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Evaluation into the Effects of Lead and Cadmium on Hemoglobin and Membrane Proteins in Human Erythrocytes

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Abstract Heavy metals have negative impacts on human health including red blood cells hemolysis and increases oxidation processes, leading to many health issues. In this study, several blood samples were collected from people who work in North Oil Company, Kirkuk, Iraq. In addition to people who live, work away from oil fields as the control group. This investigation looks at the impact of various concentrations of cadmium and / or lead individually and combined (Cd:Pb) on erythrocytes hemolysis. The findings demonstrated that hemolysis produced by the (100 mM) were (72.0, 88.80, and 96.90%) for (Cd, Pb, and Cd: Pb) respectively. Vitamins (B2 and C) were applied with different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 mol/L).to investigate the reduction of hemolysis percentage. The findings showed that both vitamins have positive effect in reducing the hemolysis percentage. Vitamin C has even more significant impact in reducing the hemolysis compared to Vitamin B2. In conclusion, cadmium and lead can cause erythrocyte hemolysis individually or combined, additionally, vitamins C and B2 were proved to in reducing hemolysis effectively.

Key Words hemolysis, heavy metals, cadmium, lead, vitamins

1. Introduction

The reproductive health of indigenous populations is significantly impacted by environmental degradation caused by the exploitation of natural gas and oil. Consequently, polluted drinking water containing physical, chemical, and heavy metal contaminants can give rise to severe health issues, including anemia, kidney failure, weakened immune system [1]. Both natural and artificial sources can contaminate areas with heavy metals. A significant amount of the planet has been contaminated by mining and smelting activities as well as agriculture. [2]. In addition, the ingestion of plants or animals contaminated with these toxic metals has harmful effects on human health such as reproductive systems [3], prostate gland [4], breast cancer development [5], endocrine function [6], neuroendocrine system [7], thyroid function, and can also contribute to obesity [8]. High amounts of heavy metal elements like lead, chromium, zinc, copper, cadmium, manganese, and nickel are directly linked to these disorders. [9]. One major reason for concern is the existence of harmful heavy metals that are not biodegradable in the environment. as even low doses can result in severe harm to the human body [10]. They could resist chemical and biological changes, causing them to build up in vital organs like the kidney, liver, and nervous system. This accumulation can result in toxicity and harm to these tissues [11]. Water reservoirs and pipes experience heightened contamination levels during extraction and transportation operations [1], [12]. Ribarov and Benov [13] have conducted a study to investigate the connection between the destructive effect of heavy metal ions on red blood cells. They explored the correlation between hemolysis caused by metal ions and the production of malonaldehyde. Because it is a component of the Earth's crust, lead (Pb) is a naturally occurring element that can be found in trace amounts in plants, water, and soil. Although Pb itself is relatively stationary, any human interference can render it highly toxic. Sources of environmental Pb contamination encompass air, food, and water [14]. It is a notable source of environmental pollution and elicits pathophysiological reactions in various organs [15]. Although 1 μg/g is the maximum daily consumption limit for lead, prolonged exposure to low quantities can be harmful to human health. Elevated levels of Pb in the body have been linked to an extended duration of puberty in females [16].

On the other hand, Cadmium (Cd) is present in small amounts within the Earth's crust, typically as an impurity found in zinc or Pb deposits. It is mostly created as a byprod-

uct when zinc or lead melts [17]. Immediate exposure to Cd can lead to liver damage, while chronic intoxication manifests as dysfunction in the proximal renal tubules, resulting in a greater loss of urine, calcium, phosphate, low molecular weight proteins, and other materials [18]. Cadmium binds to metallothionein's sulfhydryl groups within the circulation. Approximately 30% of Cd is stored in the liver, another 30% in the kidneys, and the remainder, which has a half-life of 25 years, is dispersed throughout the body. [19]. Oxidative stress brought on by cadmium causes tissue damage. [18]. It also interferes with mitochondrial function, which leads to apoptosis, and hinders heme production [20]. Cadmium triggers the activation of fibroblast growth factor 23, which in turn leads to phosphaturia and reduced phosphate uptake. This process contributes to the development of osteomalacia in bones [21]. Additionally, Cd induces hemolysis, causing the body to become more iron-rich and anemic [22]. Additionally, it increases the synthesis of nonspecific antibodies while lowering the proliferation of lymphocytes and the generation of antigen-specific antibodies [23]. Significant harm to lymphocytes and erythrocytes in the bone marrow was seen during Cd exposure in a research by Suljevic et al. [24]. This study's main goal was to compare the percentage of injured blood cells, including erythrocytes, to healthy blood cells, as earlier research has shown how heavy metals affect blood cells.

