The role of some heavy metal pollution in idiopathic recurrent spontaneous abortion

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Research Article

Keywords: antioxidants, heavy metals, lipid peroxidation, mussels, pregnancy

Posted Date: November 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2264562/v1

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Abstract

Background: Environmental pollution with heavy metal (HM) have been associated with human reproductive failure. Oxidative stress and disruption of homeostatic regulation of reproductive hormones has been implicated in HM induced reproductive toxicity. The HM, indices of oxidative stress, reproductive hormone and red cell indices in women with recurrent pregnancy loss (RPL) in relation to exposure to HM contaminated mussels were assessed in this study.

Methods: Seventy-six women (20-35 years) categorized into 18 fertile women without RPL (control group), and Groups I, II and III comprising 24, 18 and 16 women with RPL (2, 3, and >3 abortions respectively) were studied. Whole blood samples were collected for the estimation of cadmium (Cd), lead (Pb), metallothionein (rbcMT), malondialdehyde (MDA), reduced glutathione (GSH), progesterone, haemoglobin (Hb), mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH). Mussel samples were collected for Cd, Pb and metallothionein (MT) estimation.

Results: The cadmium content of mussels were above the maximum limit. Women with RPL (Groups I-III) had higher Cd, Pb, rbcMT and MDA and lower catalase, GSH, P4, Hb, MCV and MCH compared to Group I (p=<0.001). Negative associations were observed between Cd and catalase (r=-0.320, p=0.014), GSH (r=-0.359, p=-0.006) and MCV (r=-0.391, p=-0.002) respectively and between Pb and GSH (r=-0.501, p=<0.001), Hb (r=-0.289, p=0.028), MCV (r=-0.394, p=0.002) and MCH (r=-0.277, p=0.036) respectively in women with RPL. Positive correlations were observed between Cd and Pb (r=0.533, p=<0.001), rbcMT with Cd (r=0.312, p=0.017), Pb (r=0.488, p=<0.000) and MDA (r=0.282, p=0.032) respectively in women with RPL.

Conclusion: Elevated cadmium levels in mussels in conjunction with increased HM, metallothionein, MDA and reduced antioxidants, progesterone and red cell indices observed in women with RPL suggest that HM induced oxidative stress and hormonal imbalance may be implicated in recurrent pregnancy loss.

Introduction

Disposal of industrial waste products into water bodies has led to pollution of aquatic environment with heavy metals which deposits and accumulate in sediments and bodies of marine life and subsequently through the food chain eventually reach humans (Amalo LF et al., 2021). Aquatic organisms as the green mussels because of their low mobility and filter feeding characteristics are targets for accumulation and contamination with pollutants in the aquatic habitat (Rusydi, Savira, Putra, Dewiyanti, & Defira, 2021). The HM content of mussels has been shown to be a reflection of the heavy metal content of their habitat, hence their utilization as bioindicators in monitoring environmental contamination with HM (Amalo LF et al., 2021; Rusydi et al., 2021). Consumption of heavy metal contaminated sea food has been associated with deleterious health consequences including reproductive impairment at the genetic, epigenetic, and biochemical levels (Dutta, Gorain, Choudhury, Roychoudhury, & Sengupta, 2021; Salamat, Movahedinia, Etemadi-Deylami, & Mohammadi, 2015). These metals impact upon the female reproduction at all stages of its regulation and function of development, maturation, or endocrine functions, and are linked to the increasing cases of infertility and recurrent pregnancy loss in women (Dutta et al., 2021). Loss of pregnancies consecutively (three or more times) before 20 weeks gestation in the first trimester with fetal weight >500 g is defined as recurrent pregnancy loss (RPL) (Giannubilo et al., 2012). Besides chromosomal abnormalities, genetic factors, uterine and endocrine abnormalities, chronic exposure to heavy metals have been associated with spontaneous abortions, as well as pre-term deliveries and stillbirths. The metalloestrogen cadmium (Cd) has been linked to retardation of fetal development and spontaneous abortions while lead (Pb) levels over a certain threshold have been associated with implantation failure and early pregnancy loss with strong teratogenic impact (Alrashed et al., 2021; Dutta et al., 2021). Elevated Lead and cadmium levels has been associated with premature rupture of placental membranes, premature delivery and miscarriage (MK., 2019; Taylor, Tilling, Golding, & Emond, 2016). Heavy metals have been shown to negatively influence hypothalamic-pituitary-gonadal (HPG) axis thereby causing deleterious alterations in menstrual cycle, ovulation and hormonal homeostasis (Bhatia, Singh, & Kumar, 2015). Mechanisms ranging from disruption of metabolic pathways, displacement of essential elements, inhibition of enzymes and hormone functions, suppression of antioxidant
defense and generation of reactive oxygen species leading to peroxidation of biomolecules and oxidative stress have been described as probable mechanisms of HM induced multiple organ toxicity (Mudgal V, Madaan N, Mudgal A, Singh R, & S., 2010; Rehman, Fatima, Waheed, & Akash, 2018). Oxidative DNA damage accruing from metal induced OS have been implicated in fetal abnormalities and pregnancy termination (Duhig, Chappell, & Shennan, 2016). Higher levels of such HM as antimony and arsenic have been reported in women with RPL (Alrashed et al., 2021). Reduction in antioxidants and increased lipid peroxidation and oxidative DNA damage have been reported in women with recurrent pregnancy loss (Zejnullahu, Zejnullahu, & Kosumi, 2021).

