Original Research Article

Oxidative Stress Pathway Mechanisms Induced by Four Individual Heavy Metals (As, Hg, Cd and Pb) and their Quaternary on MCF-7 Breast cancer cells

Abstract

Cell death induced by the production of reactive oxygen species (ROS) has largely been associated with the activation of oxidative stress pathway; however, the direct mechanism(s) involved are unknown. This study evaluates the oxidative stress pathways by which four heavy metals (As, Hg, Cd and Pb) administered singly and as a quaternary mixture induce cytotoxic effects on MCF-7 breast cancer cells, in the presence and absence of cellular antioxidant, glutathione (GSH). Cells were exposed to 21.7µg/ml of the individual metals and the mixture and assayed after 5 hr. Cellular levels of nonspecific ROS, superoxide anion ($O_2^{\bullet}$), mitochondria membrane potential (MMP), and GSH were assayed using flow cytometry-FACScalibur equipped with cell quest pro for data collection. Results showed that, in the presence of cellular GSH, As and Pb induced cytotoxicity by reducing the MMP while Cd, and Hg were cytotoxic by the production of mostly superoxide anions and nonspecific ROS. The mixture exhibited cytotoxicity by decreasing the cellular MMP as well as producing ROS and $O_2^{\bullet}$. When the synthesis of cellular glutathione was inhibited, all five treatments damaged the mitochondria membrane and depleted basal GSH. In addition to depleting the basal GSH and causing damage to the mitochondria membrane, Cd, As, and Pb also elicited the production of ROS.

Keywords: Heavy metals; Cytotoxicity; Glutathione; Reactive oxygen species; Superoxide anion; MCF-7 cells.

Introduction

Exposure to environmental contaminants such as heavy metals can pose serious health threats to humans ATSDR [1]. Heavy metals are among the most abundant and persistent environmental inorganic pollutants because they cannot degrade readily Castro-Gonzalez and Mendez- Armenta [2]. They bioaccumulate through multiple trophic levels in food chains (Seebaugh et al. [3]. Heavy metals, especially the non-essential metals which are included in the composition of biological systems, may induce deleterious effects like cell death and redox signaling (Ryter et al. [4] on organisms as well as cause adverse effect in the environment. In addition, some heavy metals are considered priority pollutants due to their biological and ecological effects ATSDR [1], while others are involved in human carcinogenesis Valko et al. [5]. Although the exact molecular mechanisms of metal-induced carcinogenesis are not fully understood, it is possible that most of the oxidative stress-induced damage is mediated by free radical attacks

It has been shown that most metals exhibit the ability to produce reactive oxygen species (ROS) (Galaris and Evangelou [6], Leonard et al. [7], Flora et al. [8]. The formation of ROS in the cells induce lipid peroxidation and DNA damage, deplete sulfhydryl groups, as well as alter signal transduction pathways and calcium homeostasis (Cerutti PA. [9], Stohs and Bagchi [10], Valko et al. [11]. ROS or free radicals are usually extremely reactive and when generated in the intracellular spaces, they are able to attack and modify all main cellular constituents. Metal ions can cause cellular damage indirectly by lowering the level of glutathione (GSH) Hartwig [12] Kasprzak et al. [13]. GSH, the most abundant nonprotein sulfhydryl in most cells, acts as a scavenger for various electrophiles and free radicals, and as such plays an important role against oxidative damage. Reduced glutathione can react directly with ROS and can act as a substrate in the glutathione peroxidase (GPX) - mediated break down of Hydrogen peroxide (H_2O_2). GSH can bind with some heavy metals to form a Metal–GSH complex which results in the excretion of the toxic metals; although, it leads to the depletion of intracellular GSH Quig [14]. Cellular defense against toxic onset can be impaired when GSH is depleted and may lead to cell injury and death.

It is more likely that several metals exist together and their individual toxicities are exhibited simultaneously and interactively. Studies have shown that interactions that occur during exposure to heavy metal mixtures may result in additive, synergistic or antagonistic effects Ishaque et al. [15]. Exposure to metal mixtures may even lead to new effects that have not been shown in single chemical exposures.

Several studies have shown the effects of a single element on a selected cell line, however, studies comparing the effects of several heavy metals and their mixtures on the same cell line are limited. Egiebor et al. [16], in their studies determined the kinetic signature of toxicity of four heavy metals (As, Cd, Hg, and Pb) and their mixture (MIX) on MCF-7 cells, within a concentration range (0.34µg/ml- 21.7µg/ml) for 96 hr. They showed that the onset of cell death occurred after about five hours of exposure to the highest concentration (21.7µg/ml) of the four heavy metals and their mixture. This study was therefore conducted in order to understand the underlying molecular mechanism(s) induced by the metals and their mixture by investigating the cellular regulation of ROS, superoxide anions (O_2•), GSH and MMP in MCF-7 breast cancer cells, when the cellular GSH is present or inhibited.

