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Modulation of Cadmium Induced Apoptotic, Cancer and Inflammation Related Cytokines by Diallyl Disulfide in Rat Liver Cells

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Abstract

Cadmium (Cd), an environmental heavy metal pollutant, is one of the high risk factors for human diseases due to the exponential increase of its use in industrial processes and products during the last 50 years. It deposits mainly in the liver and leads to various diseases including cancer. Currently, there is a growing attention to explore natural compounds for the prevention of unfavorable effects of Cd in humans. Diallyl disulfide (DADS), an organosulfur compound in garlic is used as a prophylactic compound for various diseases in many countries. In this study, the modulatory effect of DADS against Cd toxicity was evaluated on the viability and cytokine proteins expression in cadmium chloride ($CdCl_2$) treated normal rat liver CRL1439 cells. The liver cells were treated with $CdCl_2$ for 24 h with or without DADS pre-treatment for 2 h and viability was measured using the crystal violet dye uptake assay. The cytokines protein expression was measured after 6 h of $CdCl_2$ treatment using the RayBiotech human cytokine array 7 kit. The DADS pre-treatment dramatically increased the viability of $CdCl_2$ treated cells to 52.0 ± 3.1% in comparison to $CdCl_2$ alone treated cells (31.4 ± 2.2%). The $CdCl_2$ treatment upregulated 8 inflammatory, 3 apoptosis, and 9 cancer pathway cytokines and downregulated 3 inflammatory pathway cytokines in liver cells. However, DADS pre-treatment attenuated all the cytokines except two cytokines affected by the $CdCl_2$ treatment. The present study clearly demonstrated the protective effect of DADS against Cd toxicity through the modulation of cytokines expression in the $CdCl_2$ treated liver cells.

Keywords

Cadmium chloride, Liver cells, Diallyl disulfide, Viability, Cytokines

Abbreviations

Cd: Cadmium; $CdCl_2$: Cadmium chloride; DADS: Diallyl disulfide; IL: Interleukin

Introduction

Cadmium (Cd) is one of the economically valuable heavy metals utilized in a variety of products that are used in our daily life [1]. On the other hand, it causes a serious concern to both animal and human's health worldwide as an environmental pollutant [2,3]. Large quantities of Cd compounds are discharged into the environment from industrial activities and fossil fuels as industrial waste into the water, air, and soil leading to contamination of edible plants and seafood [4,5]. Studies have reported that two thirds of the Cd exposure through the diet is attributed to contaminated vegetables and one third to animal products [6]. Thus, ingestible food and water are the major sources of Cd exposure for nonsmokers with

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the Cd intake from food and water estimated between 50 to 100 µg per day. Previous report showed that the estimated weekly intake value for Cd via rice consumption even ranged from 20 to 82 µg Cd per kg bodyweight [7]. The foods with highest Cd concentration are shellfish, offal products, and certain seeds, but the most common sources of dietary Cd exposure are cereals, potatoes, root crops, and vegetables (around 80%).

Upon absorption, Cd forms complexes with biomolecules and produces toxicity in various organs. Cd accumulates mostly in the liver due to its role in metal homeostasis and detoxification via the first bypass. Analyses of mammalian organs have shown more Cd accumulation in liver and kidney than in other parts of the body [5,8]. It was reported that environmental Cd exposure was associated with hepatic necro-inflammation in both men and women [9]. In the same study, it was reported that individuals in the top quartile of creatinine-corrected urinary Cd had over a threefold increased risk of liver disease mortality. The liver injury is characterized by increased serum levels of hepatic transaminases and massive necrosis of hepatocytes [10]. In addition to the induction of necrosis, cadmium has been reported to induce apoptosis in liver cells [11]. Our previous reports showed that Cd induce cytokines which play an important role in Cd-induced toxicity in lung cells [12-14].