Spontaneous abortion is largely becoming a public health concern for developing countries. Approximately 10% of the clinically diagnosed pregnancies result in spontaneous abortion (MK., 2019). Despite intensive and thorough investigations in this field, most of the RPL cases are with unidentified causes. Several studies have investigated the role of dietary and environmental exposure to HM in the etiology of human reproductive failure including idiopathic recurrent spontaneous abortion based on its well documented association with reproductive toxicity (Alrashed et al., 2021; Bhatia et al., 2015; Dutta et al., 2021). Yet, fewer studies have examined the relationship between exposure to HM contaminated sea food with increasing incidence of recurrent pregnancy loss. Assessment of the HM content of mussels, a bio-indicator of environmental HM contamination in relation to HM levels and other biomarkers of HM induced toxicity in women with idiopathic recurrent abortion may be vital in ascertaining the underlying pathologic mechanism between chronic exposure to HM and idiopathic recurrent spontaneous abortion.

**Subjects And Methods**

**Study design**

This case study involving women with recurrent spontaneous abortions and their corresponding control counterparts was carried out in the coastal area of Alexandria using random sampling method. Informed consent was sought and obtained from the volunteers before recruitment into the study after ethical committee approved the study protocol.

**Selection of subjects**

A total of seventy-six (76) apparently healthy female subjects with an average age 20-35 years were recruited into the study and divided into four groups.

**A) Control subjects:** Comprised of 18 normal healthy fertile women who had no history of recurrent spontaneous abortion. All had at least one living child and their pregnancy proceed successfully giving full term healthy newborn.

**B) Volunteer subjects**

**Group I:** Comprised of 24 women who had experienced at least 2 successive unexplained recurrent spontaneous abortion up to 20 weeks gestational age.

**Group II:** Comprised of 18 women who had experienced at least 3 successive unexplained recurrent spontaneous abortion up to 20 weeks gestational age.

**Group III:** Comprised of 16 women who had experienced at least >3 successive unexplained recurrent spontaneous abortion up to 20 weeks gestational age.

A semi-structure questionnaire was administered to all participants in the study for history of past and present ailment with emphasis on toxoplasmosis, rubella, herpes simplex. Patient/ control women with uterine abnormalities, endocrinal irregularities (diabetes mellitus, thyroid dysfunction), hypertension, liver diseases, urinary tract insult, smoking, working in industrial area with metal exposure were all excluded from the study.
Sample collection

a. Blood Samples

Ten milliliters of venous blood samples were withdrawn from all subjects and collected into two tubes: 6.5 ml of blood were collected into tube containing anticoagulant (EDTA); 1 ml whole blood was used to measure (Haemoglobin, MCH and MCV), 0.5 ml whole blood was used to measure heavy metals (lead and cadmium), 0.5 ml whole blood was used to measure reduced glutathione and the remaining blood was then centrifuged at 4,000 rpm for 15 minutes at 4°C and plasma for catalase enzyme activity assay. 0.5 ml of the separated red blood cells was used to determine metallothionein concentration. The remaining blood was then collected into tube without any anticoagulant and centrifuged at 4,000 rpm for 15 minutes at room temperature and serum separation for the estimation of malondialdehyde and progesterone.

b. Mussel samples:

Mussel samples were collected from Mediterranean Sea "Alexandria coast". The mussel samples were opened raw, and the flesh was scraped out of the shell with plastic scalpel. Mussel gills and digestive glands were rapidly dissected out and then stored at -80°C for determination of cadmium, lead and metallothionein.

