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Evaluation of some heavy metals in women with an unexplained recurrent pregnancy loss

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Abstract:

Recurrent pregnancy loss (RPL) is one of the most frustrating problems in reproductive medicine because the etiology is often unknown. Recent studies suggest the role of environmental chemicals in the pathogenesis of RPL. The objective of this study was to investigate the possible role of some heavy metals (lead and cadmium) as risk factors of RPL by determining their levels in normal and unexplained RPL pregnancies. This cross-sectional study included 47 cases with definite diagnosis RPL and 30 pregnant controls with normal obstetric history. Blood levels of heavy metals (lead and cadmium) were measured using atomic absorption spectrometer with graphite furnace. The results showed significantly higher levels of lead and cadmium in RPL group than those levels in the control group. These levels were higher in women who lived in urban areas and those exposed to cigarette smoke than women who lived in rural areas and non smokers, respectively. In conclusion: This study suggests that high blood levels of lead and cadmium are associated with increased risk of RPL.

Keywords Environmental chemicals, lead, cadmium, recurrent pregnancy loss (RPL).

1. INTRODUCTION

Recurrent pregnancy loss (RPL), defined as the loss of three or more consecutive pregnancies, affects 1% of couples trying to conceive [1]. Risk factors for recurrent miscarriage include epidemiological factors, antiphospholipid syndrome, genetic factors, anatomical anomalies, endocrine disorders, immune factors, infective agents and Inherited thrombophilic defects. However, in about half of the cases no definite cause could be detected [2**].** Previous studies showed an association between exposure to environmental chemicals as heavy metals and poor reproductive outcome, including spontaneous abortion. They possibly act as endocrine disruptors [3]. Millions of environmental pollutants which are found in air and water as well as chemicals used at home may pose a risk of adverse effects on pregnancy outcomes [4]. The increasing percentage of young married females that join the working force in their reproductive ages makes them and their offspring at a particular risk to these hazards [5]. Pregnant women may inhale, ingest or occasionally absorb these pollutants from their skin [6]. These pollutants may affect the outcome of pregnancy directly e.g. spontaneous abortion [5], [7, 8]. Kannan et al. [9] described potential biologic pathways including, systemic oxidative stress and inflammation, changes in blood coagulation, endothelial function and hemodynamic responses. Generally, the environmental factors show their effects through direct or indirect mechanisms [10]. Lead (Pb) is among the first studied environmental hazards with common sources that include occupational exposure, deteriorating lead paint, lead-containing gasoline, pesticides, groundwater and lead-containing products such as cigarette smoking, newspaper and kohl [11-13]**.** The routes of entry of lead to human body may be inhalation, ingestion or occasionally skin contact [11], [14]. About 15% of ingested inorganic lead is absorbed. This percentage is higher in children, pregnant women and people with deficiencies of zinc or iron [15]. Llop et al. [16] found that daily intake of iron and zinc significantly decreased cord blood lead levels. Also, lead absorption is inversely related to dietary calcium [17]. Sub toxic levels of lead could increase the incidence of early membrane rupture [18]. It is able to cross the placenta and has many adverse effects on pregnant women including RPL [19]. Cadmium(Cd) is another common environmental pollutant that is gained a public attention due to the world wide increase in the discard of electronic waste containing this toxic metal such as cell phones and computers [20-22]. Human intoxication with cadmium results mainly from cigarette smoking due to its high Cd content [23].Cigarette smoking increases indoor Cd concentrations and the average daily exposure from cigarette smoking (20 cigarettes a day) is 2-4μg of Cd [24]. Sorkun et al. [25] reported that smoking increased Cd levels in placenta. In non-smokers and non-occupationally exposed people, food is the main source of Cd toxicity [26]. Recent reports of cadmium's use in children and adult jewelry and artist pigments highlight other possible routes of human exposure [27-29]. It may disrupt the female reproductive functions, among other effects, by its potent oestrogen like activities [30], stimulation of ovarian progesterone biosynthesis at low concentrations and inhibition of ovarian and placental progesterone synthesis at high concentrations [31]. The aim of the current study is to evaluate the possible association between exposure to some environmental toxins as lead and cadmium and unexplained RPL.

