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Impact of heavy metal pollution on idiopathic recurrent spontaneous abortion

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ABSTRACT

Environmental pollution with heavy metal (HM) may be associated with human reproductive failure where pollutants accumulate in marine organisms and sediment and are subsequently transferred to humans through the food chain. Seventy-six women (20-35 years) were 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, hemoglobin (Hb), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). Mussel samples were collected for Cd, Pb, and metallothionein (MT) estimation. The cadmium content of mussels was above the maximum limit. Women with RPL (Groups I-III) had higher Cd, Pb, rbcMT, and MDA and lower catalase, GSH, P_4, Hb, MCV, and MCH compared to the control group (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, 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 (RPL).

INTRODUCTION

Disposal of industrial waste products into water bodies has led to the pollution of aquatic environments with heavy metals which deposit and accumulate in sediments and bodies of marine life and subsequently through the food chain, eventually reaching humans (**Vardhan *et al.*, 2019**). Because of their low mobility and filter-feeding characteristics, aquatic organisms such as green mussels are targets for accumulation and

contamination with pollutants such as heavy metals, polychlorinated Biphenyles, and polyaromatic hydrocarbons in the aquatic habitat **(Rusydi *et al.*, 2021; Mohamed *et al.*, 2022**; **El-Sikaily *et al.*, 2023)**. The HM content of mussels has been shown to reflect 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**). In this context, the consumption of heavy metal-contaminated food has been associated with deleterious health consequences, including reproductive impairment at the genetic, epigenetic, and biochemical levels **(Salamat *et al.*, 2015**; **Dutta *et al.*, 2021)**. These metals impact female reproduction at all stages of its regulation and function, for instance, in 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, and 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 the 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).** Moreover, elevated Lead and cadmium levels have been associated with premature rupture of placental membranes, premature delivery, and miscarriage **(Taylor *et al.*, 2016; MK., 2019)**. Heavy metals have been shown to negatively influence the hypothalamic-pituitary-gonadal (HPG) axis, thereby causing deleterious alterations in the menstrual cycle, ovulation, and hormonal homeostasis **(Bhatia *et al.*, 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 *et al.*, 2010; Rehman *et al.*, 2018)**. Oxidative DNA damage occurring from metal-induced OS has been implicated in fetal abnormalities and pregnancy termination **(Duhig *et al.*, 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 *et al.*, 2021).**

Spontaneous abortion is largely becoming a public health concern for developing countries. Approximately 10% of clinically diagnosed pregnancies end with spontaneous abortion **(MK., 2019).** Despite intensive and thorough investigations in this field, most RPL cases have 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 **(Bhatia *et al.*, 2015; Alrashed *et al.*, 2021; Dutta *et al.*, 2021)**. However, fewer studies have examined the relationship between exposure to HM-contaminated seafood and the increasing incidence of recurrent pregnancy loss.

Aim of this study: this study was designed to investigate the possible connection between exposure to heavy metals and RPL through an assessment of the (1) HM content in mussels as a bio-indicator of environmental HM contamination and as an inexpensive dietary intake species. (2) The HM indices of oxidative stress, reproductive hormone, and red cell indices in women with recurrent pregnancy loss (RPL).

MATERIALS AND METHODS

Subjects and Methods

Study design

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

Selection of subjects

A total of seventy-six (76) healthy female subjects with an age range of 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 proceeded successfully, giving full-term healthy newborns.

B) Volunteer subjects

Group I: Comprised of 24 women with at least two successive unexplained recurrent spontaneous abortions up to 20 weeks gestational age**.**

Group II: Comprised of 18 women with at least three successive unexplained recurrent spontaneous abortions up to 20 weeks gestational age.

Group III: Comprised of 16 women with more than three successive unexplained recurrent spontaneous abortions up to 20 weeks gestational age.

A semi-structured questionnaire was administered to all participants in the study for a history of past and present ailments with emphasis on toxoplasmosis, rubella, herpes simplex, uterine abnormalities, endocrinal irregularities (diabetes mellitus, thyroid dysfunction), hypertension, liver diseases, urinary tract insult, smoking, working in an industrial area with metal exposure were all excluded from the study. Also, all subjects were asked about their intake of mussels per week.