Laboratory Methods

Oceanographic studies

Determination of metallothionein concentration in mussel samples.

Metallothionein concentration was evaluated utilizing a partially purified metalloprotein fraction obtained by acidic ethanol/chloroform fractionation of the tissue homogenate following manufacturer's instructions (Viarengo, Ponzano, Dondero, & Fabbri, 1997).

Determination of heavy metals (cadmium and lead) in mussel samples.

Cadmium and lead levels were determined by atomic absorption spectrophotometer. Briefly, each tissue sample of the examined organs was weighed separately in a clean, labeled Petri dish and was dried for several days at 70 °C to constant weight. A dry sample (0.2 g) was placed in a Teflon vessel and 4 ml of analar nitric acid was added. The vessels were tightly covered and were allowed to predigest at room temperature overnight. The digestion vessels were placed on a preheated hotplate at 80 °C for 3 h. The samples were cooled at room temperature and were then transferred to 25 ml volumetric flask. The water used was deionized. All digested solutions were analyzed by Flame Atomic Absorption Spectroscopy (Perkin Elmer Analyst 300, USA). The accuracy of the method was verified using standard reference materials (Christensen, Poulsen, & Anglov, 1992).

Biochemical analysis

Determination of Metallothionein concentration in blood.

Metallothionein concentration was evaluated utilizing a partially purified metalloprotein fraction obtained by acidic ethanol/chloroform fractionation of the blood hemolysate (Grider, Bailey, & Cousins, 1990).

Determination of plasma catalase (CAT) enzyme activity

Catalase enzyme activity was determined by spectrophotometric method. Catalase enzyme reacts with a known quantity of H₂O₂. The reaction is stopped after exactly one minute with catalase inhibitor.
In the presence of peroxidase (HRP), remaining \( \text{H}_2\text{O}_2 \) reacts with 3, 5-Dichloro-2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with a color intensity inversely proportional to the amount of catalase in the original sample (Donald W & E., 1987).

\[
\text{Catalase} \quad \quad 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\]

\[
\text{HRP} \quad 2\text{H}_2\text{O}_2 + \text{DHBS} + \text{AAP} \rightarrow \text{Quinoneimine Dye} + 4\text{H}_2\text{O}
\]

**Estimation of Malondialdehyde (MDA)**

Estimation of MDA was done using the thiobarbituric acid (TBA) assay. Malondialdehyde formed from the breakdown of polyunsaturated fatty acid serves as a convenient index for determining the extent of the peroxidation products in the body. MDA in the sample react with thiobarbituric acid to give a red coloured (MDA-TBA2) whose absorbance was measured at 532 nm. According to (Draper & Hadley, 1990).

**Estimation of Reduced Glutathione (GSH)**

Estimation of GSH was carried out following the modified standard Ellman's method. Briefly, on addition of test serum to Ellman's reagent (5-5'-dithiobis-2-nitrobenzoic acid (DNTB)), the GSH in the sample reacts with Ellman's reagent to form the chromophore 5-thionitrobenzoic acid (TNB) and GS-TNB whose absorbance is measured at 412nm and is proportional to the concentration of GSH in the sample (Beutler, Duron, & Kelly, 1963).

**Determination of Hb, MCV, and MCH.**

All blood samples were measured by a fully automated cell coulter Mindray BC-2800 auto- hematology analyzer (Abraham, 1974).

**Determination of serum Progesterone hormone.**

Serum progesterone estimation was done using enzyme linked immunosorbent assay method which is based on the principle of the specific interaction between antigens and their corresponding antibodies. Upon mixing biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competitive reaction results between the native antigen and the enzyme antigen conjugate for a limited number of antibody binding sites (Aufrère & Benson, 1976).