2. Materials and Methods

A. Subjects and Sample Collection

The study has included 45 samples divided to two groups: Group I (G1): 15 samples of individuals were exposed to elevated heavy metal concentrations while working in northern oil company and Group II (G2): 30 samples were considered as the control group, with ages ranges between (18 and 45 years) for the two groups.

A single vein puncture using a disposable syringe was used to get a total of five milliliters of intravenous blood. Heparin anticoagulant tubes were used to collect the blood samples, which were subsequently separated by centrifugation at 175 xg for 15 minutes. Packed erythrocytes remained after the buffy coat, top layer of cells, and plasma were removed during centrifugation. Three rounds of washings were performed on these erythrocytes using an isotonic phosphate buffer saline solution (PBS).

B. Ex vivo Hemolysis Assay

In an ex vivo environment, erythrocytes were exposed to a single metal at varying concentrations, starting with CdCl2 and PbCl2.

1) Cadmium Chloride CdCl2: A (100 mM) solution of CdCl2 was prepared in isotonic phosphate buffer saline solution. From this stock solution, varying amounts of CdCl2 (20, 40, 60, 80 and 100 mM) were prepared [25].
2) Lead Chloride PbCl2: PbCl2 was prepared using the same procedure of preparing CdCl2 [23].
3) Reagent (1): The preparation of isotonic phosphate buffer solution (isoPBS) with a pH of 7.4 involved dissolving 8.1 mM, 1.44 g of Na2HPO4, 1.9 mM, 0.280 g of NaH2PO4, 0.137 M, 8 g of NaCl, and 3 mM, 0.2 g of KCl in 400 ml of deionized water. The pH was then adjusted to 7.4, and the volume was then finished using deionized water to a final volume of 500 ml [23].
4) Reagent (2): A (0.5 M) solution of Vitamin B2 was prepared by dissolving Vitamin B2 in reagent (1). From this stock solution, different concentrations of Vitamin B2 (0.1, 0.2, 0.3, 0.4, and 0.5 M) were prepared [25].
5) Reagent (3): Vitamin C was prepared using the same procedure of preparing Vitamin B2 [26].

C. Exposure of Erythrocytes ex vivo to A Single Metal CdCl2 / PbCl2 [25]

A volume of 300μL of CdCl2 and/or PbCl2 solution with varying concentrations (20, 40, 60, 80, and 100 mM) was added to a 700μL volume of 20% erythrocyte suspension in a plane tube for each experiment. This resulted in a mixed volume of 1 mL. Following exposure, the samples were incubated in a shaker water bath at 37 $^{\circ}C$ for two hours. The absorbance of the supernatant was measured at 570 nm using a spectrophotometer.

The experiment was conducted with (700μL) of 20% erythrocytes suspension being exposed to (300μL) of higher concentrations of hemolysis of the single metals with (300μL) of varied concentrations of Vitamin C or Vitamin B2 (0.1, 0.2, 0.3, 0.4, and 0.5 M) respectively. The experiment was also repeated with (700μL) of 20% erythrocytes suspension exposed to (300μL) double metals combinations at varying concentrations.

D. Control Solutions

The following is how the control solutions were made:

1) Zero% hemolysis (blank): 300μL of PBS solution was added to 700 μL of erythrocyte solution.
2) 100% hemolysis: 300μL of (Tween20) was added to 700μL of erythrocyte solution.