Although the biological system protects itself by inducing the expression of antioxidant proteins to a certain extent, in the event of Cd accumulation in organs, the use of exogenous antioxidants or metal chelators provides additional protection against Cd induced oxidative stress [15,16]. The search for effective, nontoxic, natural compounds with antioxidant activity against Cd toxicity has been intensified in recent years [17,18]. Garlic (*Allium sativum*) has been used as a conventional food, in herbal therapy and in folk medicine in all parts of the world. It contains both water- and lipid-soluble organosulfur compounds (OSC) which have been reported to be responsible for its therapeutic properties. Diallyl disulfide (DADS) is one of the major organo-sulfur compounds in garlic and is highly stable and lipid-soluble [19]. However, studies related to cytokines expression in the liver during Cd-induced toxicity and the modulatory effects of natural compounds on Cd-induced inflammation are limited [20,21]. In this report, we evaluated the modulatory effect of DADS on Cd toxicity through viability studies and the study of the expression of a panel of 60 cytokine proteins which are involved in apoptosis, cancer and inflammation pathways in normal rat liver CRL 1439 epithelial cells cultured *in vitro*.

Materials and Methods

Reagents

F12K medium (1x), penicillin-streptomycin antibiotic solution (100x), amphotericin, fetal bovine serum (FBS), trypsin-EDTA solution (1x), phosphate buffered saline (PBS), cadmium chloride, 25% glutaraldehyde, diallyl disulfide (DADS) and crystal violet were purchased from Sigma-Aldrich company (St. Louis, MO, USA). The Human cytokine array 7 kit was purchased from RayBiotech, Inc. (Norcross, GA, USA).

Maintenance of cell line

A normal rat liver (catalog number CRL 1439) epithelial cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The supplied frozen cells were cultured according to ATCC instructions. In brief, 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% FBS in T-75 cm^2 tissue culture flasks at 37 °C in a 5% CO_2 incubator (Nuaire Inc, Plymouth, MN, USA).

Treatment of cells

To investigate the modulatory effect of DADS against Cd toxicity in $CdCl_2$ treated liver cells, 1×10^5 cells/well were seeded into 24 well tissue culture plate in 800 µl of complete medium and incubated overnight in a 5% CO_2 incubator at 37 °C to attain stabilization. Following the stabilization, the cells were treated with $CdCl_2$ (0 or 150 µM [27.45 ppm]) and DADS (150 µM) in a final volume of 1 ml in triplicate wells and incubated for 24 h at 37 °C in a 5% CO_2 incubator. In the co-treatment group, cells were pre-treated with DADS for 2 h prior to the treatment with $CdCl_2$. Cells incubated with only culture medium without $CdCl_2$ or DADS served as the control cells.

Cell viability assay

The viability of the cells was evaluated using the crystal violet dye uptake assay as reported earlier [22]. In brief, at the end of the treatment period, 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 (RT) 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 10 min. The crystal violet solution (400 µl of 0.1%) was added to each well and incubated for 15 min, followed by several washes and dried for 10 min. The dye was solubilized in each well with the 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 Plate Reader). The mean O.D. value of the control cells was taken as 100% and the other treated groups were calculated as a percent of the control.

Preparation of samples for cytokine array

To study the cytokines' protein expression of the

$CdCl_2$ treated liver cells and the modulatory effect of DADS, approximately 3.9×10^6 cells were plated in T-75 cm^2 flasks in complete F12K medium and then were allowed to stabilize overnight. The cells were then treated with $CdCl_2$ alone (0, 150 µM) or co-treated with 150 µM DADS and 150 µM $CdCl_2$ in triplicate T-75 cm^2 flasks. In co-treatment group, cells were pre-treated with 150 µM DADS for 2 h prior to treatment with $CdCl_2$. The flasks were incubated for 6 h only at 37 °C in a 5% CO_2 incubator to study the cytokines' expression at earlier time point. At the end of the incubation period, 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 this cell lysate was used for cytokine array analysis or stored at -80 °C for future analysis.

Protein estimation

The protein concentration was determined using the Pierce Bicinchoninic Acid (BCA) Assay (Thermo-Scientific Company, Rockford, IL, USA). The 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 vortexed to mix. 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 lysates were determined.