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 was collected into a 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 were used to determine metallothionein concentration. The remaining blood was then collected into a 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 was carried out.

b. Mussel samples:

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

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 the spectrophotometric method **(Viarengo *et al.*, 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 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 a 25 ml volumetric flask. 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 *et al.*, 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 according to the method of **(Grider *et al.*, 1990).**

Determination of plasma catalase (CAT) enzyme activity

Catalase enzyme activity was determined by the spectrophotometric method at 510 nm wavelength **(Aebi, 1984)**. Catalase enzyme reacts with a known quantity of H_2O_2 and

subsequently, the reaction is stopped after exactly one minute with a catalase inhibitor. In the presence of peroxidase (HRP), remaining H_2O_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).

$$2H_2O_2 + DHBS + AAP \xrightarrow{HRP} \text{Quinoneimine Dye} + 4H_2O$$

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 reacts with thiobarbituric acid to give a red color (MDA-TBA2) whose absorbance was measured at 532 nm. Briefly, TBA and H3PO4 were added to the serum and the reaction mixture was heated to 90^{o}C for 30 min in a temperature-controlled heating block. The reaction was stopped by placing the reaction mixture on ice, after which the concentration of MDA in the sample was read at 532 nm **(Draper & Hadley, 1990).**

Estimation of Reduced Glutathione (GSH)

Estimation of GSH was carried out following the modified standard Ellman's method. Briefly, on the 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 412 nm and is proportional to the concentration of GSH in the sample **(Beutler *et al.*, 1963).**

Determination of Hb, MCV, and MCH.

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

Determination of serum Progesterone hormone.

Serum progesterone estimation was done using the enzyme-linked immunosorbent assay (ELISA) method developed by **(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 the Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for the vision test. For normal 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 were expressed using median, minimum, and maximum. For normally distributed data, analysis of variance and post hoc test were used to determine variations among multiple

groups' means. For abnormally distributed data, Mann-Whitney and Kruskal Wallis tests were used to compare multiple groups' means. The significance of the obtained results was judged at the 5% level.

RESULTS

High Cd & Pb in mussel and women subjects affect their metallothionein levels

Fig. 1a depicts the cadmium, lead, and metallothionein content of mussels collected from the Alexandria coast of Egypt. The cadmium content of the mussels studied was higher than the EU maximum limits while the lead levels were below the maximum limit (E.U., 2006). A measurable amount of metallothionein was detected in the mussel tissues studied (Fig. 1a). Heavy metal concentration was also measured in women of the control group and women with different stages of pregnancy loss in groups I-II and the results showed that there is a significant difference in Cd and Pb levels between women of the control group and women of other groups I-III (Fig. 1b &). Also, there was a significant positive correlation between Cd and Pb levels in those women ($r=0.533$, $p=<0.001$ (Fig. 1d).

Heavy metals modulate sex hormone level, blood characteristics and induce oxidative stress in women subjects

The levels of Cd, Pb, catalase, GSH, MDA, rbc MT, P_4, Hb, MCV, and MCH of the control group and RPL groups (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 whereas catalase, GSH, P_4, Hb, MCV, and MCH levels were lower in RPL groups compared to the control group ($p=<0.001$). Of note, group I had lower Cd, Pb, MDA, and rbcMT and higher catalase, GSH, Hb, MCV, and MCH compared to groups II and group III. Similarly, group II had decreased Cd, Pb, MDA, and rbcMT and increased catalase, GSH, P_4, Hb, and MCV compared to group III ($P<0.05$).

Cd & Pb levels are significantly correlated with enzyme levels in aborted women subjects

The relationship between Cd, Pb, MDA, and rbcMT was depicted in Figs. 2a-f. Oxidative stress markers MDA and rbcMT were significantly higher in women groups I-III than in the control group (Fig. 2a&d). On the other hand, the antioxidant activity of catalase and GSH were significantly lower between the same groups (figure 2g&j). Cd and Pb had no significant correlation with MDA (Fig. 2b&c). On the other hand, significant positive correlations were observed between rbcMT with Cd ($r=0.312$, $p=0.017$) (Fig. 2e), with Pb ($r=0.488$, $p=<0.000$) (Fig. 2f) and with MDA ($r=0.282$, $p=0.032$) (Fig. 2m) respectively in women with recurrent pregnancy loss. Negative associations were observed between Cd and catalase ($r=-0.320$, $p=0.014$), and GSH ($r=-0.359$, $p=-0.006$) (Fig. 2h). Although Pb showed no significant correlation with catalase

(Fig. 2I) it showed a significant negative association with GSH (r=-0.501, p=<0.001) (Fig. 2l).