**Statistical Analysis**

Data were analyzed using IBM SPSS software package version 20.0. The distributions of quantitative variables were tested for normality using Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for vision test. For normally data distribution, parametric tests were applied. For data not normally distributed, non-parametric tests were used. Quantitative data were described using mean and standard deviation for normally distributed data while abnormally distributed data was expressed using median, minimum and maximum. For normally distributed data, analysis of variance and post hoc test was used determine variations among multiple groups means. For abnormally distributed data, Mann-Whitney and Kruskal Wallis test was used to compare multiple groups means. Significance of the obtained results was judged at the 5% level.

**Results**

**Heavy metals and metallothionein content of mussels and women**
Figure 1a, depicts the cadmium, lead and metallothionein content of mussels collected from Alexandria coast Egypt. The cadmium content of mussels studied were higher than the EU maximum limits while the lead levels were below the maximum limit [20]. A measurable amount of metallothionein was demonstrated in mussel tissues studied (figure 1a). Heavy metal concentration was also measured in women of control group and women with different stages of pregnancy loss groups I-II and results showed that there is a significant difference in Cd and Pb levels between women of control group and women of other groups I-III (figure 1b &1c). There was a significant positive correlation between Cd and Pb levels in those women also (r=0.533, p=<0.001 (figure 1d).

Heavy metals, indices of oxidative stress, reproductive hormone and red blood cell indices in women without recurrent pregnancy loss (RPL) (control group) and women with RPL (groups III).

The Cd, Pb, catalase, GSH, MDA, rbc MT, P₄, Hb, MCV and MCH of women without recurrent pregnancy loss (RPL) (control group) and women with RPL (groups I-III) are shown in table 1. Significant variations were observed in the levels of all indices among the four groups studied (p=<0.001). The Cd, Pb, rbc MT and MDA levels were significantly higher and catalase, GSH, P₄, Hb, MCV and MCH levels lower in women with RPL (GROUPS I-III) compared to women without RPL (control group) (p=<0.001). Group I had lower Cd, Pb, MDA and rbcMT and higher catalase, GSH, Hb, MCV and MCH compared to groups II and group III while group II had decreased Cd, Pb, MDA and rbcMT and increased catalase, GSH, P₄, Hb and MCV compared to group III (P<0.05).

Associations between Cd, Pb, and oxidative stress indices & antioxidant indices (MD, rbcMT, catalase and GSH) in women with recurrent pregnancy loss

Oxidative stress markers MDA and rbcMT were significantly higher in women groups I-III than the control group (figure 2a&d). On the other hand, antioxidant activity of catalase and GSH were significantly lower between the same groups (figure 2 g&j).

The relationship between Cd, Pb, MDA and rbcMT were depicted in figures 2a-f). Cd and Pb had no significant correlation with MDA (figure 2 b&c). on the other hand, significant positive correlations were observed between rbcMT with Cd (r=0.312, p=0.017) (figure 2 e), with Pb (r=0.488, p=<0.000) (figure 2f) and with MDA (r=0.282, p=0.032) (figure 2m) respectively in women with recurrent pregnancy loss. Negative associations were observed between Cd and catalase (r=-0.320, p=0.014), GSH (r=-0.359, p=-0.006) (figure 2h). Although Pb showed no significance correlation with catalase (figure 2I) it showed a Significant negative association with GSH (r=-0.501, p=<0.001) (figure 2l).

Relationship between Pb, Cd and red cell indices in women with recurrent pregnancy loss

Figures 3, shows the difference between different women groups in Hb blood content, MCH and MCV. The three blood indices were significantly lower in women groups I-III than the control group (figure 3a, d&g). Furthermore, the association between Pb, Cd and red cell indices in women with recurrent pregnancy loss is presented in figure 3. Significant negative associations were observed between Pb and Hb (r=-0.289, p=0.028) (figure 3c), while Cd showed no correlation with Hb (figure 3b) in women with recurrent pregnancy loss. Pb showed a significant negative correlation with MCH (r=-0.277, p=0.036) (figure 3f) and Cd had no correlation with MCH (figure 3e) between women with RPL. Cd showed significant negative correlation with MCV (r=-0.391, p=-0.002) (figure 3h), MCV (r=-0.394, p=0.002) (figure 3i) and MCH respectively in women with recurrent pregnancy loss.