Cytokine array analysis

The cytokines' protein expression was determined in cell lysate using Ray Biotech's Human Cytokine Antibody Array 7 kit (catalog # AAH-CYT-7). The array study 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 (total volume 1 ml) for 2 h at RT. 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 RT and then washed. Furthermore, the membranes were incubated with the HRP-conjugated streptavidin secondary antibodies at RT for 2 h, washed and finally incubated with detection buffer for 5 min for development. The chemiluminiscence of the arrays was then detected using Alpha Innotech's FluorChem FC2 machine and was analyzed by Alpha Ease FC software.

Statistical analysis

The viability data were presented as mean ± standard deviation (SD, n = 3). All treated cells data were presented as percentage values in comparison to the untreated control (100%). The data were analyzed for significance by one-way ANOVA, and then compared by Tukey's multiple comparison tests, using the GraphPad Prism Software, version 3.00 (GraphPad Software, Inc., San Diego, CA, USA). Differences with the respective untreated control were considered

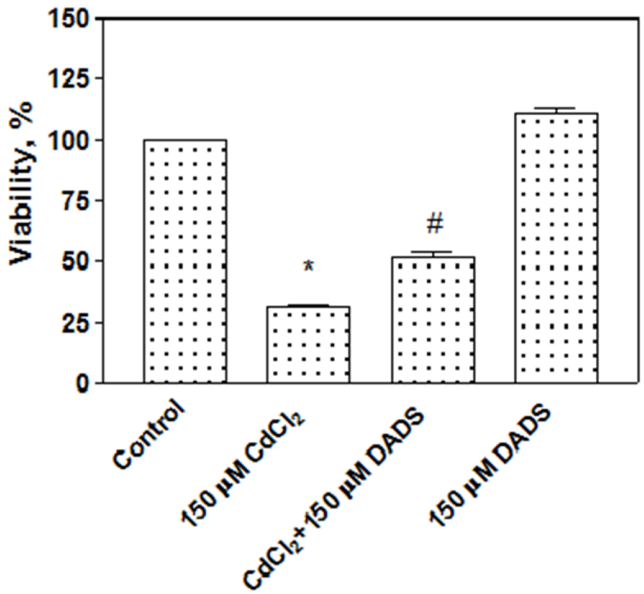

Figure 1: Modulation of DADS on the viability in $CdCl_2$ treated rat normal liver cells. All values are mean ± SEM (n = 3). Statistically (Tukey's Multiple Comparison Test) different from the control ($^*p < 0.001$) and from the 150 µM $CdCl_2$ alone ($^\#p < 0.01$) were shown.

statistically significant when $p < 0.05$. For cytokine array, statistical analyses were performed using Minitab and Microsoft Excel. For pairwise comparisons, we performed both one-tailed and two-tailed two-sample hypothesis tests for the difference in the mean intensity values amongst corresponding cytokines exposed to cadmium (Cd) versus the control baseline cytokines and corresponding cytokines exposed to Cd and DADS (Cd + DADS) versus the control baseline cytokines. All Two-Sample Hypothesis tests were performed at the ($\alpha = 0.05$) level of significance. Here, a statistical significance results in P-value < 0.05; thus, yielding a rejection of the null hypothesis in favor of the alternative hypothesis [23].

Results

Modulatory effect of DADS on the viability of $CdCl_2$ treated rat liver cells

The modulatory effect of DADS against Cd toxicity was examined through the viability assay and the results are shown in Figure 1. The cells treated with 150 μM $CdCl_2$ alone showed a significant decrease (31.4 ± 2.2%) ($P < 0.05$) in cell viability, in comparison to untreated control cells (100%). However, the viability of the cells pre-treated with DADS (150 μM) for 2 h followed by 150 μM $CdCl_2$ for 24 h was increased to 52.0 ± 3.1% ($P < 0.05$) in comparison to the cells treated with 150 μM $CdCl_2$ alone for the same duration. Furthermore, cells treated with 150 μM DADS alone did not show toxicity (110.9 ± 3.9%) rather it slightly promoted cell proliferation (Figure 1). The viability data clearly showed a significant modulatory effect of DADS against Cd toxicity in rat liver cells.