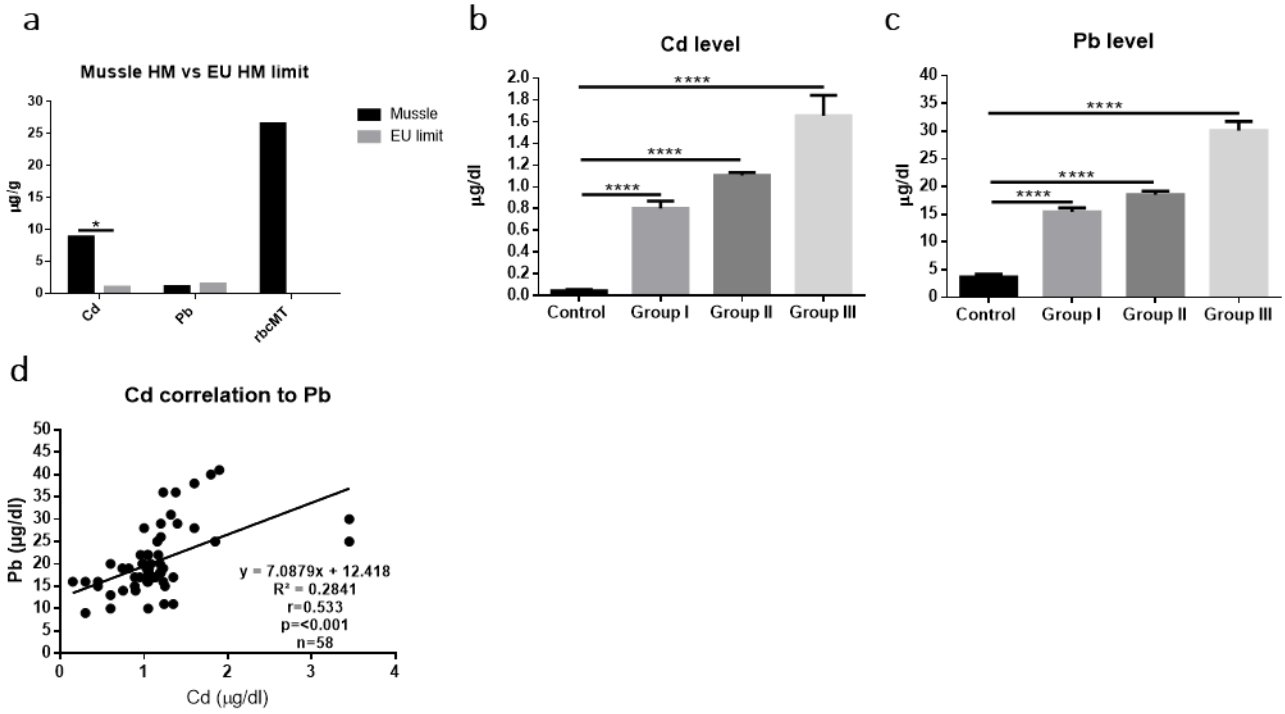

Fig. 1: **Cadmium (Cd) and lead (Pb) concentrations in different studied groups.** The mean concentration of Cd, Pb, and metallothionein in mussels and EU maximum limits (a) and in women without recurrent pregnancy loss (control) and women with pregnancy loss (group I, II, and III) (b&c). Cd and Pb correlation plot show 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 $p \leq 0.001$.

Cd & Pb level is linked with changes in red blood cell parameters

Fig. 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 in the control group (Figs. 3a, d &g). Furthermore, the association between Pb, Cd, and red cell indices in women with recurrent pregnancy loss is presented in Fig. 3. Significant negative associations (r=-0.289, p=0.028) were observed between Pb and Hb (Fig. 3c), while Cd showed no correlation with Hb (Fig. 3b) in women with recurrent pregnancy loss. Pb showed a significant negative correlation (r=-0.277, p=0.036) with MCH (Fig. 3f) and Cd did not correlate with MCH (Fig. 3e) between women with RPL. Cd showed a significant negative correlation (r=-0.391, p=-0.002) with MCV (Fig. 3h) and MCH (r=-0.394, p=0.002) in women with recurrent pregnancy loss (Fig. 3i).