Hemolysis percentage was estimated as follows [25]:

$$\%Hemolysis = \frac{(Sample - Blank)}{(100\%Hemolysis - Blank)} \times 100$$

E. Exposure of Erythrocytes ex vivo to A Double Metal CdCl2 : PbCl2

A volume of 150μL CdCl2 (100 mM) and 150μL PbCl2 (100 mM) were mixed, and different concentrations of (20, 40, 60, and 80 mM) were prepared. For each concentration, (300μL) of each mixture solution was mixed with 700μL of erythrocytes. Following exposure, the samples were incubated in a shaker water bath at 37 $^{\circ}C$ for two hours. A spectrophotometer set at (570 nm) was used to measure the absorbance of the supernatant [27].

F. Estimation of Cd and Pb Using ICP Technique

Vasconcelos et al. [28] and Tripathi et al. [26] techniques were employed in this investigation. Standard Solutions and Reagents BDH provided the ultrapure TCA. Perkin Elmer (USA) standard solutions containing 1,000 ppm for each element under test were used to generate working standards for inductively coupled plasma optical emission spectrometry (ICP-OES) analysis. Analytical grade reagents made up the remaining batch. To reduce the possibility of dust and ambient air contamination, all work was conducted on a clean bench.

G. Statistical Analysis

GraphPad Prism version 7.1.3 was used to analyze the data and get the P value, SEM, and SD.

H. Results and Discussion

Over the past few decades, there has been an increase in heavy metal pollution in the environment, which poses major threats to all biological systems, particularly those that include food and drinking water. Two examples of heavy metals that are bad for your health are cadmium and lead. The first goal of this study is to evaluate the Cd and Pb toxicity in erythrocytes of G1 (workers in Northern Oil Company) compared to G2 (people who live and work away from oil fields) indicated that there was higher toxic effect of these two heavy metals. It can be noticed that Cd and Pb revealed varying levels of toxicity, with Pb being the most toxic. The hazardous element cadmium is non-biodegradable and can have a number of negative consequences on blood [29]. The diet may contribute to mercury pollution since it originates from both the environment and different food sources [29]. The food chain then allows cadmium to pass from other animals to people, where it damages the lungs [30], liver [31], and kidneys [32] and causes hypertension [33]. Cadmium is accumulated in numerous organs day after day because of chronic exposure to low amounts of cadmium, causing negative impacts on the kidneys [32], liver [31], and testes [3].

Continuous exposure to this hazardous metal caused inflammatory infiltration and hepatocyte necrosis. [34]. In addition to affecting metabolism, lead is neurotoxic and can damage hemopoiesis, renal function, the neurological system, and the gastrointestinal tract [34]. It also has an impact on human's central nervous system leading to many disorders that are related to the activity of human as well as the quotient intelligent that is lowered due to the exposure to lead [35]. While approximately 95% of lead is deposited as insoluble phosphate in skeletal bones, the distribution of lead in the body is first determined by blood flow into different organs. Azeez and Zainal have determined the hemolytic effect of some heavy metals including arsenic, lead, cadmium, copper, and mercury in pregnant women using the ICP technique [36].

The concentration of the Cd and Pb in the blood of Groups I and II were illustrated as (mean ± SD) in Table 1 using Inductively Coupled Plasma (ICP) technique.

Metal	Group I (n=15) mean + SD	Group II (n=30) mean + SD	P value
Cd ng / ml	1.959 ± 0.916	10.34 ± 8.042	($P \leq 0.05$)
Pb μg / dL	6.609 ± 4.971	27.53 ± 26.53	($P \leq 0.05$)

Table 1: The concentrations of Cd and Pb in the blood of both studied groups using ICP

Concentration (mM)	Hemolysis%		
	Cd	Pb	Cd: Pb
20	1.91%	2.76%	2.87%
40	15.9%	9.08%	15.26%
60	28.02%	25.60%	31.90%
80	48.07%	45.90%	88%
100	72%	88.80%	96.90%
Average	34.428	33.184	46.986