Modulation of DADS on cytokines expression in $CdCl_2$ treated rat liver cells

The cytokines' protein expression of cells treated with $CdCl_2$ alone, DADS alone and a combination of $CdCl_2$ and DADS in normal rat liver epithelial cells was examined and compared with the cytokines' expression of untreated control cells using Ray Biotech Cytokine Array 7. The upregulation is defined as the expression level that is 30% above the control cells expression, while downregulation is defined as the expression level 30% below the control cells expression (based on both one and two tailed tests). The regulated cytokines were grouped according to the pathway in which they function namely apoptosis, cancer or inflammation.

In the $CdCl_2$ alone treated cells, 8 inflammatory, 3 apoptosis and 9 cancer pathway cytokines were upregulated, while 3 inflammatory pathway cytokines were downregulated. The upregulated cytokines involved in inflammation pathway were: NT-4, I-TAC, sTNF-R2, Lymphotactin, GITR-LIGAND, MSP-α, MIF, and IL-12 P40 (Figure 2); in apoptosis pathway: bFGF, IGF-1R, and Fas/TNFRSF6/APO (Figure 3); and in cancer pathway: TRAIL-R4, dtk, IGFBP6, VEGF-D, FGF-4, IGFBP-3, TECK, CTACK, and sTNF R1 (Figure 4). The downregulated cytokines involved in inflammatory pathway were: Hepatocyte growth factor (HGF), IL-8 and HCC-4 (Figure 2).

The pre-treatment of the cells with 150 μM DADS for 2 h prior to $CdCl_2$ treatment resulted in the downregulation of 8 inflammatory, 3 apoptosis and 7 cancer cytokines that were involved in the pathways in comparison to cells treated with $CdCl_2$ alone (Figure 2, Figure 3 and Figure 4). However, the TECK and CTACK cytokines involved in inflammation were more upregulated in the pre-treated cells than cells treated with $CdCl_2$ alone (Figure 2). Furthermore, downregulated inflammatory cytokines (HGF, IL-8 and HCC-4) in $CdCl_2$ alone treat-

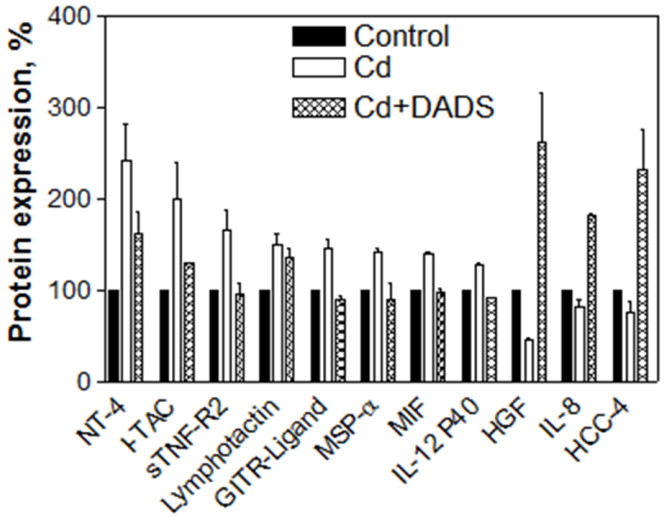

Figure 2: Effect of $CdCl_2$ on inflammation related cytokines and their modulation by DADS pre-treatment in liver cells. All values are represented as mean ± SEM (n = 2).