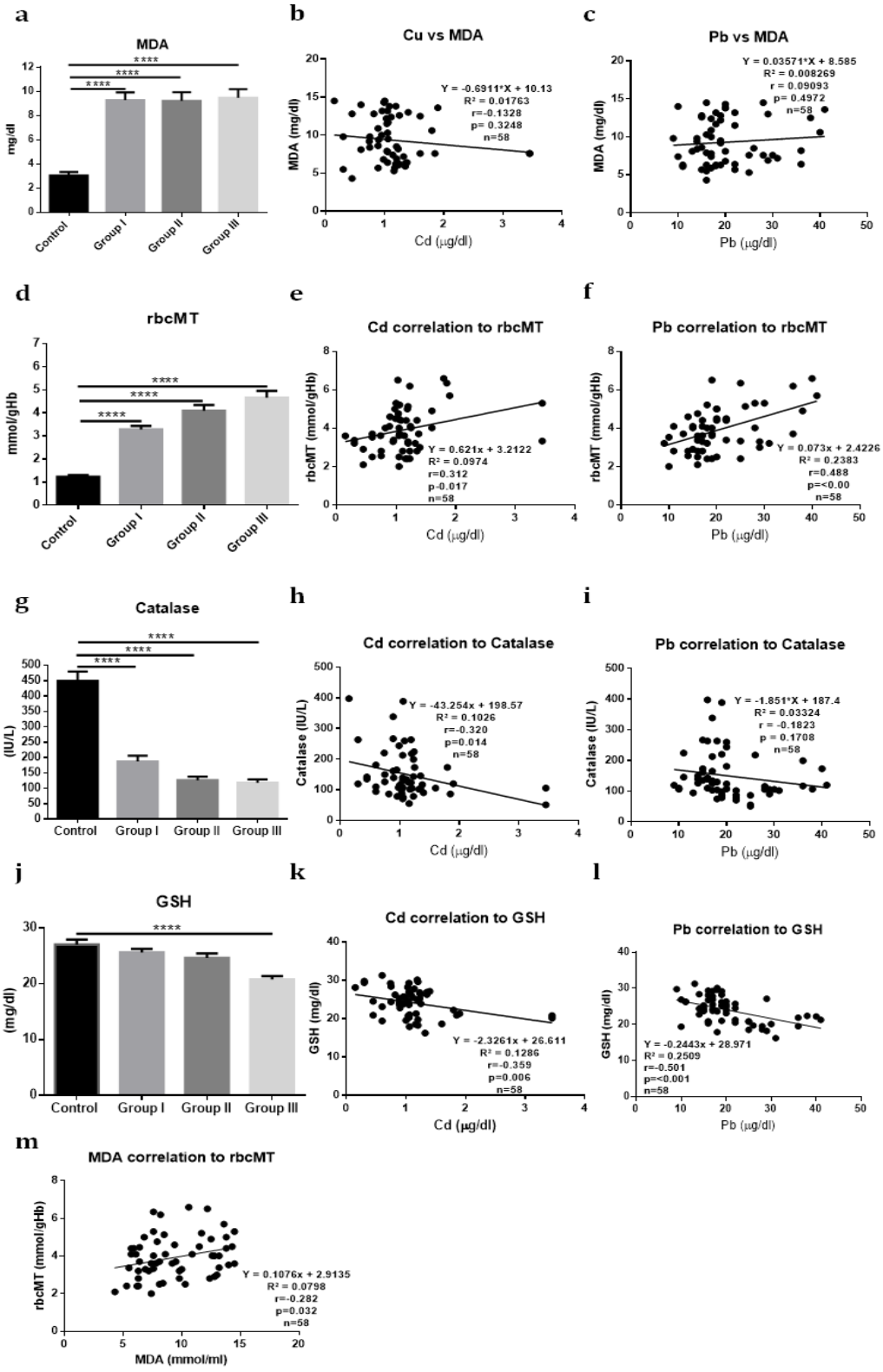

Fig. 2: **Impacts of Cd and Pd accumulation on oxidative stress status in different women group.** Oxidative stress enzymes and their correlation to Cd and Pb concentration in different women groups; MDA (a, b&c), rbcMT (d, e&f), catalase (g, h&i) and GSH (j, k&l). MDA and rbcMT correlation is shown in Fig. (m). Correlation Fig.s were added to show the positive and negative relationship between Cd and Pb to each index. n (control = 18, group I = 24, group II = 18 and group III = 16). Statistical significance $p \leq 0.001$.

