. 4).

In addition to the cytokine levels measured in the lysate, level of the cytokines in the cell culture medium of the above mentioned treatments were also measured using the same method as lysate. In the CdCl₂ alone treated cell culture medium, 13 cytokine levels were significantly up regulated and 4 cytokines were significantly down regulated in comparison to the control cells cell culture medium (Figs. 5, 6). In the cell culture medium of the co-treated cells with MiADMS and CdCl₂, 8 (IL-13, leptin, MCP-3, rantes, SCF, SDF-1, TARC, and TGF- β 1) cytokine levels out of the 13 cytokines that were up regulated in the CdCl₂ alone treatment were decreased and 3 cytokine (GM-CSF, IL-5, and IL-6) levels were increased (Fig. 5). The levels of angiogenin, IGFBP-4, IGF-1 and NAP-2 cytokines in the cell culture medium of CdCl₂ alone treated cells were significantly down regulated in comparison to the cell culture medium of the control cells, while they were up regulated in the cell culture medium of co-treated cells with MiADMS and CdCl₂ (Fig. 6). These results clearly show the chemo-protective effect of MiADMS on the level of various cytokines expressed in the CdCl₂ treated lung cells.

Validation of Array Results by ELISA

The ELISA assay was used to validate the data of cytokines expression analysis by the array. IL-1 α cytokine was one of the highly regulated cytokines in the cytokines expression analysis by the array. In the cell lysate of 75 μ M CdCl₂ alone treated cells, the cytokine IL-1 α expression increased to $281.7 \pm 10.4\%$ in comparison to control cells (100%, Fig. 7), while in the cells co-treated with MiADMS and 75 μ M CdCl₂, the expression of IL-1 α was decreased to $141.7 \pm 7.6\%$ (Fig. 7). The similar pattern of expression was observed earlier in the array (Fig. 3A). This result clearly validates the data of cytokines expression analysis by the array.

DISCUSSION

The prevalent exposure of humans due to the increased level of cadmium in the environment has prompted the testing of various chemicals that can ameliorate the effects of cadmium toxicity and their mode of action in cell lines or animal models. Previously, we reported various studies on cadmium toxicity, and the protective effect of different antioxidant or chelating compounds that reduce the toxic effect of cadmium on the liver cells (Forrester et al., 2000; Badisa et al., 2008; Odewumi et al., 2011a; Odewumi et al., 2011b). Since inhalation is the main route of the cadmium exposure in the humans due to cigarette smoke and the fumes containing cadmium that was released into the environment from various industries. Further, cadmium toxicity has been linked to lung cancer and various pulmonary

diseases (Kirschvink et al., 2005; Nawrot et al., 2006). Hence in this report, we studied the chemo protective of MiADMS against cadmium toxicity in the lung cells. Human lung A549 cells were chosen because they display many differentiated features of lung alveolar cells and was used by many researchers in the cadmium toxicity studies (Schwerdtle et al., 2010; Kundu et al., 2011).

In our previous studies with liver cells, we studied the chemo-protective effect of MiADMS against CdCl₂ toxicity at the concurrent and 2, 4 or 6 h post-treatment period on the viability and found that concurrent MiADMS treatment showed higher protection on the viability and decreased with increase of post-treatment time (Odewumi et al., 2011b). It was also noticed that the tetrazolium salts react with MiADMS or N-acetyl cysteine and showed the positive color (data not shown) and therefore crystal violet dye was used for the viability assay. Hence, in this report, we studied the chemo-protective effect of MiADMS at the concurrent or same time against CdCl₂ toxicity in the lung cells. The viability and morphology of the cells cotreated with cadmium chloride and MiADMS showed an increase in viability as well as restoration of the morphology in comparison to cells treated with 75 µM CdCl₂ alone (Figs. 1, 2). These results clearly showed the chemo-protective role of MiADMS against cadmium chloride toxicity in lung cells. The chemoprotective effect of MiADMS is due to its ability to act as a metal chelator by binding to the cadmium or as an antioxidant (Jones et al., 1992, Odewumi et.al. 2011b). This observation is consistent with previous studies which showed MiADMS preventing cadmium and lead induced toxicity in the liver, renal, and brain of rats or in rat liver cells (Tandon et al., 2002; Swaran et al., 2003; Tandon et al., 2003; Odewumi et al., 2011b).

Lungs react to airway insults through inflammation response by secreting inflammatory mediators such as cytokines (Borošková et al., 2007; Kataranovski et al., 2009; Låg et al., 2010). The induction of cytokines expression by cadmium chloride in lung cells and the alteration of cytokine levels in cells co-treated with MiADMS and cadmium chloride were investigated using Ray Biotech antibody membrane arrays. The Human Antibody Array 6 was used to quantify the expression of 60 different cytokines simultaneously in the lysate and medium of the control cells, cells treated with cadmium chloride alone and co- treated with MiADMS and cadmium chloride for 24 h. It was found that in the lysate of cells treated with cadmium chloride alone, level of 22 cytokines that were associated with proliferation like fit-3, FGF-6, FGF-7, IGF-1, IGFBP-1 and inflammatory immune response like eotaxin-2, fractalkine, and anti-inflammatory cytokine like IL-10 were highly up regulated and level of 15 cytokines that were associated with chemotaxis like MCP-3, MCP-4, MDC, MIG, MIP-1α, and MIP-1δ and regulators of growth factors like IGFBP-2, IGFBP-4 and PARC were highly down regulated (Figs. 3, 4). Furthermore, from the above data, we can hypothesize that the high expression of growth factors and anti-inflammatory cytokines in the cadmium chloride alone treated cells was to resist the cadmium chloride toxicity. In addition, chemokines which are responsible for inflammatory response and protection of the cells against tissue damage were highly down regulated in the cadmium alone treated cells which suggest the cells damage due to cadmium toxicity. Interestingly, the up regulated cytokines in the cadmium chloride alone treated cells were down regulated in the cells co-treated with MiADMS and cadmium chloride and vice-versa. This result clearly showed the chemo-protective mechanism of MiADMS which was reflected in the cytokine expression