Materials and methods

Chemicals

The following chemicals were used for the analyses: Atomic Absorption standards (Acros Organic, New Jersey) consisting of Arsenic 1mg/L, 2% KOH, Cadmium 1mg/L 0.5N nitric acid, Lead 1mg/L 2% nitric acid and Mercury 1mg/L in 10% nitric acid. L-Buthionine Sulfoximine (LBSO) was purchased from Toronto Research Chemicals (North York, ON Canada). Rhodamine 123 fluorescent dye (Sigma; Ex/Em=507nm/529nm), 2, 7-

Dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen Molecular Probes, Eugene, OR). 5-chloromethylfluorescein diacetate (CMFDA, Invitrogen Molecular Probes), dihydroethidium (DHE) (Ex/Em = 518 nm/605 nm) (Invitrogen Molecular Probes).

Cell lines and culturing reagent

MCF-7 cell lines were obtained from American Type Culture Collection (ATTC) (Manassas, VA). Minimum Essential Medium (MEM) alpha 1x, Dulbecco's Phosphate Buffered Saline (PBS), MEM without phenol, and Penicillin Streptomycin were obtained from GIBCO Invitrogen (Grand Island, NY). Trypsin-EDTA and Fetal Bovine Serum (FBS) were obtained from ATTC (Manassas, VA).

Chemical Preparations

To prepare 100 µg/ml Stock solutions of each heavy metal, 1ml from 1mg/ml solution of each metal was added to 9mls of PBS respectively. 50µl of the stock solution was added to 180µl of media to obtain a final concentration of 21.7µg/ml. To prepare the quaternary mixture of all four metals, 1ml each was taken from the 100 µg/ml stock solutions of all four heavy metals to make a total of 4ml. 200µl of this mixture was then added to 30µl of media to give a final concentration of 21.7µg/ml for the mixture. 1µg/ml of H_2O_2 was used as positive control for the production of non-specific ROS and superoxide anion

Cell culture

MCF-7 cells were cultured in MEM supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin in six well plates using general techniques for cell cultures described in Tchounwou et al. [17]. Cells were incubated at 37 ^{o}C in a 5% CO_2 Incubator. The cells were harvested with a solution of trypsin-EDTA while in their logarithmic phase of growth and maintained in these culture conditions for all experiments.

Inhibition of cellular glutathione with LBSO

LBSO irreversibly inhibits gamma glutamylcysteine synthetase, which is the rate limiting enzyme of GSH synthesis and thereby inhibits GSH synthesis Anderson and Reynolds [18]. To determine the appropriate concentration of LBSO that inhibits GSH synthesis, MCF-7 cells were treated with a concentration range (0.5mM - 20mM) of LBSO in a 96-well cell culture plate. The effective concentration which inhibited GSH Synthesis and did not kill more than 5% of the cells was determined at 2.5mM. To inhibit cellular GSH, MCF-7 cells were incubated in 2.5mM of LBSO in MEM supplemented with 10% FBS and 1% penicillin streptomycin for 24 hours.

5 hr Exposure Studies

Previous studies by Egiebor et al. [16], showed that cell death became evident after a 5 hr cell exposure to the high concentrations (21.7 µg/ml) of each metals studied. This exsposure time was therefore used in the current studies to determine the molecular pathway of cell death at 5 hr. MCF-7 cells were exposed to concentration of 21.7µg/ml of each metal singly and as a quaternary mixture of all four of the heavy metal for 5 hr. The exposure was repeated when glutathione production was inhibited by pre-treating MCF-7 cells with 2.5mM L-Buthionine sulphoximine (LBSO) for 24 hr.

Measurement of intracellular glutathione (GSH) content in MCF-7 cells and LBSO pretreated MCF-7 cells.

GSH levels were analyzed in MCF-7 cells and in LBSO pretreated MCF-7 cells using 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes) Han et al. [19]. Cells were exposed to 21.7 µg/ml of the individual metal and the quaternary mixture of the metals (As, Cd, Hg, Pb and Mix) and subsequently exposed to CMFDA dye for 45 min. The procedure was repeated using cells that were pretreated with LBSO for 24 hr. CMFDA fluorescence intensity was determined using a FACScalibur flow cytometer (Becton Dickinson) and calculated with Cell Quest pro software. 10,000 events were collected for each sample.

Measurement of mitochondrial membrane potential (MMP) in MCF-7 cells and LBSO pretreated MCF-7 cells.

The mitochondrial membrane potential was measured using the Rhodamine 123 fluorescent dye. This is a cell-permeable lipophilic cationic dye which distributes electrophoretically into the mitochondrial matrix and fluoresces due to the trans membrane potential that the organelles maintain. MMP can be determined by the florescence intensity due to the uptake of the dye. Loss of MMP will result in loss of the dye and therefore, the fluorescence intensity Scaduto and Grotyohann, [20]. Briefly, cells were exposed to 21.7 µg/ml of the individual and the quaternary mixture of the metals (As, Cd, Hg, Pb and Mix) and subsequently exposed to the Rhodamine 123 fluorescent dye for 45 min. The same procedure was repeated using cells that were pretreated with LBSO for 24 hr. Rhodamine 123 fluorescence intensity was determined using a FACScalibur cytometer (Becton Dickinson) and calculated with CellQuest pro software. 10,000 events were collected for each sample.