Discussion

Pollution of the coastal environment and aquatic life with heavy metals and other chemical pollutants from uncontrolled anthropogenic activities has become a global public health issue. Heavy metal contamination of aquatic organisms would eventually translate to deleterious human health consequences through the food chain. Environmental and dietary exposure to HM have been associated with multiple organ and systemic toxicities including nephrotoxicity, hepatotoxicity, immunotoxicity, neurotoxicity, reproductive failure, genotoxicity and carcinogenesis (El-Sikaily A & M., 2021; Fuerst, 2019;
Gade, Comfort, & Re, 2021; Kim, Ock, Moon, & Park, 2021; Lee, Min, & Min, 2020; Moody, Coca, & Sanders, 2018; Rehman et al., 2018). Mechanisms involving oxidative stress and oxidative DNA damage has been implicated as probable biological pathways of HM induced organ toxicities (Sharma, Singh, & Siddiqi, 2014). Exposure to HM contaminated mussels in relation to redox imbalance among women with recurrent pregnancy loss were assessed in this study.

Our study demonstrated that cadmium levels were higher than the EU maximum limits, lead levels below the maximum limit ("European Union (EU). Commission regulation (EC) No 1881/2006. Setting maximum levels for certain contaminants in foodstuff. L 364/ 5-24.,” 2006) and elevated levels of metallothionein in mussel tissues studied. Our findings are in accordance with previous studies which had demonstrated higher levels of HM as cadmium, lead, copper, chromium, and zinc and measurable amount of metallothionein in mussel tissues collected from Abu Qir Bay (A. El Nemr, Khaled, Moneer, & El Sikaily, 2012; A. M. El Nemr, El Sikaily, & Khaled, 2007; Saad, El-Sikaily, & Kassem, 2017). Higher Cd content of mussels observed may result from pollution of coastal waters accruing from waste disposal from the community, diverse industrial process, sea transportation, and oil spills (Abdel Ghani, El Zokm, Shobier, Othman, & Shreadah, 2013). These activities have high potential to release waste containing heavy metals leading to HM contamination of coastal waters (Amalo LF et al., 2021). Heavy metals are not biologically biodegradable, hence form deposits and bioaccumulate in sediments, are absorbed and bioaccumulate in the bodies of marine life and through the food chain undergo environmental biomagnification and eventually reach humans (Rusydi et al., 2021). Mussels because of their slow mobility and filter feeding characteristics have the potential to accumulate heavy metals present in their environment. These characteristics may be responsible for high Cd levels observed in mussel tissues studied (B.Y. Kamaruzzaman et al., 2011). Mussels are commonly used to assess the ecotoxicological effects of the products released by anthropogenic activities. The concentration of metal in the tissue of mussels has been shown to increase concomitantly with the HM levels of the habitat (B.Y. Kamaruzzaman et al., 2011; Rusydi et al., 2021). Elevated MT in mussel may result from bioaccumulation of HM in the tissues. MT is a heavy-metal-binding protein mostly synthesized by aquatic organisms in response to the presence of heavy metals. (Chen et al., 2014). Due to their inducibility by heavy metals, metallothionein are usually considered as important specific biomarker to detect organism response to inorganic pollutants such as Cd, Hg, Cu, and Zn present in the aquatic environment (Amalo LF et al., 2021).

The biochemical and functional characteristics of metallothionein enables to protect cell structures from non-specific interactions with heavy metal cations and to detoxify metal excess penetrating into the cell (Chen et al., 2014; Rusydi et al., 2021).

Higher Cd, Pb and rbcMT were demonstrated in women with RPL compared to women without RPL. Elevated levels of Cd and Pb have been reported in women with recurrent miscarriages and abortions by previous studies (El-Maali NA, El-Baz MAH, Roshty AS, & AF, 2015; MK., 2019; Omeljaniuk et al., 2018) and may highlight the possible role of subclinical Cd and Pb toxicity in the development of RPL. Studies showing evidence of detrimental effects of HM on fetal development and pregnancy outcome have been documented (K. Turan, A. Arslan, K. Uçkan, H. Demir, & C. Demir, 2019; Wang et al., 2020). HM as Cd, Hg, As and Pb has been shown to cross the placental barrier and accumulate in embryo tissues, inhibit intracellular calcium and magnesium ions Ca$^{2+}$ and Mg$^{2+}$, disrupt cell division, spindle formation and cell cycle which will eventually lead to cell death, fetal malformation and fetal loss (Wang et al., 2020). HM can also cause placental defects, leading to placental malfunction with a compromised capability to support embryonic growth. Cadmium and lead have been implicated in undermining ovum quality, normal ovum division, implantation of embryo leading to reduced pregnancy rates and an increase in fetal malformations, spontaneous abortion, and premature birth (Al-Saleh, Shinwari, Mashhour, Mohamed Gel, & Rabah, 2011). In addition, HM has been linked with disruption of the hypothalamic-pituitary-gonadal (HPG) axis, DNA methylation in neonates and chromosomal aberrations leadig to impaired embryonic development, and abortion (Li, Chen, Li, & Tollefsbol, 2019).