levels in the co-treated cells with MiADMS and CdCl₂. The cytokines expressed in the cadmium chloride alone treated cells were similar to the cytokines expressed in chronic treatment reported in earlier study (Feghali et al., 1997). A study done using low concentration of cadmium (3-6 µM) in lung cells reported alterations in inflammation related genes expression and an increased release of IL-6 and MIP-2/CXCL2 from the epithelial cells and MIP-2, IL-1beta and TNF-alpha from alveolar macrophages (Låg et al., 2010). Our present study is the first report to investigate the effect of cadmium and the chemo-preventive effect of MiADMS on various cytokines expression at the protein level by antibody array.

IL-1α is one of the highly regulated cytokine in cadmium chloride alone treated cells and was selected to validate the array results. It plays specific role in the onset of inflammatory process that regulates expression of other cytokines and chemokines in the cells against infection or insult (Barksby et al., 2007; Grivennikov et al., 2006). The result of ELISA experiment validated the result obtained from Cytokine Antibody Array analysis with the same pattern of IL-1α cytokine expression in the cadmium chloride alone and co-treated cells (Fig. 3A, 7).

CONCLUSIONS

In conclusion, our present study showed that MiADMS compound increased the viability and restored the morphology of lung cells treated with cadmium chloride. The cytokine array analysis showed that cadmium chloride altered the expression of various inflammatory cytokines that were important for inflammatory response in the presence of insult and cotreatment with MiADMS reversed the altered cytokines expression. The present study clearly demonstrated the chemo protective effect of MiADMS in the human lung A549 cells treated with cadmium chloride.

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Fig. 1. Chemo-protective effect of MiADMS on the morphology of CdCl_2 treated cells
The cells were treated with CdCl_2 (0, 50, 75 and 100 μM) alone, MiADMS (300 μM) alone, or MiADMS and 75 μM CdCl_2 for 24 h. The images were taken with a Kodak digital camera under the Nikon Diaphot phase contrast microscope with 10x objective.

Fig. 2. Chemo-protective effect of MiADMS on the viability of CdCl₂ treated cells

The cells were treated with CdCl₂ (0, 50, 75 and 100 μM) alone, MiADMS (300 μM) alone or MiADMS and 75 μM CdCl₂ for 24 h. The viability was measured by crystal violet assay.

*Significantly different compared to control ($p < 0.05$). #Significantly different compared to CdCl₂ alone ($p < 0.05$).

A

B

Fig. 3. Up-regulated cytokines expression in the cell lysate of 75 μ M CdCl₂ alone treated cells and their alteration in MiADMS and 75 μ M CdCl₂ co-treated cells
The cells were treated with CdCl₂ (0, 75 μ M) alone, or MiADMS and 75 μ M CdCl₂ for 24 h. The cells were lysed and cytokines expressions in the cell lysate were measured by Ray Biotech Human Cytokine arrays 6. *Significantly different compared to control (p < 0.05). #Significantly different compared to CdCl₂ alone (p < 0.05).

Fig. 4.

Down-regulated cytokines expression in the cell lysate of 75 μ M CdCl₂ alone treated cells and their alteration in MiADMS and 75 μ M CdCl₂ co-treated cells. The cells were treated with CdCl₂ (0, 75 μ M) alone, or MiADMS and 75 μ M CdCl₂ for 24 h. The cells were lysed and cytokines expressions in the cell lysate were measured by Ray Biotech Human Cytokine array 6. *Significantly different compared to control ($p < 0.05$). #Significantly different compared to CdCl₂ alone ($p < 0.05$).

Fig. 5.

Up-regulated cytokines expression in the cell culture medium of 75 μ M CdCl₂ alone treated cells and their alteration in MiADMS and 75 μ M CdCl₂ co-treated cells. The cells were treated with CdCl₂ (0, 75 μ M) alone, or MiADMS and 75 μ M CdCl₂ for 24 h. The cells were lysed and cytokines expressions in the cell lysate were measured by Ray Biotech Human Cytokine array 6. *Significantly different compared to control ($p < 0.05$). #Significantly different compared to CdCl₂ alone ($p < 0.05$).

Fig. 6. Down-regulated cytokines expression in the cell culture medium of 75 μ M CdCl₂ alone treated cells and their alteration in MiADMS and 75 μ M CdCl₂ co-treated cells. The cells were treated with CdCl₂ (0, 75 μ M) alone, or MiADMS and 75 μ M CdCl₂ for 24 h. The cells were lysed and cytokines expressions in the cell lysate were measured by Ray Biotech Human Cytokine array 6. *Significantly different compared to control ($p < 0.05$). [#]Significantly different compared to CdCl₂ alone ($p < 0.05$).

Fig. 7.

ELISA confirmation of IL-1 α expression in the cell lysate of 75 μ M CdCl₂ alone treated and MiADMS and 75 μ M CdCl₂ co-treated cells. The cells were treated with CdCl₂ (0, 75 μ M) alone, or MiADMS and 75 μ M CdCl₂ for 24 h. The cells were lysed and cytokine expression in the cell lysate was measured by Ray Biotech Human IL-1 α Cytokine ELISA kit.

*Significantly different compared to control ($p < 0.05$). #Significantly different compared to CdCl₂ alone ($p < 0.05$).