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Assessment Hemolytic Effect of Lead, Cadmium and Copper on Erythrocytes of Pregnant Women

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Abstract **Background:** Heavy metals are naturally occurring elements characterized by their elevated density and atomic weight. These elements are present in varying proportions within the Earth's soil and rock formations, their distribution contingent upon geographical location. Heavy metals, such as arsenic, lead, cadmium, copper, and mercury, have been identified as substances that can adversely affect the reproductive system. These heavy metals are particularly harmful to the developing fetus. **Objective:** to assess the hemolytic effect of lead cadmium copper on the erythrocytes of pregnant women and study reduced glutathione concentration in pregnant women with anemia compared with healthy pregnant. **Material and method:** Blood samples were collected from pregnant women around Kirkuk governorate, Iraq. In addition to 30 samples for glutathione estimation (15 from anemia pregnant women and 15 from control). The study concerns the effect of different concentrations of single metal (20, 40, 60, 80, and 100) mM from Pb, (20, 15,10,5, and 1) mM from Cd, and (10, 7, 5, 3 and 1) mM from Cu individually on the erythrocytes hemolysis. Also, this study concerns the effect of the combined metals on the hemolysis of erythrocytes, which represent double (Cd:Pb), (Pb:Cu) & (Cd:Cu), and triple (Pb:Cd:Cu) metals with different concentrations on erythrocytes hemolysis. Glutathione concentration estimated in three trimesters in pregnant with anemia compared to healthy pregnant. **Results:** The results showed that the hemolysis achieved by accessing the concentration of the metals, with (100) mM pb the range of hemolysis is between (94-96)% of the three trimesters, with (20) mM Cd the range of hemolysis is between (89-99)% of the three trimesters and (10) mM Cu the range of hemolysis is between (65-76)% of 3 trimesters, effect of the metals in first trimester Cd(99%)>>Pb(94%)>>Cu(65%), second trimester Cd(97%)>>Pb(96%)>>Cu(76%) and third trimester Pb(96%)>>Cd(89%)>>Cu(72%). The levels of GSH that there were indicated non-significant ($p \geq 0.05$) difference in all patients and subdivided trimesters compared to the control.

Key Words Hemolysis, Heavy metals, Cadmium, Lead, Copper

1. Introduction

Heavy metals exist naturally and possess a significant density, atomic weight, and specific gravity (about five times greater than water) [1]. Various elements can be found on the Earth, exhibiting varying proportions in soils and rocks, contingent upon their specific geographical distribution. Rocks undergo weathering processes, leading to their liberation and subsequent soil formation. This soil is then subject to biogeochemical cycling, ultimately resulting in the reformation of rocks in a continuous natural cycle [2]. Organic contaminants may break down into innocuous chemicals, but inorganic heavy metals cannot. Thus, they persist and build up in the soil [3]. The extensive range of industrial, agricultural, domestic, medical, and technical applications has resulted in their widespread presence in the environment, prompting apprehension regarding their possible impacts on human health and the ecosystem. The toxicity of substances is influenced by various elements, such as the quantity administered, the specific chemical composition, and the exposure method.

Additionally, individual characteristics such as age, gender, nutritional condition, and genetic predisposition can also contribute to the level of toxicity. The minerals As, Pb, Cr, Cd, and Hg are considered to be among the most hazardous to human health due to their significant toxicity levels. These metallic elements possess systemic toxicity, recognized for impairing several organs, even when exposed to low amounts. Various severe illnesses, including tumors, eyelid edema, gastrointestinal disorders, congestion of nasal mucous membranes and pharynx, head congestion, as well as muscular, neurological, and genetic abnormalities resulting

from exposure to certain heavy metals, have been extensively reported [4]. Hence, the surveillance of heavy metals holds significance in evaluating the safety implications for human health and the environment [5]. Copper plays a crucial role in certain vital cupro-enzymes for sustaining life. For instance, ceruloplasmin, a protein containing copper, facilitates the catalytic conversion of ferric ions to the ferrous form. This enzymatic activity enhances the absorption of iron from the gastrointestinal tract.