Table 2: Hemolytic effect of single metals Cd ,Pb and combined metals Cd : Pb in human RBC

It can be clearly seen from (Table 1) that the concentrations of the two studied metals Cd and Pb are significantly high in Group I (10.34 ± 8.042 ng/ml) and (10.34 ± 8.042) for Cd and Pb respectively Group II (1.959 ± 0.916 ng/ml) and (6.609 ± 4.971 μg/dL) for Cd and Pb respectively, and both metals were significantly high in the Group I ($p \leq 0.05$). It can be noticed that Cd and Pb revealed varying levels of toxicity, with Pb being the most toxic. Many health hazards have been linked to heavy metal toxicity, which has been shown to be a serious hazard. The second goal of this study is an attempt to explore the harmful ex vivo effects of Cd and Pb on erythrocytes. To determine the metal concentration at which hemolysis occurs, erythrocytes were exposed to increasing concentrations of each metal separately and in combination with varying quantities of Cd and Pb.

When double metal Cd:Pb combinations were statistically high (96.9%) compared to single metal hemolysis (72.0%) and (88.8%) for the hemolysis caused by Cd and Pb respectively, where substantial differences were found. The % hemolysis of Cd:Pb differs greatly from the % hemolysis generated by the single metals (Cd or Pb alone) that make them up. Hemolysis percentage caused by lower concentrations of Cd, Pb, and the combined metals Cd:Pb were increasing subjected to the effect depending on each concentration. The least % hemolysis has been seen when using the lowest concentration (20 mM) of the single or double metal Cd, Pb or Cd:Pb, as the achieved % hemolysis reached (1.91, 2.76 and 2.87%) respectively, as shown in Table 2.

Table 3 showed the average % hemolysis of erythrocyte suspension in group I erythrocytes via single and double metal exposure, these results were calculated through the statistical analysis of the data collected.

When double metal Cd: Pb combinations were statistically compared to single metals in Group I, substantial differences were found. The % hemolysis of Cd:Pb is very different from the hemolysis that each of the constituent metals (Cd or Pb) produces. Hemolysis results reached (34.428, 33.184, and

Heavy Metal	Cd	Pb	Cd: Pb
Average Hemolysis %	34.428	33.184	46.986
SEM	20.99	18.75	23.83

Table 3: The average hemolysis% of single and double metal on human erythrocytes Group I

Metal	Cd:Pb
Synergistic*	—-
Antagonistic**	0.41
Additive***	—-

Table 4: MDR calculation based on hemolysis assay

46.986%) for Cd, Pb, and Cd:Pb respectively.

Although there were statistically significant differences between single and double metal Cd:Pb combination, this does not always imply a biological relationship. Furthermore, statistical analysis cannot distinguish between differences caused by synergistic or antagonistic effects. As a result, The Model Deviation Ration (MDR) analysis was developed to identify metal combinations, pesticides, and pharmaceuticals with equivalent biological effects. The MDR results showed a value of (0.41), with an antagonistic behaviour, even when compared to these made by Batool et al [37]. The latter study found that the MDR was equal to 0.245 for the combined metals Fe:Cu:Ni that caused the hemolysis and treated after that using vitamins C and B2 to reduce the % hemolysis. To determine whether a combination of metals has an additive, antagonistic, or synergistic effect, utilize the MDR values in Table 4.