Measurement of intracellular nonspecific ROS and O_2 Concentration in MCF7 cells and LBSO pretreated MCF-7 cells.

Intracellular nonspecific ROS such as H_2O_2, •OH and ONOO• were measured using the oxidation-sensitive fluorescent probe dye, 2, 7-Dichlorodihydrofluorescein diacetate

(H2DCFDA) (Invitrogen Molecular Probes, Eugene, OR), H2DCF-DA, a permeable dye, is cleaved to form non-fluorescent dichlorofluorescein (DCFH) in the cells, and is oxidized to fluorescent dichlorofluorescein (DCF) by ROS. To study intracellular superoxide anion, Dihydroethidium (DHE) (Ex/Em = 518 nm/605 nm) (Invitrogen Molecular Probes), a fluorogenic probe, which is highly selective for O_2• among ROS was used. DHE permeates the cell and reacts with superoxide anion to form ethidium, which then reacts with deoxyribonucleic acid, to give red fluorescence. In this study, cells were exposed to 21.7 µg/ml of the individual and quaternary mixture of the metals (As, Cd, Hg, Pb, Mix and H_2O_2). The cells were subsequently exposed to H_2DCFDA and DHE fluorescent dye respectively for 45 min. The procedure was repeated using cells that were pretreated with LBSO for 24 hr. For each sample, 10,000 events were collected. ROS and O_2• levels were calculated with CellQuest pro software.

Statistical Analyses

The results represent the mean of three independent replicates. Microsoft Excel was use to analyze the data. Analyses of variance (ANOVA) with post hoc analysis using Tukeys HSD test was carried out. Statistical significance was defined as $p < 0.05$.

Results

Effects of LBSO and/or metals and mixture on mitochondria membrane potential (MMP) in MCF-7 cells

After 5 hr of exposure (Fig. 1a), As, MIX and Pb induced significant decreases in mitochondria membrane potential in MCF-7 cells. Cells treated with Hg and Cd on the other hand showed significant increases in the mitochondria membrane potential. In contrast, when cellular GSH was inhibited by pretreating the cells with LBSO (Fig. 1b), all five treatments induced significant reduction of the mitochondria membrane potential of MCF-7 cells. Hg induced the most effect on the cellular MMP followed by the quaternary mixture and Cd respectively.

Effects of LBSO and/or metals and mixture on intracellular nonspecific ROS production in MCF-7 cells

Assays to determine the effects of the four individual chemicals and their quaternary mixture on ROS production in MCF-7 cells using H_2DCFDA fluorescence dye showed ROS production in cells exposed to Cd, Hg, and mixture of all four metals (Fig. 2a). Hg stimulated the most production of ROS Its ROS production was significantly higher than the positive control (H_2O_2) and was about four times higher than the control. ROS production in both cadmium and mixture

were also significantly higher than the control. Cells exposed to As and Pb did not show any significant ROS production. When cells were pretreated with LBSO, the results (Fig. 2b) showed that cell exposed to Pb and Cd induced a significant production of ROS. The production of ROS in LBSO pretreated cells exposed to Pb was significantly higher than that produced by the positive control. In contrast, Hg, As and Mix did not induce any significant ROS production (Fig. 2b) in LBSO pretreated cells.

Effects of LBSO and/or metals and mixture on the production of superoxide anions ($O_2^{\bullet}$) in MCF7 cells.

The results of the effects of the heavy metals and their mixture on superoxide anion ($O_2^{\bullet}$) production using DHE dye are shown in Fig. 3a. Hg, Cd, and Mix induced significant production of $O_2^{\bullet}$. The production of $O_2^{\bullet}$ was similar in cells exposed to Hg and the positive control (H_2O_2). Cells exposed to As and Pb did not induce any significant production of $O_2^{\bullet}$. When cellular GHS was scavenged (Fig 3b), there were significant production of $O_2^{\bullet}$ in cells exposed to Cd, As, and Pb. No significant production of superoxide anions was observed in cells exposed to Hg and the Mix.

Effects of LBSO and/or metals and mixture on glutathione production in MCF7 cells.

In the presence of cellular GSH, the production of GSH in cells exposed to Cd and Hg were similar and significantly higher than the control (Fig. 4a). However, MCF 7 cells exposed to Pb did not induce a significant production of cellular GSH. Cells exposed to As and Mix were similar to the control. When MCF 7 cells were treated with LBSO before being exposed to metal treatments (Fig. 4b), it was observed that all five treatments induced significant decreases in basal GSH as compared to the control.