2. Materials and methods

2.1 Chemical and Solution

2.1.1 Chemicals

Standard lead and cadmium **.**lead and cadmium chloride with high purity and nitricacid (65%) was sigma Aldrich pure grade. Ultrapure water used was from Milli-Q system model: Milli-Q Gradient A10, Elix

3UV and Tank 60L, Serial NO: F7AN24007K, F7BN902741, USA.

2.1.2 Solution Preparation

2.1.2.1 Standard solution, Stock

these solutions prepared from pure standard materials for each lead and cadmium from CPA chem with NIST SRM No 3128 lot 030721 for lead and NIST SRM No 3108 lot 060531 for cadmium (1000ppm) and dissolved them in 1% HNO_3(65%).

2.1.2.2 Standard solution, Secondary Dilution –use the stock standard solution to prepare secondary dilution standard solution in 1% HNO_3 (65%).and prepare a series of secondary solutions (10 ppm,1ppm,50 ppb) and check frequently before preparing calibration standard.

2.1.3 Sample Preparation

2.1.3.1 Sample Collection A venous blood samples (10 ml) were collected aseptically from women with recurrent pregnant loss (from the Outpatient clinic of women Health Hospital, Assiut University. via vein puncture in a sterile collecting tube containing K_2EDTA. as anti coagulant.

2.1.3.2 Sample Preservation and Storing

The samples were stored at -20°C and maintained at this temperature until the analyst is used it for the extraction process.

2.1.3.3 Working Procedure

Stored samples are removed from the fridge and allow them to equilibrate equilibrate with room temperature. A microwave –assisted acid digestion procedure was carried out to achieve a shorter digestion time .For the digest on of biological samples, blood samples (0.5 ml) were directly taken into Teflon PTFE flasks (Kartell).Added to each flask 2ml of a freshly prepared mixture of concentrated HNO_3-H_2O_2(2:1,v/v). The flasks were kept for 10 min at room temperature and then placed in a covered PTFE container. This was then heated following a one-stage digestion program at 80% of total power (900W), for 2-4 min for blood samples. Thereafter, the digestion flasks were cooled and the resulting solution was evaporated to a semidried mass to remove excess acid, and then diluted up to 10.0ml in volumetric flasks with 0.1M nitric acid.

2.1.4 Instrumentation

Graphite Atomic Absorption Spectrophotometer (Model ContrAA 700 High Resolution Continuum Source Atomic Absorption Spectrometer) directs concentration readout and an argon gas used for the element determinations. The light source used was xenon lamp which was used at a wavelength of 283.3 and 228.8 nm for lead and cadmium respectively.

2.1.5 Patients and methods

This cross sectional study was carried out on 77 women recruited from the outpatient clinic of the Women Health Hospital, Assiut University, Egypt. Women were enrolled into the study between January 2010 and January 2011. They were classified into 2 groups: 47 cases with definite diagnosis of missed abortion and history of two or more events of RPL and 30 controls with normal first trimester pregnancy and normal obstetric history. Women with hormonal disorders (uncontrolled diabetis ,uncontrolled thyroid dysfunction), uterine abnormalities (uterus subseptus, uterus fibroids, adhesions), anti-phospholipid syndrome, immunological causes of miscarriages, hypertension, bacterial vaginosis, TORCH infections, tuberculosis, carriers of chromosomal translocation, active smokers were excluded from both the groups. The spouses of these women were also with normal karyotype, normal sperm count and normal sperm morphology. The women we included in this study were of relatively homogenous group and they were similar in terms of demographical characteristics such as age, weight, BMI, food habits, drinking water supply, living style and socioeconomic status. Cases with RPL meeting these exclusion criteria are termed idiopathic RPL.