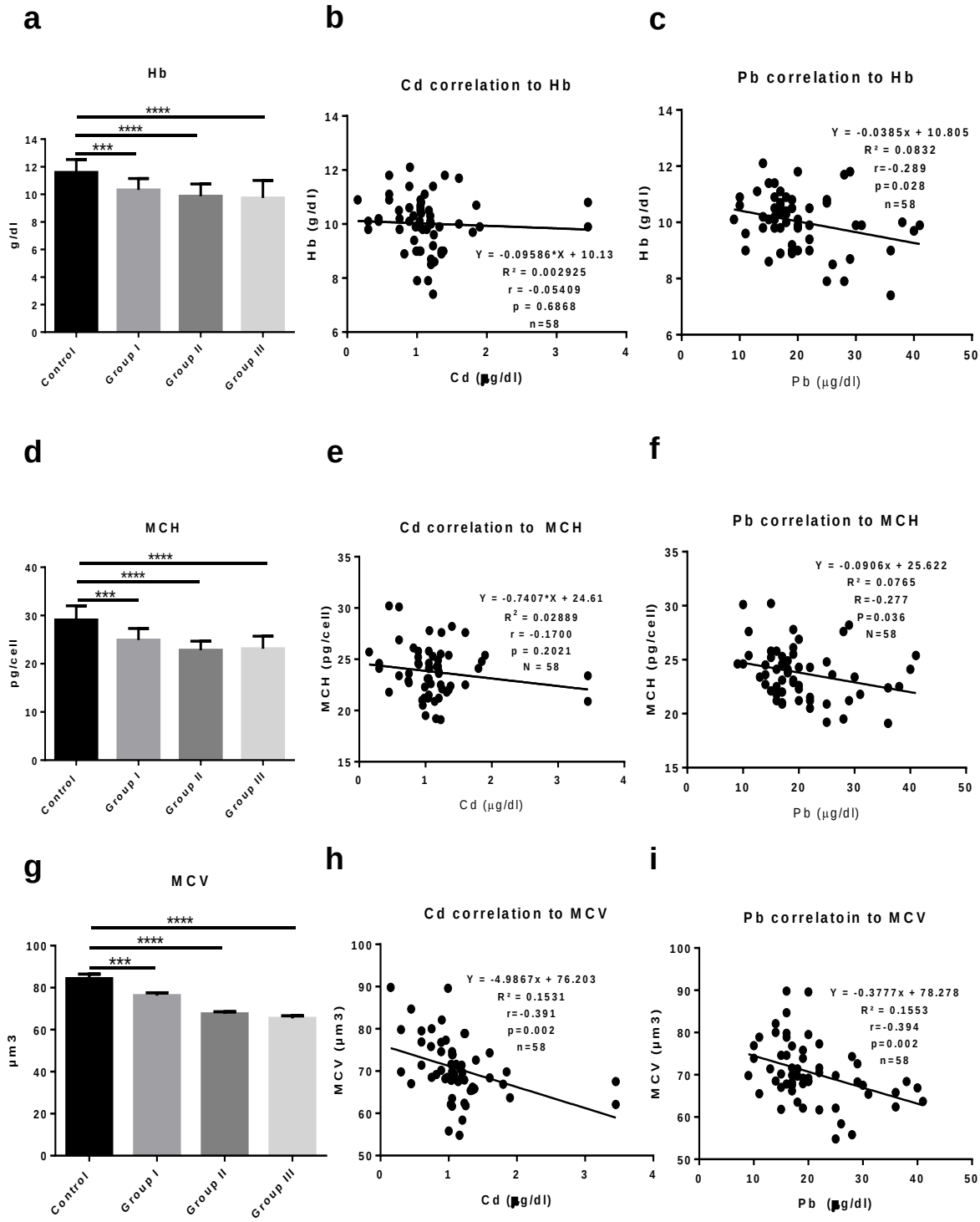

Fig. 3: **Toxic impacts of Cd and Pd on blood parameters in different women groups.** Blood indices changes and their correlation to Cd and Pb concentration in different women groups; Hb (a, b&c), MCH (d, e&f), and MCV (g, h&i). Blood samples were collected from women groups and subjected to analysis of blood Hb, MCH, and MCV. Correlation Fig.s 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 $p \leq 0.001$.

