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, but 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 their 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 mixture and assayed after 5 hr. Cellular levels of non specific ROS, superoxide anion ($O_2^{\bullet}$), mitochondria membrane potential (MMP), and GSH were assayed using the 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 Non specific 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. 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], and 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 apoptosis and redox signaling (Ryter et al.[4] on organisms as well as cause ecological disturbances. In addition, some heavy metals are considered priority pollutants due to their biological and ecological effects ATSDR, 2014 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 repeatedly 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, therefore 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 might 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 seen in single chemical exposures.

Numerous literatures include investigations on the effects of a single element on a selected cell line. Studies comparing several heavy metals and their mixture acting on the same cell line are few. In our previous study Egiebor et al. [16], we 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. In the study, it was noted 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. Hence, In order to understand the underlying molecular mechanism(s) induced by the metals and their mixture, this study investigated the cellular regulation of ROS, superoxide anions (O_2•), GSH and MMP using MCF-7 breast cancer cells in the presence and inhibition of cellular GSH

Materials and methods

Chemicals

The following chemicals were purchased 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 purchased 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 purchased from GIBCO Invitrogen (Grand Island, NY). Trypsin-EDTA and Fetal Bovine Serum (FBS) were purchased 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 get 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 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 methodology

MCF-7 cells were cultured in MEM supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin in six well plates following 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, 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 to be 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

From our previous study Egiebor et al. [16], cell death became evident after a 5 hr cell exposure to the high concentrations (21.7 µg/ml) of each metals studied. Hence, we studied the molecular pathway of cell death at 5 hr. Briefly, 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. This same 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]. Briefly, cells were exposed to 21.7 µg/ml of the individual metal and quaternary mixture of the metals (As, Cd, Hg, Pb and Mix) and subsequently exposed to CMFDA dye for 45 min. This same 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 (Sigma; Ex/Em = 507 nm/529 nm), which is a cell-permeable lipophilic cationic dye that is taken up by mitochondria in proportion to the change in mitochondria membrane potential (MMP) (Scaduto and Grotyohann, 1999). Briefly, cells were exposed to 21.7 µg/ml of the individual and quaternary mixture of the metals (As, Cd, Hg, Pb and Mix) and subsequently exposed to the Rhodamine 123 fluorescent dye for 45 min. This 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 like 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, which gets 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 reacts with deoxyribonucleic acid, to give red fluorescence. For 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. This same 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 decrease in mitochondria membrane potential in MCF-7 cells, but cells treated with Hg and Cd did not show any decrease in MMP; instead, there was a significant increase 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

Assay to determine the effects of the four individual chemicals and their quaternary mixture on ROS production in MCF-7 cells was performed using H_2DCFDA fluorescence dye. ROS production was observed in cells exposed to Cd, Hg, and mixture (Fig. 2a) of all four metals. Hg stimulated the most production of ROS; its ROS production was about four times higher than the control and significantly higher than the positive control (H_2O_2). 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 ROS in Pb exposed LBSO pretreated cells 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).

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

To study the effects of the heavy metals and their mixture on superoxide anion ($O_2^{\bullet}$) production, the DHE dye was used. From the results (Fig. 3a), Hg, Cd, and Mix induced significant production of $O_2^{\bullet}$. The production of $O_2^{\bullet}$ were similar in cells exposed to Hg and the positive control (H_2O_2). Cell exposure 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 were observed in cells exposed to Hg and the mMix.

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

Cellular GSH has been shown to be crucial for cell proliferation, cell cycle progression and apoptosis Schnelldorfer et al.[20]. The intensity of CMF fluorescence has been shown to be well correlated with biochemically estimated content of GSH in the cell Chikahisa et al. [21]. In the presence ot 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 they were exposed to metal treatments (Fig. 4b), it was noticed that all five treatment induced significant decreases in basal GSH as compared to the control.

Discussion

Oxidative stress occurs as a result of disturbance in the balance between the production of ROS and antioxidant defenses. Loss of mitochondria membrane potential is a biomarker for oxidative stress and it occurs when the electrochemical gradient across the mitochondria membrane collapses. Studies have shown that the loss of MMP due to metal intoxication is an early event in mitochondria-mediated apoptosis Takahashi et al. [22]. Our result 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 researcher who indicated that heavy metals like Arsenic Baysan et al. [23] and lead Pal et al. [24] damaged the mitochondria membrane in different cell lines. Studies by Kumar et al. [25] noted that arsenic trioxide can

activate the intrinsic pathway of cell death by modulating the expression and translocation of apoptotic molecules and decreasing the mitochondrial membrane potential in leukemia cells.