Discussion

Oxidative stress is a situation that occurs when the production of reactive oxygen species or free radicals is greater than the body's ability to detoxify the reactive intermediates. This imbalance leads to oxidative damage to proteins, molecules, and genes within the body. Loss of MMP is a biomarker for oxidative stress and it occurs when the electrochemical gradient across the mitochondria membrane in a cell collapses. Studies have shown that the loss of MMP due to metal intoxication is an early event in mitochondria-mediated apoptosis Takahashi et al. [21]. Results of this study indicates that arsenic, lead, and the mixture of all four metals induced the loss of mitochondria membrane potential in MCF-7 cells. This agrees with the findings of other studies that have shown that heavy metals such as As Baysan et al. [22] and Pb Pal et al. [23] can

damage the mitochondria membrane in different cell lines. Studies by Kumar et al. [24] noted that in leukemia cells, arsenic trioxide can activate the intrinsic pathway of cell death by modulating the expression and translocation of apoptotic molecules thus decreasing the mitochondrial membrane potential.

Metals can upset the oxidation-reduction equilibrium in cells and cellular equilibrium disturbance can lead to increased ROS production Tchounwou. [25]. Oxidative stress has been considered as one of the major mechanisms behind heavy metal toxicity. Heavy metals produce free radicals which have the ability to cause lipid peroxidation, DNA damage, oxidation of sulfhydryl groups of proteins, and several other effects Valko [5]. Studies have shown increased ROS levels during metal-induced cell death in acute promyelocyte leukemia (APL), acute myeloid leukemia Uslu et al. [26] and cervical cancer cells Kang et al. [27]. Our studies indicated a significant increase in nonspecific ROS and superoxide anions production in cells exposed to Cd, Hg, and the mixture of all four metals. This finding is in accordance with the results of studies by Szuster-Ciesielska [28] who showed that Cd induced the production of ROS in cell cultures. Some studies have indicated the production of ROS by cells exposed to arsenic Shi et al. [29] and lead Pal et al. [23] However, our results indicate that cells exposed to arsenic and lead did not produce significant amount of ROS and superoxide anions. This is in agreement with the results of studies by Han et al. [30] which did not show any increase in ROS production when A549 cells were exposed to high doses (20, 30 or 50 μM) of arsenic trioxide. Similarly, Stacchiotti et al. [31] did not find any increased production of ROS in NRK-52E cell line exposed to lead, even at high concentration (20um). They suggested that the low level of nonspecific ROS and superoxide anion production in Pb exposed cells may be because lead's affinity to SH-groups is not as strong as that of other heavy metals (As, Hg and Cd).

Apart from inducing oxidative stress, exposure to heavy metals share several primary mechanisms of toxicity, including reaction with intracellular thiols and changes in mitochondrial membrane potential Wang and Fowler [32]. If the cells are not eliminated by apoptosis or necrosis, they may be able to express a series of events that favor their survival. Cellular GSH has been shown to be crucial for cell proliferation, cell cycle progression and apoptosis Schnelldorfer et al. [33]. The intensity of CMF fluorescence has been shown to be well correlated with biochemically estimated content of GSH in the cell Chikahisa et al. [34] The synthesis of antioxidant molecules such as GSH represents a mechanism of cell protection against heavy metal intoxication Sabolic [35]. GSH is a non-protein tripeptide which serves as a natural antioxidant and reducing agent. It helps protects the body systems from the effects of ROS Iwama et al., [36]. The results of this study indicated that when cellular GSH was intact, cells exposed to Cd and Hg elicited increased production of GSH. These findings are in accordance with previous studies which showed that heavy metals such as Cd and Hg induced an increase in the concentration of GSH in mammalian cells Lash and Zalups [37] and fish tissues Thomas and Juedes [38]. Cell exposure to heavy metals induce the production of ROS and it is speculated that increased production of ROS and superoxide anion may lead to a corresponding increase in the cellular GSH production in an attempt to attain cellular equilibrium.

When cells were pretreated with LBSO before metal exposure, the production of ROS was significant in cells exposed to Cd and Pb only. However, all treatments induced significant

decrease in the mitochondria membrane potential as well as basal GHS. The decrease in MMP and basal GHS was particularly obvious in cells exposed to Hg and the quaternary mixture. Research has shown that Hg has one of the strongest affinities for GSH and is able to form Hg-GSH complex, Franco et al. [39]. A single Hg ion can bind to and cause irreversible excretion of two GSH molecules. The release of GSH-Hg conjugates result in stronger activity of the free Hg ions disturbing GSH metabolism, and ultimately cell death Franco et al. [39]. Results of this study revealed that the heavy metals and mixture studied were more toxic when cellular glutathione was inhibited

This study showed that, heavy metals induce oxidative stress via different mechanisms. Primarily, arsenics and lead induced cytotoxicity by reducing the mitochondria membrane potential while Cd, and Hg were cytotoxic by the production of mostly superoxide anions and nonspecific ROS. The mechanism of the mixture induced oxidative stress includes damage to mitochondria membrane, as well as superoxide anion and ROS production. When the synthesis of cellular glutathione was inhibited, all five treatments damaged the mitochondria membrane and depleted basal GSH. Cd, Pb, and As also elicited the production of ROS. This study is the first to show the possible oxidative stress mechanism induced by four metals and their quaternary mixture (As, Cd, Hg, Pb and Mix) on MCF-7 breast cancer cell line.