**Table 1. Comparison of HM, indices of oxidative stress, reproductive hormone and red cell indices in women without recurrent pregnancy loss (RPL) (control Group) and women with RPL (Groups I-III).**
<table>
<thead>
<tr>
<th>Index</th>
<th>Control</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>$P$-value</th>
<th>Control vs Grp I</th>
<th>Control vs Grp II</th>
<th>Control vs Grp III</th>
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<td>n=18</td>
<td>n=16</td>
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**Heavy metals**

- **Cd (µg/dl)**
  - Control: 0.05±0.05
  - Group I: 0.80±0.32
  - Group II: 1.11±0.12
  - Group III: 1.65±0.75
  - $P$-value: <0.001<sup>a</sup>

- **Pb (µg/dl)**
  - Control: 3.72±1.96
  - Group I: 15.42±3.52
  - Group II: 18.55±2.61
  - Group III: 30.13±6.54
  - $P$-value: <0.001<sup>a</sup>

**Indices of oxidative stress**

- **Catalase (IU/L)**
  - Control: 449.70±128.08
  - Group I: 187.67±51.38
  - Group II: 126.35±43.96
  - Group III: 118.52±43.96
  - $P$-value: <0.001<sup>a</sup>

- **GSH (mg/dl)**
  - Control: 27.02±3.85
  - Group I: 25.60±3.28
  - Group II: 24.65±3.35
  - Group III: 20.78±2.47
  - $P$-value: <0.001<sup>a</sup>, 0.166, 0.046<sup>b</sup>, <0.001<sup>b</sup>

- **MDA (mg/dl)**
  - Control: 3.04±1.21
  - Group I: 9.28±3.06
  - Group II: 9.22±3.00
  - Group III: 9.46±2.90
  - $P$-value: <0.001<sup>a</sup>, <0.001<sup>b</sup>, <0.001<sup>b</sup>, <0.001<sup>b</sup>

- **rbcMT (mmol/gHb)**
  - Control: 1.23±0.32
  - Group I: 3.28±0.75
  - Group II: 4.09±1.05
  - Group III: 4.65±1.19
  - $P$-value: <0.001<sup>a</sup>, <0.001<sup>b</sup>, <0.001<sup>b</sup>, <0.001<sup>b</sup>

**Reproductive hormone**

- **P<sub>4</sub> (ng/dl)**
  - Control: 13.54±11.91
  - Group I: 2.18±2.36
  - Group II: 2.50±2.24
  - Group III: 1.83±1.80
  - $P$-value: <0.001<sup>a</sup>, <0.001<sup>b</sup>, <0.001<sup>b</sup>, <0.001<sup>b</sup>

**Red cell indices**

- **Hb (g/dl)**
  - Control: 11.59±0.93
  - Group I: 10.32±0.82
  - Group II: 9.87±0.88
  - Group III: 9.73±1.27
  - $P$-value: <0.001<sup>a</sup>, <0.001<sup>b</sup>, <0.001<sup>b</sup>, <0.001<sup>b</sup>

- **MCV (µm<sup>3</sup>)**
  - Control: 94.17±14.57
  - Group I: 76.24±6.38
  - Group II: 67.53±4.17
  - Group III: 65.41±4.87
  - $P$-value: <0.001<sup>a</sup>, 0.006<sup>b</sup>, <0.001<sup>b</sup>, <0.001<sup>b</sup>

- **MCH (pg/cell)**
  - Control: 29.08±2.91
  - Group I: 24.90±2.41
  - Group II: 22.82±1.85
  - Group III: 23.13±2.57
  - $P$-value: <0.001<sup>a</sup>, <0.001<sup>b</sup>, <0.001<sup>b</sup>, <0.001<sup>b</sup>

Data presented as mean±SD, *<sup>a</sup> = indicates significant variations among the groups at $P < 0.05$, <sup>b</sup> = $P$ values from Kruskal-Wallis test, Pb = lead, GSH = reduced glutathione, MDA = malondialdehyde, rbcMT = red cell metallothionein, P<sub>4</sub> = progesterone, Hb = haemoglobin, MCV = mean corpuscular volume, MCH = mean corpuscular haemoglobin.