Metals can upset the oxidation-reduction equilibrium in cells and cellular equilibrium disturbance can lead to increased ROS production Tchounwou. [26]. Studies have indicated increased ROS levels during metal-induced cell death in acute promyelocyte leukemia (APL), and acute myeloid leukemia Uslu et al. [27] cervical cancer cells Kang et al.[28]. Our studies indicate a significant increase in non specific 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 [29] 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. [30] and lead Pal et al. [24] 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 works by Han et al.[31] which did not reveal any increase in ROS production when high doses (20, 30 or 50 μM) of arsenic trioxicde was exposed to A549 cell. Stacchiotti et al. [32] also did not find increased production of ROS in NRK-52E cell line exposed to lead, even at high concentration (20um). They suggested that the low level of non specific 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).

Exposure to heavy metals share several primary mechanisms of toxicity, including oxidative stress, reaction with intracellular thiols and changes in mitochondrial membrane potential Wang and Fowler [33]. If the cells are not eliminated by apoptosis or necrosis, they may be able to express a series of events that favor their survival. The synthesis of antioxidant molecules like GSH represents a mechanism of cell protection against heavy metal intoxication Sabolic [34] 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., [35]. Our study investigated the effects of heavy metals on the cells in the presence and absence of GSH. Our results indicate that when cellular GSH was intact, cells exposed to Cd and Hg elicited increased production of GSH. This finding is in accordance with previous studies which showed that heavy metals such as Cd and Hg induced increase in the concentration of GSH in mammalian Lash and Zalups [36] and fish tissues Thomas and Juedes [37]. Cell exposure to heavy metals induce the production of ROS and it is speculated that increased production of ROS and superoxide anion may have lead to a corresponding increase in the cellular GSH production; the cell's attempt to attain 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. 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. [38]. 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. [38].This research

revealed that the heavy metals and mixture studied were more toxic when cellular glutathione was inhibited

In conclusion, 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 Non specific ROS. The mechanism of the mixture induce oxidative stress include 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. Our group is the first to study 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≤.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≤.05.

Figure 2a Effects of individual metals and their quaternary mixtures on non specific 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≤.05.

Figure 2b. Effects of individual metals and their quaternary mixtures on non specific 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.

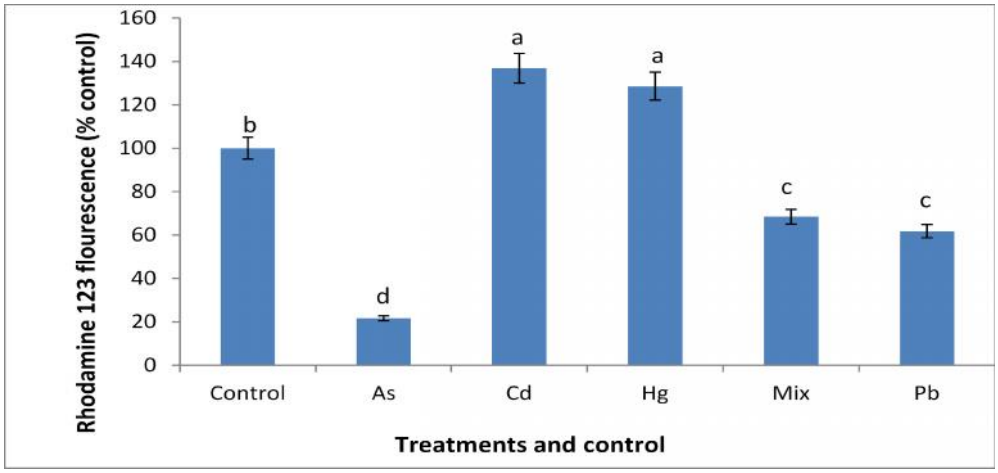
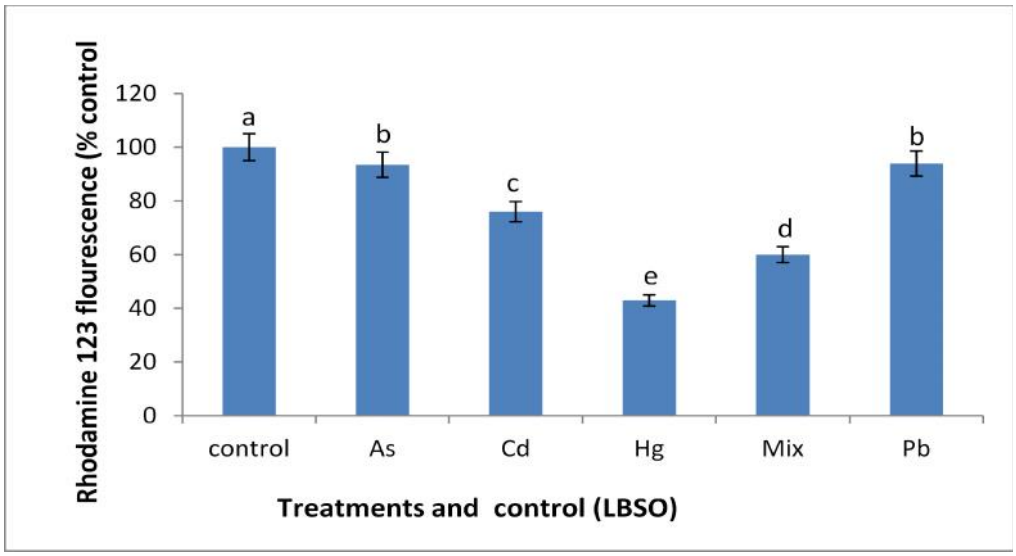