Additionally, it contributes to iron mobilization from tissue reserves into the plasma [6]. Copper deficiency throughout the prenatal and embryonic stages can lead to significant morphological and metabolic problems. Multiple documents provide mounting evidence of a notable prevalence of developmental abnormalities, potentially attributed to insufficient nutrition, particularly about copper and embryonic fetal development [7]. Lead is a hazardous heavy metal found in the Earth's crust and widely dispersed and mobilized throughout the environment [7], [8]. Human exposure to lead (Pb) can arise from multiple causes, including battery recycling, the use of lead-based paints, industrial activities such as lead smelting and coal combustion, and the presence of lead-containing pipes or lead-based solder in water supply systems, electrical grids, and bearings, among others. Lead is associated with various biochemical, physiological, and behavioral dysfunctions [9]. Several studies have demonstrated that lead (Pb) induces oxidative damage in various tissues, including the kidneys, heart, reproductive organs, brain, and erythrocytes [10]. Cadmium is classified as a heavy metal and is considered a non-essential element, lacking any recognized physiological role in the human body. However, even at low concentrations, cadmium can display hazardous properties owing to its limited excretion rate. The interaction between the biological system and Cd results in cellular toxicity [11], [12]. The substance gradually builds up inside the skeletal structure, hepatic system, pulmonary organs, renal organs, and neural tissues of the human body, resulting in their impairment and disruption of normal functioning [13], [14]. Cadmium exacerbates renal injury due to its limited renal excretion, as the kidneys serve as the primary site of cadmium accumulation. The substance is conveyed via the circulatory system by albumin and subsequently binds to metallothionein within the liver [15].

The present study investigated the impact of Pb on erythrocytes in an in vitro setting. The results revealed that Pb induces an elevation in intracellular free Ca levels, triggers lipid oxidation, and diminishes glutathione and glutathione oxidation levels. These effects were observed to be both time- and dose-dependent [16]–[18].

Erythrocytes have been recognized as a pertinent cellular model for investigating the cellular-level effects of metal combinations [19]–[21]. Therefore, this study aimed to assess the hemolytic effect of Pb, Cd, and Cu of pregnant women with and without anemia and estimate the glutathione concentration in the anemia pregnant women compared to the control group.

2. Materials and Methods

A. *Subjects and Sample Collection*

This study was done in Kirkuk City, North of Iraq, from September to December 2022. The study includes 27 samples divided into three trimesters (1st, second, and third). The samples were collected from pregnant women without any complications from exposure to heavy metals with different concentrations. All the participants were within the age ranges between (17-43) years. A total of (5 mL) of venous blood was taken through vein puncture with a disposable syringe, and the blood samples were collected using heparin anticoagulant tubes and then separated through centrifugation at (175) Xg for (15) minutes. After centrifugation, The plasma, buffy coat, and uppermost layer of cells were eliminated, resulting in the retention of densely packed erythrocytes. The erythrocytes underwent a triple wash using an isotonic phosphate buffer saline solution (iPBS) with a pH of 7.4 and a concentration of 0.1M. The iPBS solution was prepared by dissolving 8.1 mM (1.44 g) of Na_2HPO_4, 1.9 mM (0.280 g) of Na_2HPO_4, 0.137 M (8 g) of NaCl, and three mM (0.2 g) of KCl in 400 ml of deionized water. The pH of the solution was adjusted to 7.4, and the volume was then brought to 500 ml using deionized water.

Ex vivo Hemolysis Assay [22]

To expose erythrocytes to a single metal in an ex vivo setting, a range of concentrations was used, from $CdCl_2$, $PbCl_2$ and $CuSO_45H_2O$.Preparing (20) mM solution in iPBS solution. From each prepared stock solution, different concentrations of $CdCl_2$ and $PbCl_2$ (1, 5, 10, 15 and 20) mM, and $CuSO_4.5H_2O$ (1, 3, 5, 7 and 10) mM were prepared.