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List of Figure

Figure 1a. Effects of individual metals and their quaternary mixture on mitochondria membrane potential after 5hr of exposure. Cells were exposed to heavy metals and their mixtures at the concentration of 21.7ppm. Mitochondria membrane potential was analyzed using flow cytometry and the bar chart represents the mean and error of three replicates. Treatments with the same letters are not significantly different. $P \leq .05$.

Figure 1b. Effects of individual metals and their quaternary mixtures on mitochondria membrane potential of LBSO Pretreated MCF7 cells after 5hrs of exposure. Cells were pretreated with LBSO then exposed to heavy metals and their mixtures of 21.7ppm. mitochondria membrane potential was analyzed using flow cytometry and the bar chart represents the mean and error of three replicates. Treatments with the same letters are not significantly different. $P \leq .05$.

Figure 2a Effects of individual metals and their quaternary mixtures on nonspecific ROS production after 5hrs of exposure. Cells were exposed to heavy metals and their mixtures at the concentration of 21.7ppm. MCF7 cells were stained with H2DCFDA 123 and analyzed by flow cytometry and the bar chart represents the mean and error of three replicates. Treatments with the same letters are not significantly different. $P \leq .05$.

Figure 2b. Effects of individual metals and their quaternary mixtures on nonspecific ROS production in LBSO pretreated MCF7 cell after 5hrs of exposure. Cells were pretreated with LBSO then exposed to heavy metals and their mixtures at the concentration of 21.7ppm. Mitochondria membrane potential was analyzed using flow cytometry and the bar chart represents the mean and error of three replicate. Treatments with the same letters are not significantly different. P≤.05.

Figure 3a. Effects of individual metals and their quaternary mixtures on superoxide anion production after 5hr of exposure. Cells were exposed to heavy metals and their mixtures at the concentration of 21.7ppm. Superoxide anion was analyzed using flow cytometry and the bar chart represents the mean and error of three replicates. Treatments with the same letters are not significantly different. P≤.05.

Figure 3b. Effects of individual metals and their quaternary mixtures on superoxide anions production in LBSO pretreated MCF7 cell after 5hrs of exposure. Cells were pretreated with LBSO then exposed to heavy metals and their mixtures at the concentration of 21.7ppm. superoxide anion was analyzed using flow cytometry and the bar chart represents the mean and error of three replicates. Treatments with the same letters are not significantly different. P≤.05.

Figure 4a. Effects of individual metals and their quaternary mixtures on glutathione production after 5hrs of exposure. Cells were exposed to heavy metals and their mixtures at the concentration of 21.7ppm. Cellular GSH was analyzed using flow cytometry and the bar chart represents the mean and error of three replicates. Treatments with the same letters are not significantly different. P≤.05.

Figure 4b. Effects of individual metals and their quaternary mixtures on cellular Glutathione production in LBSO pretreated MCF7 cell after 5hrs of exposure. Cells were pretreated with LBSO then exposed to heavy metals and their mixtures at the concentration of 21.7ppm. GSH was analyzed using flow cytometry and the bar chart represents the mean and error of three replicates. Treatments with the same letters are not significantly different. P≤.05.