Positive associations were also observed between Cd and Pb, Cd with rbcMT, Pb with rbcMT and MDA with rbcMT respectively in women with recurrent pregnancy loss. Higher metallothionein observed in this study corroborates our previous findings (Saad et al., 2017). Metallothionein has been shown to be a useful biomarker for the prediction of heavy metal toxicity and adverse biological outcome as its synthesis has been shown to be induced by exposure to HM. Upon heavy metals stimuli, metallothionein genes are rapidly transcriptionally activated and function in protecting cells from damage (Chen et al., 2014; Saad et al., 2017). Higher levels of Cd and Pb observed in women with RPL may have elicited higher MT levels also observed in these women. Induction of methalothionein synthesis by exposure to HM may also explain the significant positive associations observed between rbcMT and the HMs in this study. Strong correlation between MT expression and environmental heavy metal burden has been previously reported (A. M. El Nemr et al., 2007). Evidence from in vivo and invitro studies have shown that Metallothionein is thought to play a major role in systemic heavy metal detoxification and neutralization of ROS (Saad et al., 2017). MT function in heavy metal detoxification primarily depends on the high affinity binding between the heavy metals and MTs, leading to the sequestration of metals away from critical macromolecules (Chen et al., 2014; Saad et al., 2017). Besides HM, oxidative stress and lipid peroxidation has been shown to induce the expression of MT gene (Chen et al., 2014). This may therefore explain the positive association observed between
MT and MDA in this study. Higher levels of MDA observed in women with RPL may be related to HM induced OS and subsequent peroxidation of membrane lipids and other biomolecule. Malondialdehyde (MDA) is formed from the breakdown of polyunsaturated fatty acids (PUFA) and it serves as a convenient index for determining the extent of lipid peroxidation (Nsonwu-Anyawu, Ndudi Idenyi, et al., 2022; Saad et al., 2017). The observed positive association between Cd and Pb is an indication that these two metals function in synergy in the precipitation of multiple organ dysfunctions. The synergistic interactions between metals and minerals have been implicated in human diseases (Nsonwu-Anyawu, Icha, et al., 2022).

Our study also demonstrated lower catalase, GSH and P4 in women with RPL compared to women without RPL. A similar observation has been made by previous studies (Al-Sheikh et al., 2019; Ghneim & Alshebly, 2016; Zejnullahu et al., 2021). Lower levels of GSH, catalase and P4 observed in women with RPL may be related to higher levels of Cd and Pb also observed in these women. This observation is supported by the concomitant negative associations observed between the HMs and these antioxidants (Cd and catalase, Cd and GSH and between Pb and GSH). Exposure to cadmium and lead has been shown to increase the generation of free radicals i.e., reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl and superoxide radicals and reactive nitrogen species (RNS). Enhanced generation of ROS can overwhelm cells intrinsic antioxidant defenses that may result in oxidative stress which has been reported to influence the female reproductive system adversely (Rehman et al., 2018). Reactive oxygen species affect multiple physiological processes from oocyte maturation to fertilization, embryo development and pregnancy outcome (Agarwal, Gupta, & Sharma, 2005; Khazaei & Aghaz, 2017). OS accruing from higher HMs results in increased lipid peroxidation, DNA damage leading to multiple organ dysfunctions (Amadi, Igweze, & Orisakwe, 2017). Depletion of systemic antioxidants and increased lipid peroxidation may be responsible for the elevated levels of MDA and concomitant decrease in GSH and catalase observed in women with RPL. Lower progesterone levels observed in this study is in agreement with observations from previous studies (Rivera-Nunez et al., 2021; Wang et al., 2020). Lower P4 levels has been attributed to dysregulated synthesis of oestrogen and progesterone induced by HM and OS (Ajayi, Charles-Davies, & Arinola, 2012; Kasim Turan, Ayse Arslan, Kazim Uçkan, Halit Demir, & Canan Demir, 2019). The decline in P4 could inhibit endometrial thickening and hinder uterine contractile function, likely leading to spontaneous abortion and adverse pregnancy outcomes (Rivera-Nunez et al., 2021).