To expose erythrocytes to a double metal in an ex vivo setting, a range of concentrations was used, from (Pb:Cd) is (20:1,40:5,60:10,80:15and100:20) mM, (Pb:Cu) is (20:1,40:3,60:5,80:7and100:10) and (Cd:Cu) is (1:1, 5:3 ,10:5 ,15:7 ,and 20:10) which Prepared in the isoPBS solution.

To expose erythrocytes to a double metal in an ex vivo setting, a range of concentrations was used, from (Pb:Cd:Cu) is (20:1:1,40:5:3,60:10:5,80:15:7and100:20:10) mM which Prepared in the isoPBS solution.

For each experiment, 700μL of 20% erythrocytes suspension was transferred to a plane tube then a volume of 300μL of $CdCl_2$, $PbCl_2$ and $CuSO_4.5H_2O$ solution with different concentrations were added to obtain a total volume of 1 ml mixture. After exposure, the samples were incubated for (2 hours) at ($37^\circ C$) in a shaker water bath. The supernatant absorbance was read using spectrophotometer at (570 nm).

Reduced glutathione estimation

Reduced glutathione (GSH) level was determined by using Uys method [21]

B. *Statistical Analysis*

The data were analyzed using GraphPad Prism 7.1.3.to estimate the P valu e, SEM and SD, the p value is significant

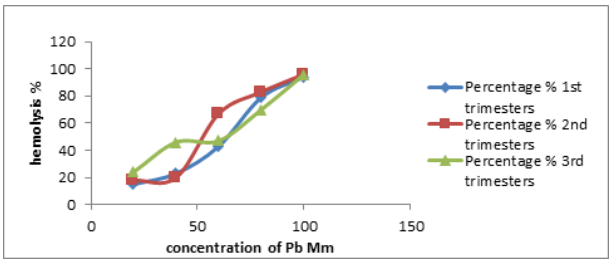

Figure 1: the hemolytic effect of different Pb concentrations on the erythrocytes of pregnant women with 3 trimesters

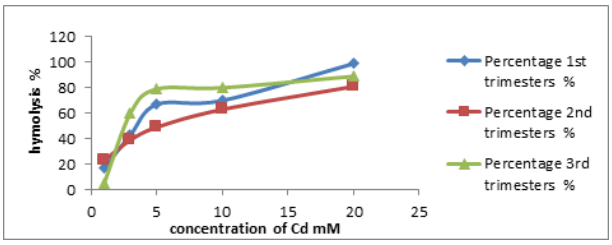

Figure 2: the hemolytic effect of different Cd concentrations on the erythrocytes of pregnant women with 3 trimesters.

(≤ 0.05) increase .

3. Results and discussion

The results of pb, Cd and Cu hemolytic effect on erythrocytes of pregnant women have been presented in the Figures 1, 2 and 3 and Tables 1, 2 and 3 respectively:

The estimation of the fraction of hemolysis resulting from the maximum percentage of breakdown for each metal was conducted utilizing the formulas derived from the distinct functions associated with metal first trimester Cd (99%) >> Pb (94%) >> Cu (65%), in second trimester Cd(97%) >> Pb(96%) >> (76%) and in third trimester Pb(96%) >> Cd(89%) >> Cu(72%) In the study, it was observed that the heavy metals Pb and Cd exhibited comparable levels of toxicity, however Cu shown the highest degree of toxicity.

This conclusion was drawn based on the visual observation of a brownish coloration in the solution. The current investigation aimed to evaluate the potential harmful impacts of lead (Pb), cadmium (Cd), and copper (Cu) on erythrocytes in an ex vivo setting. The findings of the study indicate that the introduction of various concentrations of salts con-

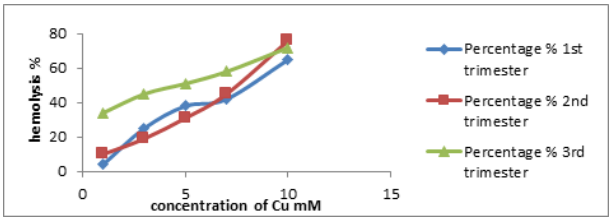

Figure 3: the hemolytic effect of different Cu concentrations on the erythrocytes of pregnant women with 3 trimesters.