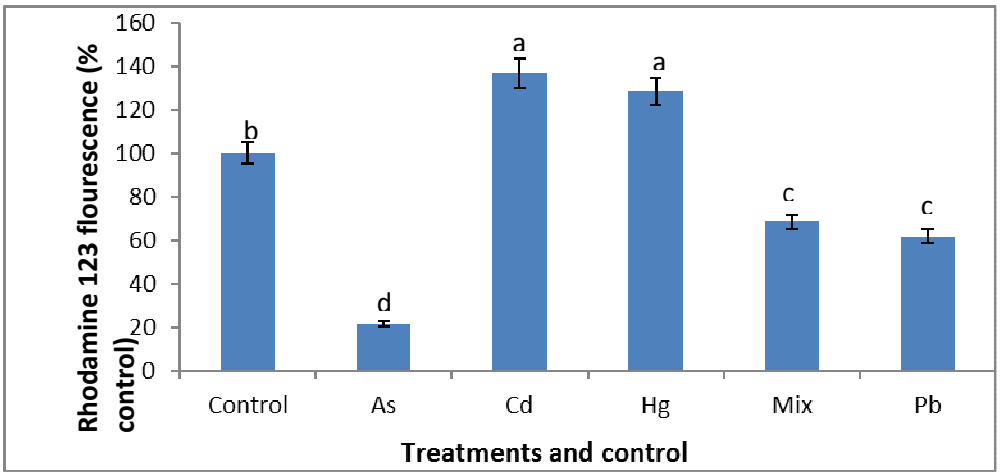
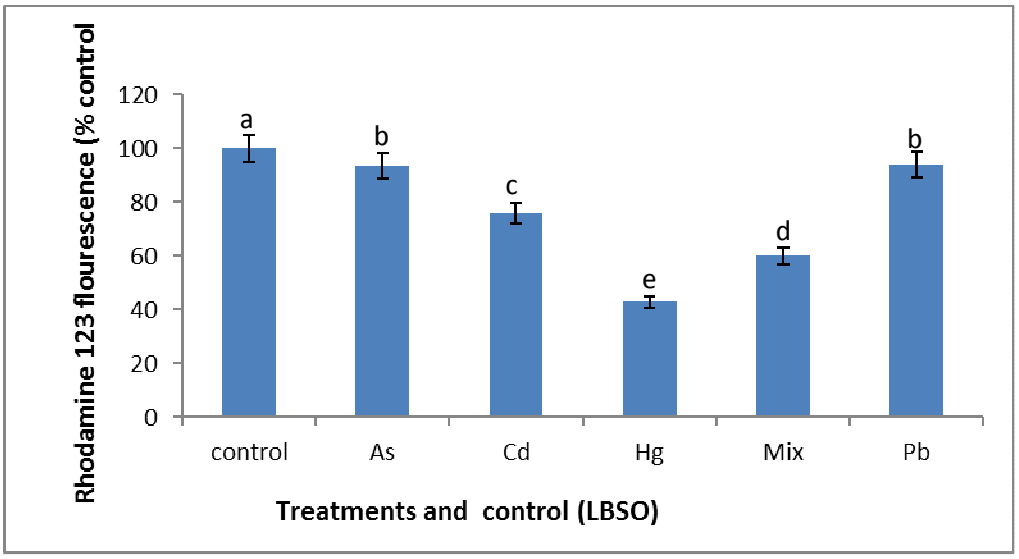


Figure 1a.



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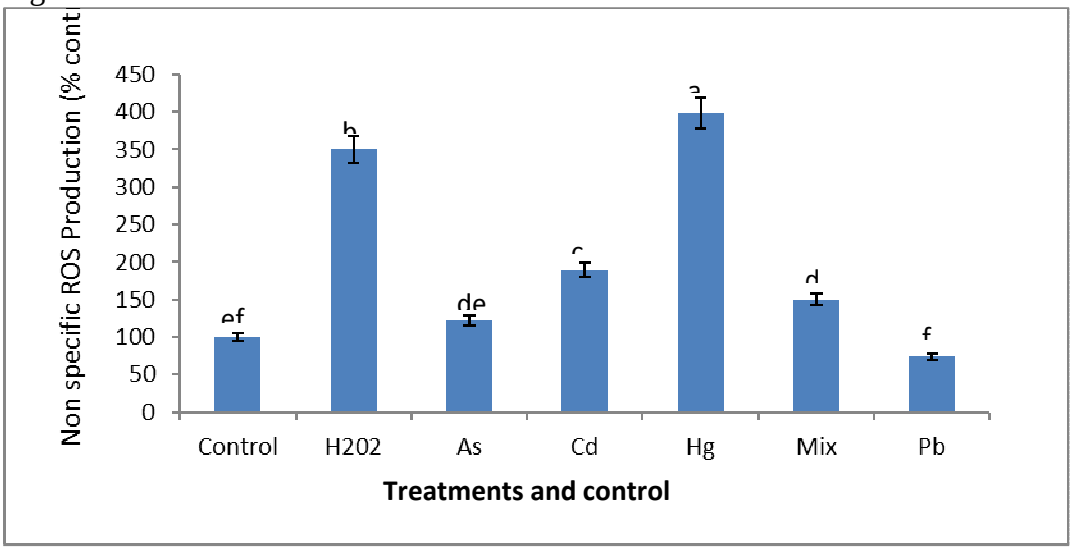

Figure 2a.