A questionnaire survey of the women was used to collect general demographic information. Cases were classified into exposed to Smoking (No.22) and non exposed (No.25) women with unexplained RPL. Informed consent was obtained from each participant woman. The study was approved by the Ethics Committee of Faculty of Medicine, Assiut University.

3. Results and discussion

The RPL and the control group were matched and there were no statistically significant differences between them as regard to residence and maternal age (Table 1).

Table 1: Socio-demographic difference between the two groups (cases vs. controls)

	Case (No=47)	Control (No=30)	P-value
Mean Age ± SD	28.1 ± 6.9	27.2 ± 6.0	0.561*
Residence			
• Rural	30 (63.8%)	22 (73.3%)	0.385**
• Urban	17 (36.2%)	8 (26.7%)	

*T-test analysis was used to compare the mean difference between the two groups
**Chi-square analysis was used to compare the difference in proportions --Significance level is considered when $p < 0.05$.

Significant increased levels of heavy metals were observed in RPL group as compared to control group (Table 2), in women who lived in urban areas as compared to those who lived in rural areas (Table 3).

Table 2: Blood levels of Lead & Cadmium (µg/L) in RPL cases as compared to their Controls.

	Case (No=47)	Control (No=30)	P-value*
	Mean± SD		
• Lead	49.15 ± 117.03	0.00 ± 0.0	<0.001**
• Cadmium	14.04 ± 33.4	5.12 ± 9.91	0.315

*Mann-Whitney non-parametric test was used to compare the mean difference between the two groups

Table 3: Comparison between Cases and control for Rural and Urban parameter.

	Cases			Control			P*	P**
	Urban	Rural	P. value	Urban	Rural	P. value		
	Mean± SD			Mean± SD				
Lead	65.4±137.5	20.4±61	0.208	0±0	0±0	NA	0.014	0.187
Cadmium	17±36.5	8.8±27.5	0.431	5.8±10.9	4.9±9.8	0.822	0.092	0.769

Mann-Whitney non-parametric test was used to compare the mean difference between the two groups

* Comparison between cases and control in rural

**Comparison between cases and control in urban
--Significance level is considered when $p < 0.05$.

Table 4: Frequency of Lead & Cadmium (µg/L) detection among patients with RPL vs. controls

	Case (No=47)	Control (No=30)	P-value
Lead			
• Exposed	7 (14.9%)	0 (0%)	0.027**
• Non-exposed	40 (85.1%)	30 (100%)	
Cadmium			
• Exposed	16(34.0%)	8(26.7%)	0.336
• Non-exposed	31 (66.0%)	22 (73.3%)	

**Fisher Exact test analysis was used to compare the difference in proportions
--Significance level is considered when $p < 0.05$.

The correlation analyses between the blood lead and cadmium levels and other risk factor within RPL group were presented in (Tables 5& 6 respectively).

Table 5: Correlation between the blood lead levels and other risk factor among the RPL cases

Parameters	Exposed group (n=7)	Non exposed group (n=40)	p-value
Maternal Age in years	27.7 ± 9.3	28.2± 6.5	0.608*
No. of Living Children	2.14 ± 2.73	1.93 ± 1.7	0.804*
No. of Abortion	3.86 ± 2.67	3.55 ± 2.8	0.942*
Residence			
• Rural	5(71.4%)	25 (62.5%)	0.501**
• Urban	2 (28.6%)	15 (37.5%)	
Exposure to Smoking			
• No	2 (28.6%)	23 (57.5%)	0.158**
• Yes	5 (71.4%)	17 (42.5%)	
Exposure to Cadmium			
• No	1 (14.3%)[&]	30 (75.0%)	0.004**
• Yes	6 (85.7%)	10 (25.0%)	

*Mann-Whitney non-parametric test was used to compare the mean difference between the two groups
**Fisher Exact test analysis was used to compare the difference in proportions
& significant difference between each item for exposed lead cases using Chi-square test
--Significance level is considered when $p<0.05$.

Table 6: Correlation between the blood cadmium levels and other risk factors among RPL Cases.