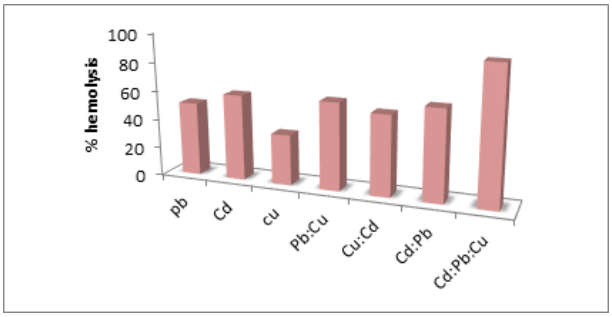

Figure 4: Percentage average hemolysis caused by each studied metals, single, double and triple combinations of human erythrocytes in 1st trimester

taining lead (Pb), cadmium (Cd), and copper (Cu) leads to an elevation in the percentage of hemolysis. It is important to note that the inclusion of heavy metal salts leads to a notable rise in the proportion of hemolysis. The presence of erythrocytes leads to a notable rise in the proportion of hemolysis. The factors above can be ascribed to mechanical injury, metabolic disturbance, and the detrimental effects of oxidative stress. The variables above contribute to lipid peroxidation in the cellular membrane and the oxidation of hemoglobin, resulting in the specific conversion of Fe+2 to Fe+3. Consequently, these processes ultimately hinder the biological functionality of hemoglobin [22], [23].

Compared to other metals, it is widely acknowledged that high levels of copper (Cu) can adversely affect human health. The addition of copper to red blood cells has been seen to lead to a substantial enhancement in the rate of erythrocyte lysis. Furthermore, it has been observed that the exposure of erythrocytes to copper (Cu) results in a color alteration from red to brown. This color change has been employed as an indicator of toxicity in conjunction with the occurrence of hemolysis. Copper (Cu) is a conjunction within Fenton's response, a chemical process that produces hydroxyl radicals (OHo). In addition, the presence of copper induces lipid peroxidation, resulting in a reduction in glutathione levels without any noticeable effect on antioxidant enzymes [24], [25].

Three model combinations in total were examined. Single (Pb, Cd and Cu) two-way combinations consist of (Cu:Cd, Cu:Pb and Cd:Pb) and triple combination includes Cd:Cu:Pb, in three 1st, 2nd, and 3rd, all presented in the Figures 4,5 and 6 respectively.

Statistical comparisons were conducted between combinations of double and triple metals and single metals, revealing substantial differences ($p \leq 0.05$). There is no significant difference in the percentage of hemolysis resulting from the combination of Pb and Cd compared to the individual metals (Pb and Cd). Copper (Cu) and lead (Pb) exhibit distinct characteristics, hence resulting in notable dissimilarities from cadmium (Cd). Specifically, introducing copper to lead led to a substantial rise in the percentage of hemolysis compared to the effects of copper in isolation. The percentage of

Concentration of Pb mM	Hemolysis% in 1st trimester	Hemolysis% in 2nd trimester	Hemolysis % in 3rd trimester
20	15%	18%	24%
40	23%	20%	46%
60	43%	67%	47%
80	79%	83%	70%
100	94%	96%	96%

Table 1: hemolytic effect of different Pb concentrations on the erythrocytes of pregnant women with 3 trimesters

Concentration of Cd mM	Hemolysis% in 1st trimester	Hemolysis% in 2nd trimester	Hemolysis% in 3rd trimester
1	17%	23%	5%
5	43%	39%	60%
10	67%	49%	79%
15	70%	81%	80%
20	99%	96%	89%

Table 2: hemolytic effect of different Cd concentrations on the erythrocytes of pregnant women with 3 trimesters

Concentrations of Cu mM	Hemolysis% in 1st trimester	Hemolysis% in 2nd trimester	Hemolysis% in 3rd trimester
1	4%	10%	10%
3	25%	19%	19%
5	38%	31%	31%
7	42%	45%	45%
10	65%	76%	76%

Table 3: hemolytic effect of different Cu concentrations on the erythrocytes of pregnant women with 3 trimesters