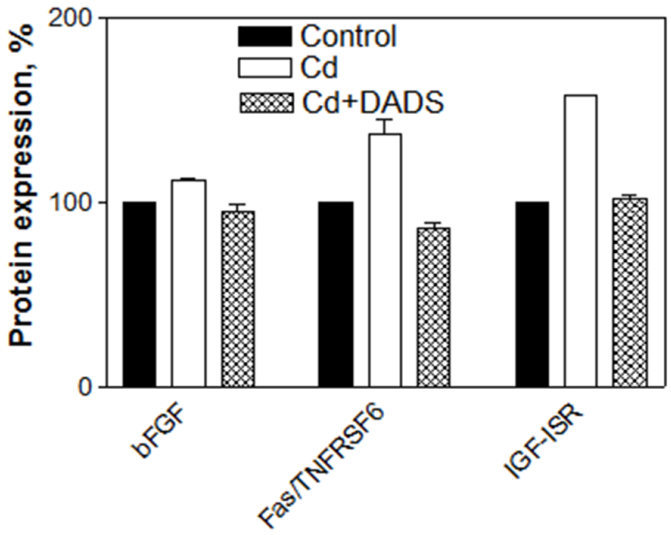

Figure 3: Effect of $CdCl_2$ on apoptosis related cytokines and their modulation by DADS pre-treatment in liver cells. All values are represented as mean ± SEM (n = 2).

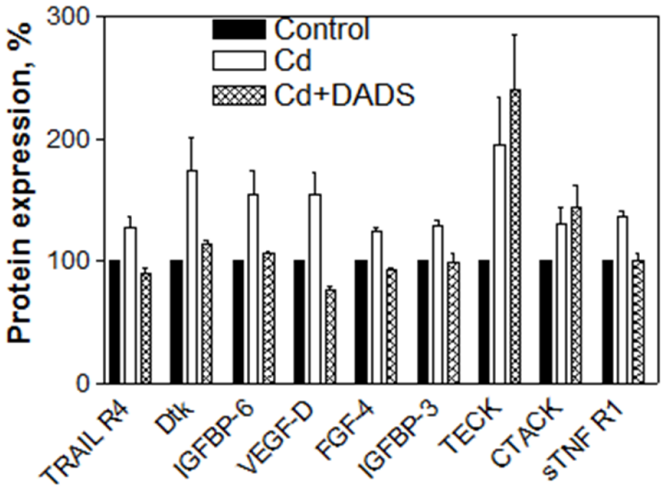

Figure 4: Effect of $CdCl_2$ on cancer related cytokines and their modulation by DADS pre-treatment in liver cells. All values are represented as mean ± SEM (n = 2).

ed cells were significantly up-regulated when pretreated with DADS (Figure 2). Thus, the results clearly showed a significant toxic effect of Cd on the cytokines expression involved in inflammation, apoptosis, and cancer pathways, while DADS pre-treatment modulated the Cd-induced cytokines in the liver epithelial cells.

Discussion

In this report, the modulatory effect of DADS was studied on the viability and cytokines protein expression in $CdCl_2$ treated liver cells. We treated the cells with 150 μM $CdCl_2$ based on our previous reports [15,16]. In our preliminary experiments, we used different concentrations of DADS for protection against Cd toxicity and 150 μM DADS was used in this study since higher concentrations showed toxicity (data not shown). The viability of the cells was measured using the cost-effective and reproducible crystal violet dye uptake assay [22]. The viability results clearly showed that $CdCl_2$ significantly reduced the viability of liver cells (Figure 1, Lane 2). The pre-treatment of cells with DADS for 2 h protected the cells against cadmium toxicity (Figure 1, Lane 3) and, DADS alone did not show any toxicity in the liver cells (Figure 1, Lane 4).

Further, we studied the effect of Cd on the cytokines expression and modulatory effect of DADS on cytokines expression using cytokine array 7 analysis. The cytokines expression study was carried out at a shorter period (6 h) for clearer understanding of the cytokines expressed in cells exposed to $CdCl_2$ within the period showing no cell death. Result from our previous study had shown no cell death at 8 h with the same $CdCl_2$ concentration