Parameters	Exposed group (n=16)	Non exposed group (n=31)	p-value
Maternal Age in years	28.63 ± 7.81	27.87 ± 6.44	0.840*
No. of Living Children	2.44 ± 2.22	1.71 ± 1.62	0.353*
No. of Abortion	3.38 ± 1.99	3.71 ± 3.10	0.923*
Residence			
• Rural	12 (75.0%)[&]	18 (58.1%)	0.206**
• Urban	4 (25.0%)	13 (41.9%)	
Exposure to Smoking			
• No	9(56.2%)	15 (48.4%)	0.503**
• Yes	7 (43.8%)	16 (51.6%)	
Exposure to Lead			
• No	10 (62.5%)	30 (96.8%)	0.004**
• Yes	6 (37.5%)	1 (3.2%)	

*Mean ± SD
*Mann-Whitney non-parametric test was used to compare the mean difference between the two groups
**Fisher Exact test analysis was used to compare the difference in proportions
& significant difference between each item for exposed cadmium cases using Chi-square test
--Significance level is considered when $p < 0.05$.

There is increase for the parameter of exposure to smoking against the lead and cadmium levels but not statistically significant (Table 7 & 8 respectively).

Table 7: Comparison between blood Lead levels (µg/L) among exposure to smoking vs.non exposure to smoking in RPL Cases.

	Exposure to smoking (n=5)	Non Exposure to smoking (n=2)	P-value*
	Mean± SD		
• Exposed to Lead (n=7)	300.3 ± 188.6	225.2 ± 168.7	0.857*

*Mann-Whitney non-parametric test was used to compare the mean difference between the two groups
--Significance level is considered when $p < 0.05$.

Table 8: Comparison between blood Cadmium levels (µg/L) among exposure to smoking vs.non exposure to smoking in RPL Cases.

	Exposure to smoking (n=7)	Non Exposure to smoking (n=9)	P-value*
	Mean± SD		
• Exposed to Cadmium (n=16)	46.7 ± 49.8	33.8 ± 46.9	0.536*

*Mann-Whitney non-parametric test was used to compare the mean difference between the two groups
--Significance level is considered when $p < 0.05$.

The calibration curves for lead and cadmium is presented in Fig. 1 A and B.

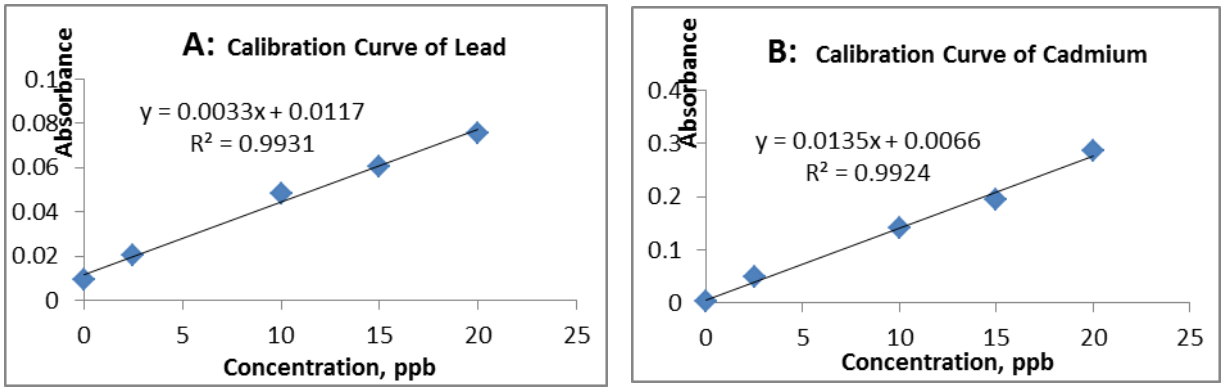

Fig. 1: Calibration curves for A: lead and B: for cadmium.

From these curves, we have calculated the detection limit of cadmium to be 3.646 µg/L and its Limit of quantitation to be 11.37 µg/L. The detection limit of lead is 2.429 µg/L and its limit of quantitation of lead is 8.763 µg/L.