DISCUSSION

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

Our study demonstrated that cadmium levels were higher than the EU maximum limits, and lead levels below the maximum limit (E.U., 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 **(El Nemr *et al.*, 2007; El Nemr *et al.*, 2012; Saad *et al.*, 2017).** The higher Cd content of mussels observed may result from the pollution of coastal waters accruing from waste disposal from the community, diverse industrial process, sea transportation, and oil spills **(Abdel Ghani *et al.*, 2013).** These activities have a 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 deposit and bioaccumulate in sediments which are subsequently absorbed and bioaccumulated in the bodies of marine life forms, 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 the high Cd levels observed in mussel tissues **(B.Y. Kamaruzzaman *et al.*, 2011)**. Mussels are commonly used to assess the eco-toxicological effects of the products released by anthropogenic activities. The concentration of metal in the tissue of mussels has been shown to increase concomitantly with their habitat HM content **(B.Y. Kamaruzzaman *et al.*, 2011; Rusydi *et al.*, 2021).** Elevated metallothionein (MT) in mussels may result from the bioaccumulation of HM in the tissues. MT is a heavy-metal-binding protein mostly synthesized by aquatic organisms in response to exposure to heavy metals **(Chen *et al.*, 2014)**. Due to their sensitivity to heavy metals, metallothionein is usually considered an 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 protect cell structures from non-specific interactions with heavy metal cations and detoxify excess metals penetrating the cells **(Chen *et al.*, 2014; Rusydi *et al.*, 2021).**

Higher Cd, Pb, and rbcMT were evident 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 *et al.*, 2015; Omeljaniuk *et al.*, 2018; MK., 2019)** and may highlight the possible role of subclinical Cd and Pb toxicity in the development of RPL. Studies showing evidence of the detrimental effects of HM on fetal development and pregnancy outcome have been documented **(K. Turan *et al.*, 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, and disrupt cell division, spindle fiber 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 ability to support embryonic growth. Cadmium and lead have been implicated in undermining ovum quality, normal ovum division, and implantation of embryos leading to reduced pregnancy rates and an increase in fetal malformations, spontaneous abortion, and premature birth **(Al-Saleh *et al.*, 2011)**. In addition, HM has been linked with disruption of the hypothalamic-pituitary-gonadal (HPG) axis, DNA methylation in neonates, and chromosomal aberrations leading to impaired embryonic development and abortion **(Li *et al.*, 2019).**

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 the present study corroborates our previous findings **(Saad *et al.*, 2017).** Metallothionein is a useful biomarker for the prediction of heavy metal toxicity and adverse biological outcome, as its synthesis is 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 metallothionein synthesis by exposure to HM may also explain the significant positive associations observed between rbcMT and the HMs in this study.

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)

Index	Control	Group I	Group II	Group III	*P*-value	Control vs Grp I *P*-value	Control vs Grp II *P*-value	Control vs Grp III *P*-value
	n=18	n=24	n=18	n=16				
Heavy metals								
Cd (µg/dl)	0.05±0.05	0.80±0.32	1.11±0.12	1.65±0.75	<0.001^{*a}	<0.001^{*b}	<0.001^{*b}	<0.001^{*b}
Pb (µg/dl)	3.72±1.96	15.42±3.52	18.55±2.61	30.13±6.54	<0.001^{*a}	<0.001^{*b}	<0.001^{*b}	<0.001^{*b}
Indices of oxidative stress								
Catalase (IU/L)	449.70± 128.08	187.67± 92.66	126.35± 51.38	118.52± 43.96	<0.001^{*a}	<0.001^{*b}	<0.001^{*b}	<0.001^{*b}
GSH (mg/dl)	27.02±3.85	25.60±3.28	24.65±3.35	20.78±2.47	<0.001^{*a}	0.166	0.046^{*b}	<0.001^{*b}
MDA (mg/dl)	3.04±1.21	9.28±3.06	9.22±3.00	9.46±2.90	<0.001^{*a}	<0.001^{*b}	<0.001^{*b}	<0.001^{*b}
rbcMT (mmol/gHb)	1.23±0.32	3.28±0.75	4.09±1.05	4.65±1.19	<0.001^{*a}	<0.001^{*b}	<0.001^{*b}	<0.001^{*b}
Reproductive hormone								
P_4 (ng/dl)	13.54±11.91	2.18±2.36	2.50±2.24	1.83±1.80	<0.001^{*a}	<0.001^{*b}	<0.001^{*b}	<0.001^{*b}
Red cell indices								
Hb (g/dl)	11.59±0.93	10.32±0.82	9.87±0.88	9.73±1.27	<0.001^{*a}	<0.001^{*b}	<0.001^{*b}	<0.001^{*b}
MCV (μm^3)	94.17±14.57	76.24±6.38	67.53±4.17	65.41±4.87	<0.001^{*a}	0.006^{*b}	<0.001^{*b}	<0.001^{*b}
MCH (pg/cell)	29.08±2.91	24.90±2.41	22.82±1.85	23.13±2.57	<0.001^{*a}	<0.001^{*b}	<0.001^{*b}	<0.001^{*b}