[22]. We studied the cytokine proteins expression in the cell lysate rather than the medium supernatant. Even though cytokines are secreted into the medium, they are diluted into the medium and hard to detect them in the cultured medium. The expressed cytokines from the cells were grouped according to their function in the cell pathological pathways namely; inflammation, apoptosis or cancer. The result from the cytokine array analysis of $CdCl_2$ alone treated cells for 6 h resulted in upregulation (NT-4, I-TAC, sTNF-R2, Lymphotactin, GITR-LIGAND, MSP-α, MIF, and IL-12 P40) and downregulation (hepatocyte growth factor (HGF), IL-8 and HCC-4 cytokines) of certain cytokines involved in inflammatory pathway (Figure 2). The other previous studies also have ascertain the involvement of the observed cytokines in our study such as NT-4 [24], I-TAC [25], sTNF-R2 [26], Lymphotactin [27], GITR-LIGAND [28], MSP-α [29], MIF [30], IL-12 P40 [31], HGF [32], IL-8 [33], HCC-4 [34] as cytokines involve in the inflammation process. Previous reports showed the Cd-induced inflammation and mitigation through anti-inflammatory compounds [35,36].

In addition to the inflammatory cytokines, another set of upregulated cytokines (bFGF, IGF-1R and Fas/TNFRSF6/APO-1 proteins) observed in $CdCl_2$ alone treated cells were involved in apoptotic pathway. Involvement of these cytokines in apoptosis has been investigated and confirmed in these studies: bFGF [37], IGF-1R [38], Fas/TNFRSF6/APO-1 [39]. Previous reports revealed that apoptosis plays an important role in Cd induced cytotoxicity and compounds which can prevent apoptosis can protect the cells against Cd toxicity [40,41].

The last set of upregulated cytokines in $CdCl_2$ alone treated cells indicated involvement in cancer pathway and they were TRAIL-R4, dtk, IGFBP6, VEGF-D, FGF-4, IGFBP-3, TECK (also known as CCL25), CTACK (also known as CCL27), and sTNF R1. In support of our findings, earlier studies have also shown the involvement of cytokines such as TRAIL-R4 [42], Dtk [43], IGFBP6 [44], VEGF-D [45], FGF-4 [46], IGFBP-3 [47], TECK [48], CTACK [49], and sTNF R1 [50] in cancer development or progression. Several reports showed that Cd exposure causes cancer in the liver [9,51,52].

The analysis of the cytokines' protein expression revealed reduction in expression of cytokines in the cells pre-treated with DADS for 2 h prior to Cd treatment except for HGF, IL-8 and HCC-4 in inflammatory pathway and TECK and CTACK in cancer pathway in comparison to the level of cytokine protein expression observed in cells treated with CdCl2 alone (Figure 2, Figure 3 and Figure 4). The modulation of cytokines by DADS pre-treatment protects the cells from Cd toxicity which is reflected in the increase of cell viability in DADS pre-treated cells (Figure 1). The result observed in this study was supported by an earlier report which demonstrated that DADS reduce cancer [53], inflammation through regulation of pro-inflammatory cytokines production by inhibiting NF-κB and MAPKs expressions in cyclophosphamide treated Rats [54] and attenuate radiation-induced apoptosis [55]. Furthermore, other studies have also reported that sulfhydryl groups in garlic are very potent in reducing the Cd-induced hepatotoxicity in rats. In addition to diallyl disulfide, another sulfhydryl compound in garlic that has been shown to confer protection against Cd toxicity in rat liver is diallyl tetrasulfide. These species of sulfhydryl groups have been unequivocally demonstrated to have protective effect(s) against Cd toxicity through increase in anti-oxidant potential [59]. Our study clearly demonstrates the modulatory effect of the diallyl disulfide compound against Cd toxicity in rat liver epithelial cells through the regulation of cytokines expression which are responsible for inflammation, apoptosis and cancer pathways.

In conclusion, this report showed that pre-treatment of cells with DADS compound exhibited protective effect against Cd toxicity through modulation of cytokine proteins expression that resulted in increased viability in $CdCl_2$ treated rat liver epithelial cells. This study suggests that use of a natural garlic compound can prevent the toxic effects of Cd in liver cells by modulating the cytokines' expression which in turn prevents cells from entering the inflammatory pathway. In addition, DADS, a major organosulfur compound in garlic has sulfhydryl groups that makes DADS a chelating agent of Cd.

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Conflict of Interest

The authors have no competing interests.

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