Table 9: Atomic Absorption Data for Patients with RPL

ID	Age	No. abortion	No. children	Exposure to Pesticides	Exposure to Smoking	Pb	Cd
1	32	12	1	NO	Yes	≤ 0.916	≤ 0.314
2	18	7	0	NO	Yes	28.565	≤ 0.314
3	20	2	0	NO	Yes	621.94	≤ 0.314
4	24	3	0	NO	Yes	5.087	≤ 0.314
5	21	3	0	NO	NO	35.413	≤ 0.314
6	25	2	3	NO	NO	≤ 0.916	≤ 0.314
7	27	3	2	NO	NO	5.637	≤ 0.314
8	18	3	0	NO	NO	78.824	22.92
9	25	2	2	Yes	Yes	≤ 0.916	14 ≤ 0.
10	24	3	0	NO	NO	≤ 0.916	≤ 0.314
11	30	2	5	NO	Yes	≤ 0.916	≤ 0.314
12	26	5	0	NO	NO	344.42	3.61
13	22	3	0	NO	Yes	≤ 0.916	≤ 0.314
14	23	2	0	NO	Yes	235.47	23.1
15	21	3	1	NO	Yes	≤ 0.916	≤ 0.314
1	30	2	2	Yes	Yes	≤ 0.916	≤ 0.314
17	24	2	0	Yes	NO	≤ 0.916	≤ 0.314
18	22	2	1	NO	NO	≤ 0.916	0.38
19	29	15	0	NO	NO	≤ 0.916	≤ 0.314
20	33	2	2	NO	NO	21.901	≤ 0.314
21	26	6	1	NO	NO	≤ 0.916	≤ 0.314
22	20	2	1	NO	Yes	≤ 0.916	≤ 0.314
23	35	8	0	NO	NO	70.019	≤ 0.314
24	22	2	1	NO	Yes	≤ 0.916	≤ 0.314
25	19	2	0	NO	Yes	131.59	1.72
26	38	3	2	NO	Yes	9.306	0.91
27	20	2	1	NO	NO	≤ 0.916	≤ 0.314
28	39	3	3	NO	NO	≤ 0.916	≤ 0.314
29	36	3	3	Yes	NO	≤ 0.916	2.72
30	39	2	4	Yes	NO	≤ 0.916	≤ 0.314
31	35	2	2	NO	Yes	≤ 0.916	≤ 0.314
32	30	2	3	NO	Yes	≤ 0.916	≤ 0.314
33	36	2	5	NO	Yes	≤ 0.916	0.72
34	24	2	2	NO	Yes	≤ 0.916	4.68
35	37	2	4	Yes	Yes	17.988	3.39
36	32	6	4	Yes	NO	≤ 0.916	≤ 0.314
37	27	4	1	NO	NO	3.986	113
38	26	2	1	NO	NO	≤ 0.916	57.9
39	37	6	4	Yes	NO	≤ 0.916	135
40	35	2	6	NO	NO	≤ 0.916	66.2
41	27	5	2	NO	NO	≤ 0.916	≤ 0.314
42	45	9	6	NO	NO	105.91	8.65
43	22	2	3	NO	Yes	80.169	3.70
44	38	2	5	NO	NO	1.48	≤ 0.314
45	22	3	0	NO	NO	≤ 0.916	10.434
46	26	2	4	NO	Yes	289.64	82.1
47	35	5	5	NO	Yes	222.93	117.78

3.1 Statistical analysis

These data were assembled in SPSS. Only normally distributed data were evaluated using parametric analysis (paired t-test and Pearson Correlation). The collected data were verified and coded by the researcher, and analyzed by using SPSS/PC (version 21). Descriptive statistics: mean, standard deviation, frequencies, percentage was calculated. Test of significances: Chi square test was used to compare the difference in distribution of frequencies among different groups. Student t-test was calculated to test the mean differences in continuous variables between groups. For non-parametric continuous variables Mann-Whitney-U-test was used. Co-linearity was investigated for the predictors involved in the analysis. Spearman's rank correlations were carried out to explore any possible co-linearity among predictors. A significant p value was considered when it is < 0.05.