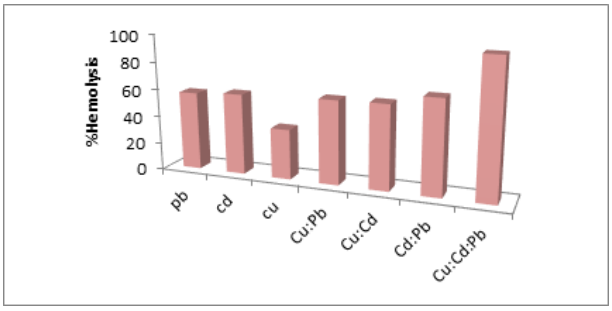

Figure 5: Percentage average hemolysis caused by each studied metals, single, double and triple combinations of human erythrocytes in 2nd trimester

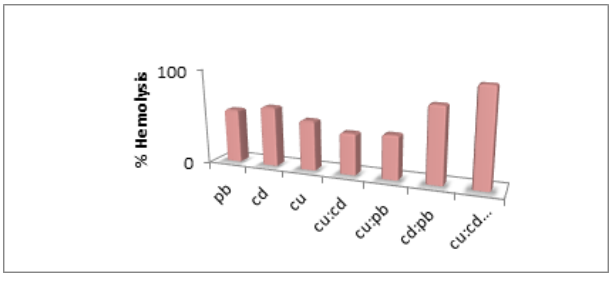

Figure 6: Percentage average hemolysis caused by each studied metals, single, double and triple combinations of human erythrocytes in 3rd trimester

hemolysis resulting from the combination of copper (Cu) and cadmium (Cd) exhibits a notable disparity when compared to the individual percentages of hemolysis induced by each metal (Cu and Cd) in isolation. The hemolytic effects of the triple metal combination, specifically the percentage of hemolysis induced by the combination of Cd, Cu, and Pb, exhibit notable distinctions when compared to the combination's individual metals (Pb, Cd, and Cu). The demonstration highlights the relative significance of hemolysis percentages caused by double metal combinations compared to the combination of three metals. While there are significant differences between Cd: Cu: Pb and Cu: Cd and Cd: Pb, there is no significant difference ($p \geq 0.05$) between Cd: Cu: Pb and Cu: Pb. This implies that adding Cu to Cd: Pb did not result in a statistically significant increase in the percentage of hemolysis. Based on the findings of Uys et al. [21], The hemolysis percentage induced by combinations of two metals, such as Mn: Ni, Mn: Pb, Cd: Ni, and Co: Mn, exhibits distinct outcomes compared to those of individual metals. Similarly, the hemolysis results of triple metal combinations, such as Cd: Mn: Pb and Cd: Co: Ni, differ from those of both single and double metal combinations.

Cytotoxic cells, also known as cytotoxic lymphocytes, play a vital role in the immune system's ability to attack tumor cells. One notable technique utilized to undermine the immune system is the upregulation of co-inhibitory ligands that selectively target receptors on the surfaces of cancer cells. This is why Model Deviation Ratio MDR analysis was created to find medications, pesticides, and, in this case, metal combinations that have biological effects that

are closely connected. The MDR results are used to assess if a metals combination has a synergistic, antagonistic, or additive impact, as cleared in Table 4:

Add% hemolysis of single metals:$Pb + Cu + Cd = 50.8 + 34.8 + 59.2 = 144.8$ Divide observed value of triple metal combination by expected additive value=MDR = 144.8 /47.8 = 3.09 calculated value synergistic interaction > 2 (if calculated value < 0.5: then antagonistic interaction)

Evaluation at a concentration that caused high % hemolysis includes, the combination of Cd:Cu in 1st trimester and 3rd trimester had the highest model (MDR) (1.865) and (2.676) respectively ,indicating an additive and synergistic toxic effect respectively while in 2nd trimester Cd:Pb had the highest model (MDR)(1.697) indicating an additive toxic effect . An antagonistic effect was not observed in this study, synergistic interaction had seen in 3 models in 1st trimester triple combination (Cd:Pb:Cu) MDR was (3.029), second and 3rd trimester double combination (Pb:Cu) & (Cu:Cd) (2.402), (2.677) respectively.