Figure 1a.



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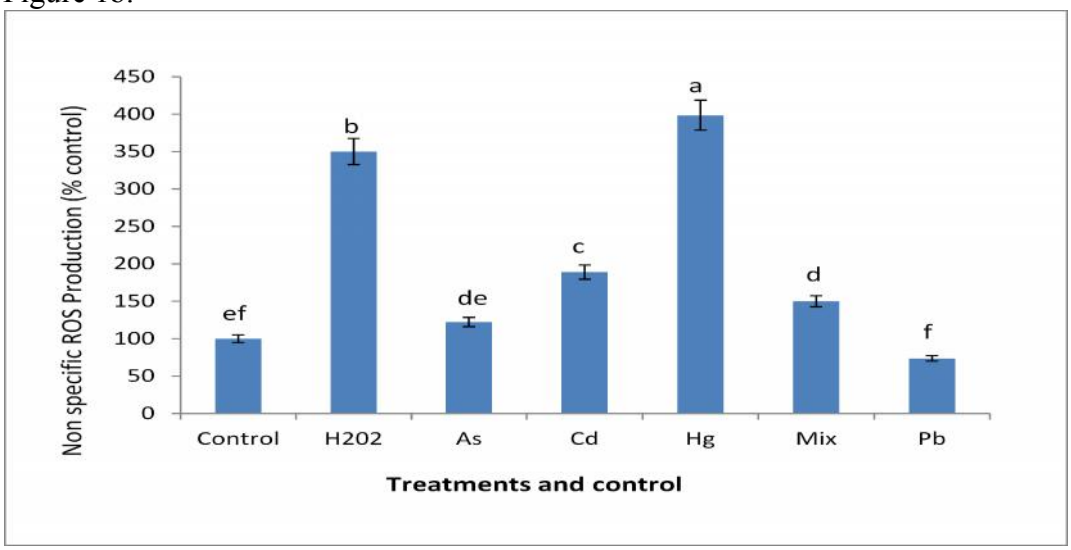

Figure 2a.