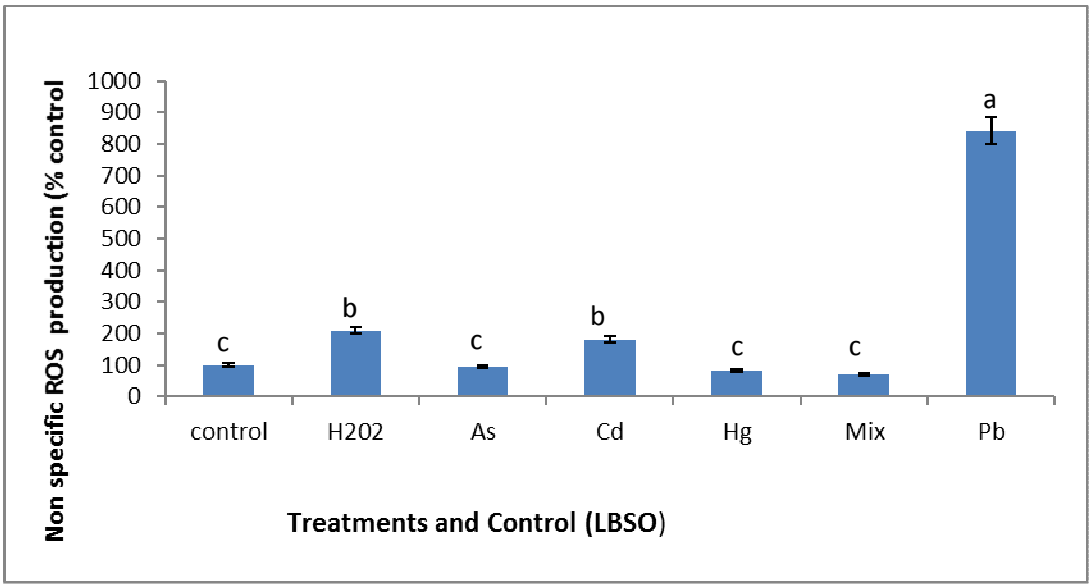

Figure 2B.

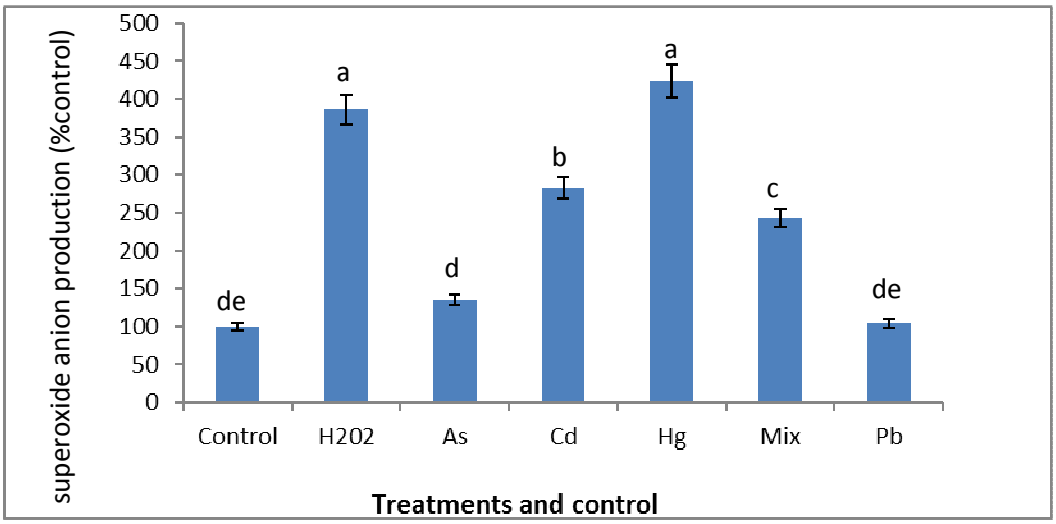

Figure 3a.

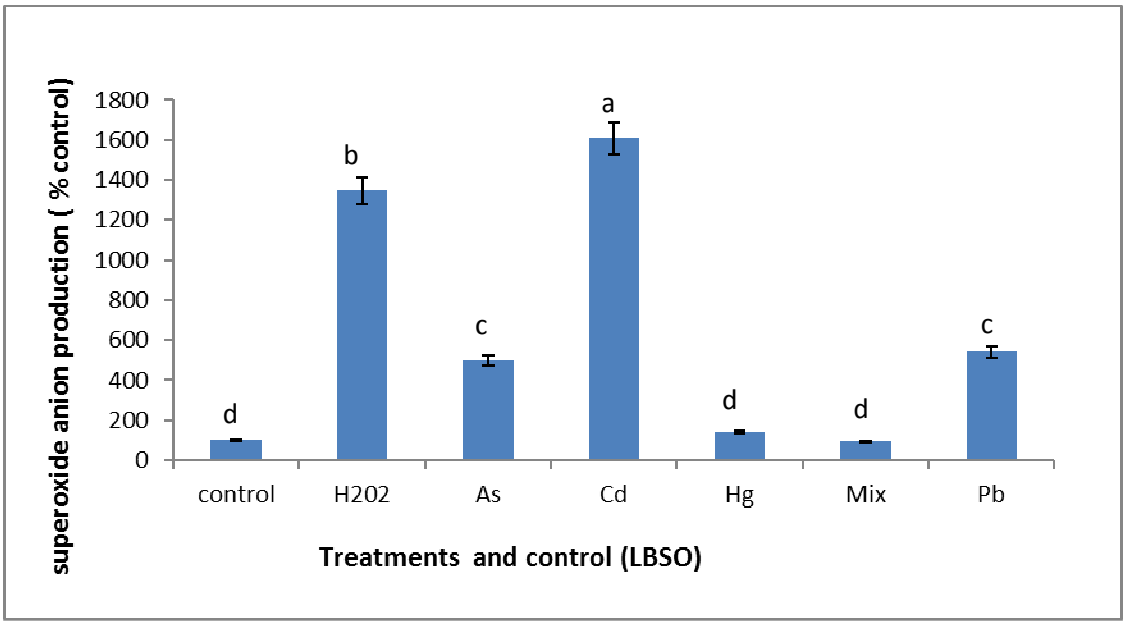

Figure 3b.