Table 4 comprehensively describes the toxicity associated with double and triple metals across three distinct groupings. Copper plays a crucial role in the human body and, thus, may have a protective impact on metal mixtures. Additionally, it has been observed that lead (Pb) and cadmium (Cd) have an additive effect when rats are simultaneously exposed to these metals [23], which aligns with the findings of the present study. The toxicity of individual metals has been well described. The metals contributing to synergism, Cd, pb, and Cu in 1st and 3rd trimesters are known to cause lipid peroxidation [23]. The process of lipid peroxidation has been found to induce cellular damage, ultimately resulting in apoptosis, as has been documented in the case of Cd and Hg [26]. It has been discovered that the presence of Cd induces alterations in rat erythrocyte membranes, leading to the occurrence of hemolysis. Cadmium has been found to exert an effect on antioxidant enzymes [23], [26]. Copper is considered an essential element, and it can be inferred that its primary function is to provide cellular protection rather than induce synergistic effects. While several metals are necessary for certain biological processes, their toxicity increases significantly when present in large amounts. Copper catalyzes the Fenton reaction, facilitating the production of hydroxyl radicals.

Additionally, it induced lipid peroxidation and resulted in a reduction of glutathione levels [23], however, it did not exert any discernible impact on the activity of the antioxidant enzymes. Metals can interact with multiple antioxidant pathways, leading to the generation of radicals and the inhibition of antioxidant molecules like glutathione and enzymes. The impacts above induce modifications in cellular homeostasis, protein composition, membrane structure, and functionality, ultimately resulting in cellular demise. The observed effect is contingent upon the concentration of metal, duration of exposure, and the specific endpoint assessed, all crucial factors accounting for the variations observed across different investigations [26]. The level of reduced glutathione cleared in the Table 5.

The GSH levels indicated a significant ($p \geq 0.05$) difference in all patients and subdivided trimesters compared to control. Cells' defense against oxidative stress is fundamentally dependent on GSH. It can function as a non-enzymatic antioxidant by directly interacting with ROS through the SH group or as a coenzyme in the enzymatic detoxification processes for ROS. According to several research studies, iron deficiency anemia patients have a lower GSH level than the control group. Besides, these decreases may be due to Pb inactivating glutathione (meant to protect cells against free radicals) by binding to sulfhydryl groups. Consequently, synthesis of GSH from cysteine via the γ-glutamyl cycle occurs, which is usually ineffective in replenishing the supply of GSH, leading to the reduction in the glutathione level [27].

Conflict of Interest

The authors declare no conflict of interests. All authors read and approved final version of the paper.

Authors Contribution

All authors contributed equally in this paper.

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1st trimester							
Metal	pb	Cd	Cu	Cu:Pb	Cu:Cd	Cd:Pb	Cu:Cd:Pb
Average	50.8	59.2	34.8	1.571	1.865	1.762	3.029
SEM	10.56	14.98	8.773	0.292	0.463	0.397	0.727
2nd trimester							
Metal	pb	Cd	cu	Cu:Pb	Cu:Cd	Cd:Pb	Cu:Cd:Pb
Average	56.8	58.6	36.2	1.537	1.554	1.697	1.546
SEM	12.8	14.01	8.100	0.382	0.388	0.424	0.386
3rd trimester							
Metal	pb	cd	cu	Cu:Pb	Cu:Cd	Cd:Pb	Cu:Cd:Pb
Average	56.6	62.6	52	2.402	2.677	1.524	1.718
SEM	12.3	11.8	13.3	0.602	0.668	0.381	0.429

Table 4: MDR results

Subject	Mean (g/l) Trimester 1st	Mean (g/l) Trimester 2nd	Mean (g/l) Trimester 3rd	Mean (g/l) In all trimester	P value
Patients	0.3041	0.300	0.315	0.306±0.013	0.107
Control	0.311	0.332	0.348	0.330±0.061	

Table 5: reduced glutathione level

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