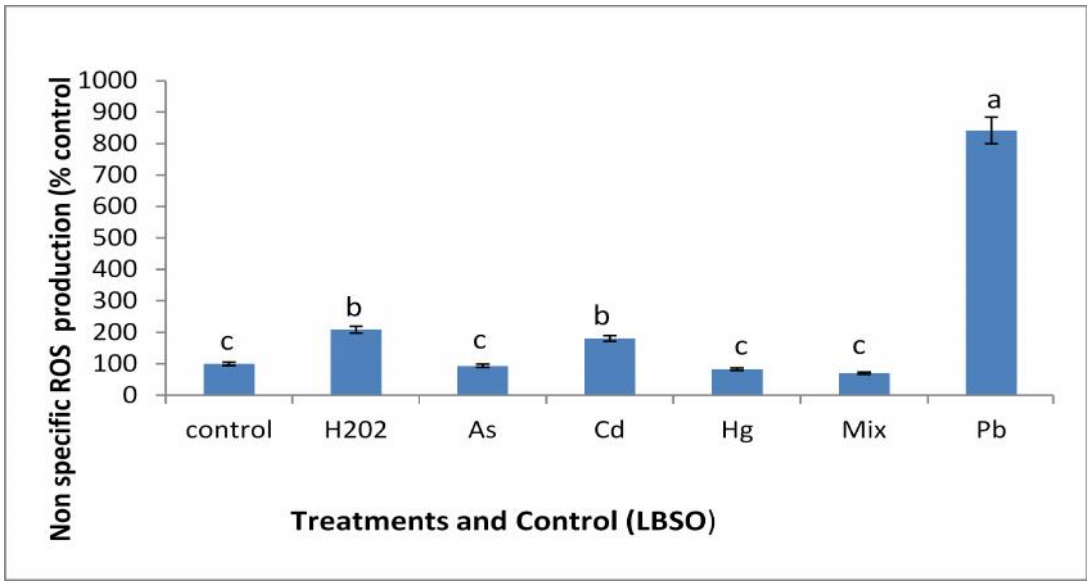

Figure 2B.

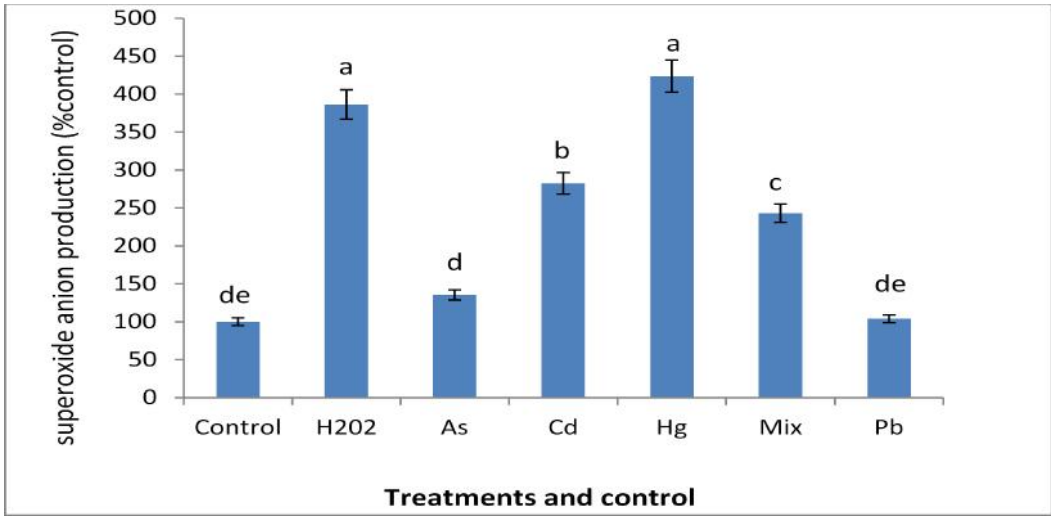

Figure 3a.

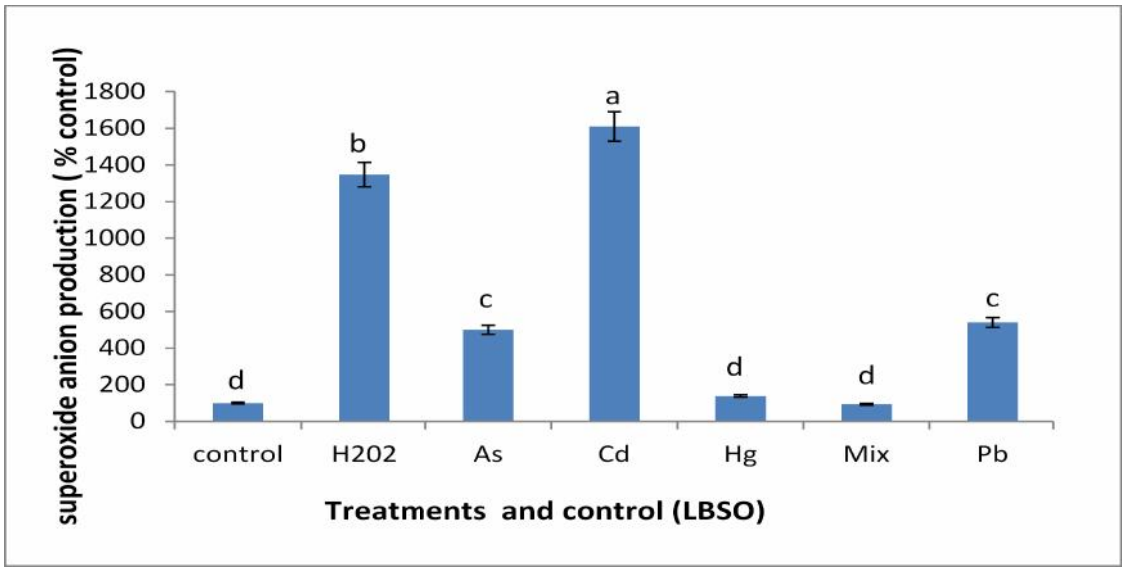

Figure 3b.