Recurrent pregnancy loss (RPL) is a condition defined as three or more consecutive abortions. Miscarriage further specifies that the abortions should occur in the first trimester [32].Currently, 70% of cases of recurrent pregnancy loss (RPL) remain unexplained. It affects 1% of couples trying to conceive. The role of the environment toxins remains in unexplained RPL is poorly understood [3]. Lead is a heavy metal with extensive history as a reproductive toxin [33]. It exerts its toxic effects on cellular functions either by its calcium mimicking effect or by inhibition of the activity of many proteins especially those involved in heme formation pathway through binding their sulfhydryl groups [11][34]. Few evidences from previous studies are available for the effect of sub toxic levels of lead on fertility and human fetus [19]. Calcium, zinc and iron deficiencies that commonly occur during pregnancy may enhance lead absorption from gastrointestinal tract that might exaggerate the status of lead toxicity [17]. In the present study, statistically significant higher maternal whole blood lead levels were found in RPL group than control group. These results were in accord with the results of Cleveland et al**.** [19], and may highlight the possible role of subclinical lead toxicity in the development of RPL.

Cadmium is a toxic metal with long history of deleterious effects [35]. It is a known placental toxicant; with limited transfer to the fetus. It exerts its effects on the fetus mostly through its induced placental dysfunctions especially in smoking women [36]. Cadmium absorption may be enhanced in pregnant women with increased nutrient demand i.e. iron, energy and protein [37]. Being cytotoxic and endocrinal disruptor, cadmium may induce disturbances in the placental nutrient and calcium transport that may lead to RPL [38]. In the present study, the levels of maternal blood cadmium were significantly higher in the RPL group than the control group. These results agreed with [31]. Different residence areas may affect the birth outcomes due to different socio-economic and work conditions [39] or in part due to higher chances of exposure to environmental pollution in urban areas [40]. In the present study, significantly higher RPL were found in pregnant women who lived in urban areas than those who lived in rural areas. The high motor exhaust and petrol stations may explain the significantly higher maternal whole blood lead levels in women who lived in urban areas than those who lived in rural urban areas in the RPL group. These results agreed with the findings of Bellinger [41]. In addition, the significantly increased blood cadmium levels that were observed in women who lived in urban than rural areas within RPL group, in the present study, may be attributed to the indoor cigarette smoking along with narrow and closed flats in urban areas. These results were in accord to [13] [42]. The impact of maternal exposure to smoking during pregnancy and its relative consequences on fetal and infant development is a well known issue [43]. Among other effects, smoking could impair placental development either directly or indirectly by reducing blood flow which can create a hypoxic environment and lead to reduced provision of oxygen and

micronutrients [44]. In the present study, the incidence of smoking was higher in RPL group than control group .These results agreed with [45]. Maternal whole blood lead levels were significantly higher in smokers than non-smoker women within RPL group which may be due to the high concentrations of lead in cigarette smoke [13] or the mobilization of lead from bone stores to blood during pregnancy especially in smokers [46]. Also, in the present study, the observed significantly higher levels of maternal blood cadmium in smokers than non-smokers were in agreement with the findings of Sorkun et al., [25]. The average daily exposure from cigarette smoking (20 cigarette /day) is 2-4μg of cadmium [24]. The positive correlations in the present study between maternal whole blood lead, maternal blood cadmium might denote common sources for these heavy metals (e.g.) smoking**[**13][47] and the risky exposure of pregnant women suffering from RPL to more than one toxic factor [48][49].

4. Conclusion

This study shows significant increases in the levels of lead and cadmium in pregnant women suffering from RPL than those with normal pregnancies. Pregnant women, exposed to tobacco smoke and other sources of pollution especially in urban areas, are at higher risk for such increases. Further studies are needed to clarify the role of heavy metals in the pathogenesis and prediction of RPL.

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