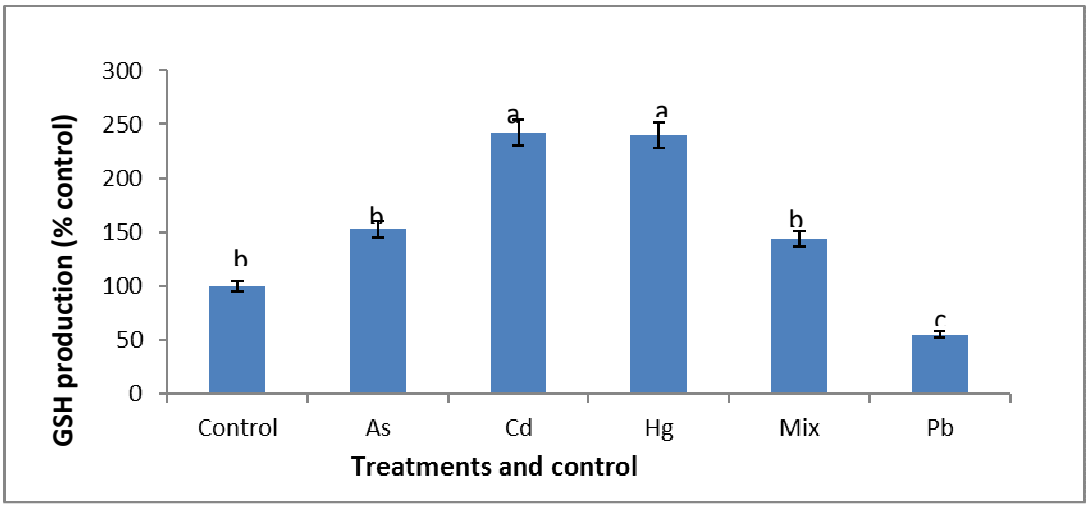

Figure 4a.

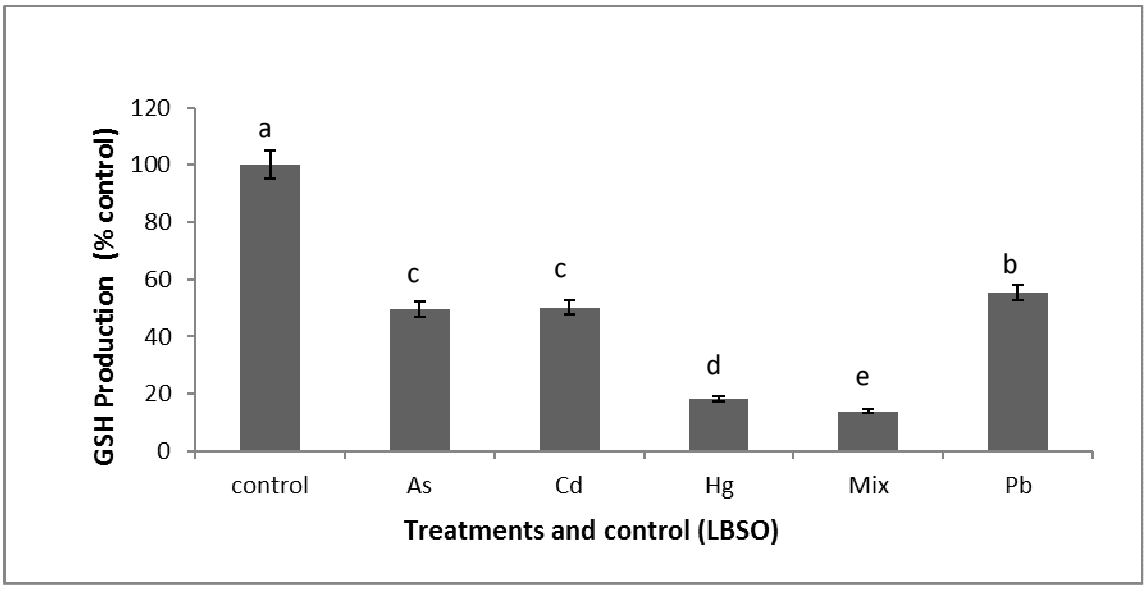
GSH Production (% control)
120
100
80
60
40
20
0
a
c
c
d
e
b
control
As
Cd
Hg
Mix
Pb
Treatments and control (LBSO)

Figure 4B.