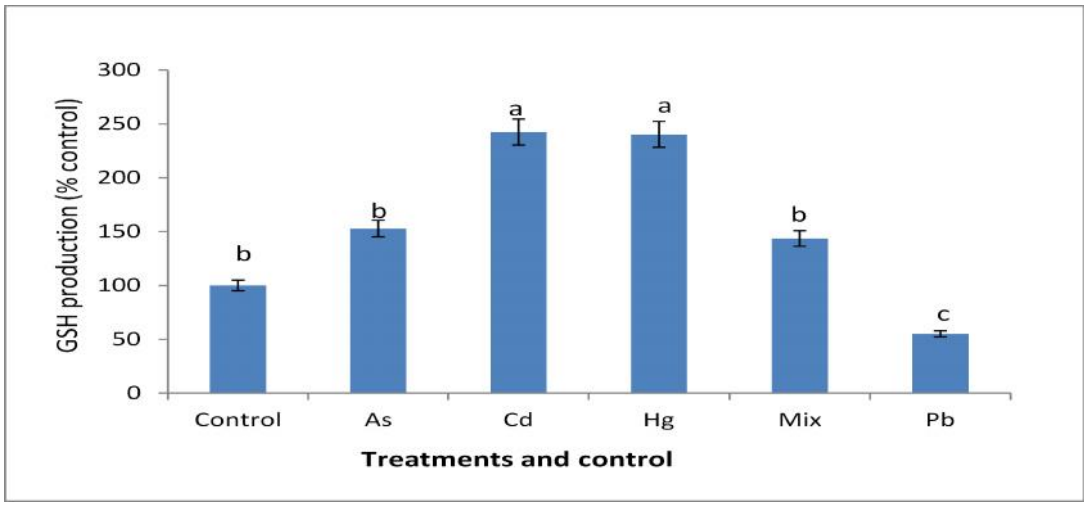

Figure 4a.

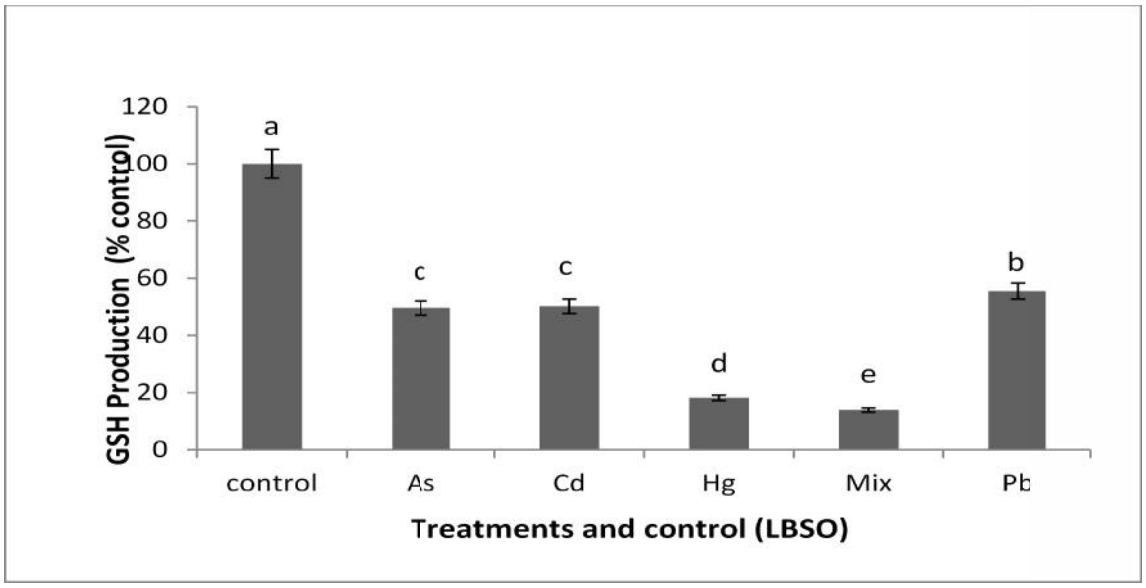
GSH Production (% control)
120
100
80
60
40
20
0
a
c
c
d
e
b
control
As
Cd
Hg
Mix
Pb
Treatments and control (LBSO)

Figure 4B.