Data presented as mean±SD**,** * = indicates significant variations among the groups at $P < 0.05$, ^{a} = *P* values from Kruskal Wallis test, ^{b}= *P* values from Mann-Whitney U test, Cd=cadmium, Pb=lead, GSH=reduced glutathione, MDA= malondialdehyde, rbcMT=red cell metallothionein, P4= progesterone, Hb=haemoglobin, MCV= mean corpuscular volume, MCH=mean corpuscular haemoglobin

The strong correlation between MT expression and the environmental heavy metal burden has been previously reported **(El Nemr *et al.*, 2007).** Evidence from in vivo and in vitro studies have shown that Metallothionein plays 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 the MT gene **(Chen *et al.*, 2014).** This may 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 biomolecules. 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 **(Saad *et al.*, 2017; Nsonwu-Anyanwu, Ndudi Idenyi, *et al.*, 2022)**. The observed positive association between Cd and Pb is indication of the synergy of these metals in relation to multiple organ dysfunctions. The synergistic interactions between

metals and minerals have been implicated in human diseases **(Nsonwu-Anyanwu, Icha, *et al.*, 2022)**.

Our study also demonstrated lower catalase, GSH, and P4 levels in women with RPL compared to women without RPL. A similar observation has been made by previous studies **(Ghneim & Alshebly, 2016; Al-Sheikh *et al.*, 2019; 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 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 *et al.*, 2005; Khazaei & Aghaz, 2017).** OS accruing from higher HMs results in increased lipid peroxidation and DNA damage leading to multiple organ dysfunctions **(Amadi *et al.*, 2017).** Depletion of systemic antioxidants and increased lipid peroxidation may be responsible for the elevated levels of MDA and a concomitant decrease in GSH and catalase observed in women with RPL. Lower progesterone levels observed in this study are in agreement with observations from previous studies **(Wang *et al.*, 2020; Rivera-Nunez *et al.*, 2021)**. Lower P4 levels have been attributed to the dysregulated synthesis of estrogen and progesterone induced by HM and OS **(Ajayi *et al.*, 2012; Kasim Turan *et al.*, 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 are 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 a reduction in red cell indices **(Rahimpoor *et al.*, 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 *et al.*, 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

In conclusion, mussels collected from the Alexandria coast of Egypt are contaminated with heavy metals. The findings of higher heavy metals in women with recurrent pregnancy loss suggest 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 aetiopathogenesis of idiopathic recurrent pregnancy loss. Higher lead levels observed in women with RPL may explain lowered red cell indices observed. Chronic exposure to lead has been associated with a reduction in red cell indices

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Declarations

Ethics approval and consent to participate

The study has been approved by the ethics committee of the Medical Research Institute, Alexandria University, and according to the code of ethics of the World Medical Association (Declaration of Helsinki) for human study. Assigned informed consent was obtained from all individuals.

Consent for publication

Not applicable

Availability of data and materials

Raw data can be provided upon reasonable request

Competing interests

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

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- Prof. Aziza Saad and Prof. Amany El-Sikaily contribute to the study conception, design, and revision of the manuscript. Dr. Mohamed Helal, Dr. Augesta and Dr. Jihan Hassan contributed to data collection and data analysis, and writing the manuscript. Dr. Hossam Azab., and Tamer Hassanein *Contribute to the sample collection and commented on previous versions of the manuscript. The 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.*

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