Women with RPL also demonstrated lower Hb, MCV and MCH compared to their control counterparts. These red cell indices also associated negatively with Pb. A previous study has demonstrated lower MCH values in RPL compared to the controls (Al-Aghbary, 2017). Higher lead levels observed in women with RPL may explain lowered red cell indices observed. Chronic exposure to lead has been associated with reduction in red cell indices (Rahimpoor, Rostami, Assari, Mirzaei, & Zare, 2020). Lead is shown to induce changes in the composition of red blood cell (RBC) membrane proteins and lipids and to inhibit hemoglobin synthesis. The biochemical basis for this effect is not known but the effect may be accompanied by inhibition in the activity of sodium and potassium dependent ATPases (Glenn, Stewart, Schwartz, & Bressler, 2001). Contrary to our findings, other studies have reported comparable levels of Hb, MVC and MCH in women with or without RPL (Amadi et al., 2017; Wang et al., 2020).

**Conclusion**

Mussels from Alexandria coast are contaminated with heavy metal. The findings of higher heavy metals in women with recurrent pregnancy loss suggests that each heavy metal has a preferred mood of action such as induced oxidative stress and disruption of reproductive hormones which is implicated in the biological pathways involved in the aetio-pathogenesis of idiopathic recurrent pregnancy loss.

**Declarations**

The authors have no competing interests to declare that are relevant to the content of this article.

**Competing Interests**
The authors have no relevant financial or non-financial interests to disclose.

**Author Contributions**

- Prof. Aziza Saad and Prof. Amany El-Sikaily contribute to study conception, design and revision of the manuscript.

- Dr. Mohamed Helal, Dr. Augesta and Dr. Jihan Hassan were contributed in data collection and data analysis and writing the manuscript.

- Dr. Hossam Azab, Tamer Hassanein Contribute in sample collection and commented on previous versions of manuscript.

- Previous draft of the manuscript was written by Dr. , Neveen Abd ElMoneim.

- All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**References**


**Figures**

**Figure 1**

Cadmium (Cd) and lead (Pb) concentration in different studied groups. Mean concentration of Cd, Pb and metallothionein in mussels and EU maximum limits (a) and in women without recurrent pregnancy loss (control) and women with pregnancy loss (group I, II and III) (b&c). Cd and Pb correlation plot showing the relation between the two studied heavy metals (d). n (mussle = 12, control = 18, group I = 24, group II = 18 and group III = 16). Statistical significance p ≤ 0.001.
Figure 2

Effect of Cd and Pd on oxidative stress and anti-oxidant indices (MDA, rbcMT, catalase and GSH) on pregnancy loss in women (control vs group I-III). Oxidative stress enzymes and their correlation to Cd and Pb concentration in different women group; MDA (a, b&c), rbcMT (d, e&f), catalase (g, h&i) and GSH (j, k&l). MDA and rbcMT correlation is shown in figure (m). Correlation figures were added to show the positive and negative relationship between Cd and Pb to each indices. n (control = 18, group I = 24, group II = 18 and group III = 16). Statistical significance p ≤ 0.001.
Figure 3

Effect of Cd and Pd on blood indices (Hb, MCH and MCV) of pregnancy loss in women (control vs group I-III). Blood indices changes and their correlation to Cd and Pb concentration in different women group; Hb (a, b&c), MCH (d, e&f) and MCV (g, h&i). Blood samples were collected from women groups and subjected to analysis of blood Hb, MCH and MCV. Correlation figures were added to show the positive and negative relationship between Cd and Pb to each indices. n (control = 18, group I = 24, group II = 18 and group III = 16). Statistical significance p ≤ 0.001.
Figure 4

Differential impact of Cd and Pb on different biological indices in women with recurrent pregnancy loss (aborted women) in comparison to control women (normal pregnant women). Briefly: elevated levels of Cd in women was associated with significant correlation with high rbcMT level and low GSH and MCV levels. On the hand, elevated Pb level is correlated with significant low levels of catalase, GSH, Hb, MCH and MCV. Each class of heavy metals exert its cumulative health impact by affecting different biological indices in different rates.