According to research, vitamin C significantly reduces the availability of metals for animals by acting as an enzyme co-factor and chain-breaking antioxidant [29], The final objective of this study was to determine how the vitamins C and B2 affected the percentage of hemolysis in erythrocytes. To this end, the subjects were exposed to increasing concentrations of each vitamin, with the concentration of Cd or Pb causing the highest percentage of hemolysis. The degree of % hemolysis was evaluated after 2 hours incubation. The highest values of % hemolysis reduction were (57.58%) and (47.78%) for (Cd:vitamin C) and (Pb:vitamin B2) respectively, when applying the concentration of (0.5 M) in Group I. While the highest % hemolysis reduction values in Group II reached (47.78% and (43.61%) for (Cd:vitamin C) and Pb:vitamin C) respectively when applying the concentration of (0.5 M) (See Table 5). As for the double metal, the highest % hemolysis reduction values were (52.23%) and (40.27%) for (Cd:Pb:vitamin B2) and (Cd:Pb:vitamin B2) respectively, when applying the concentration of (0.5 mM) (See Table 6). A study by Khan et al found that vitamins are very effective and a chain breaker antioxidant, as well as being a cofactor of the enzyme that works against the toxicity of heavy metals. The resulting values of hemolysis reduction are in good agreement with the literature [29].

In In this investigation, varied concentrations of Vit. (C and B2) treated with (Cd, Pb) resulted in a decrease in the rate of % hemolysis caused by Cd and Pb. These results support earlier research showing that micronutrients from fruits, including phenolic compounds, vitamin B2, vitamin A, and vitamin C, inhibit heavy metal-induced toxicity by reducing lipid peroxidation [26], [29], [38]. Vitamin C with Cd and Pb causes a significant decrease in % hemolysis at high concentrations where the % hemolysis is greater when using B2 vitamin, This study shows that the inhibitory effects of (Cd, Pb) metals on hemolysis resulted in a decrease in the percentage of hemolysis of vitamins C and B2. The interaction of vitamin C with erythrocytes was thought to cause membrane lipid peroxidation and hemoglobin oxidation (oxidation of Fe+2 to Fe+3) [39], [40]. If the vitamin concentration is relatively high, this mechanism results in hemolysis of the erythrocytes (low vitamin concentrations function as antioxidants). The results of this investigation were compared to other results from the literature. Batool et al showed that the lower concentrations of vitamins C and B2 had greater effects on reducing hemolysis, which is opposite to the results in this research where the highest concentrations of the vitamins had the greater effect on reducing % hemolysis [37]. Emmanuel et al. found in another investigation that the suppression of hemolysis (4.1-18%) was achieved by using very low concentrations ranged between (20 to 100 μg/mL) of C, B1, B2, B6, D and K vitamins. The same study claimed that the inhibition of protein denaturation and proteinase enzyme reached (71 and 30%) respectively [41]. Finally, vitamins such as C and E were considered to have improved results in terms of curing the unexplained genetical hemolysis effectively (Table 7) [42].

3. Conclusions

To sum up, this research concerned the induction of hemolysis process of erythrocytes caused by different concentrations of Cd and Pb (20, 40, 60, 80, and 100 mM) as individual heavy metals or combined. The highest %hemolysis (96.9%) was caused by the concentration of (100 mM) of Cd: Pb combination. This was followed by a successful attempt to reduce the hemolysis percentages using vitamins C and B2 with varying amounts (0.1, 0.2, 0.3, 0.4, and 0.5 mol/L), Where the most reduced hemolysis (60.58%) was achieved by using vitamin C along with the Cd: Pb double metal combination.

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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Conc. (mol/L)	Hemolysis % in Group I				Hemolysis % in Group II			
	Cd:Vitamin		Pb:Vitamin		Cd:Vitamin		Pb:Vitamin	
	vit.C	vit.B2	vit.C	vit.B2	vit.C	vit.B2	vit.C	vit.B2
0.1	72.31	61.24	65.53	73.42	66.29	57.73	67.8	65.49
0.2	68.45	56.59	62.93	68.89	61.30	50.61	60.41	58.49
0.3	65.57	54.15	58.87	65.06	56.47	42.94	55.01	50.1
0.4	61.43	50.02	53.13	57.44	51.44	43.27	48.55	42.30
0.5	57.58	50.22	45.02	51.45	47.78	43	43.61	37.96

Table 5: The inhibition of single heavy metals by vitamin C and vitamin B2

Conc. (mol/L)	Hemolysis % in Group I		Hemolysis % in Group II	
	vit.C	vit.B2	vit.C	vit.B2
0.1	81.09	87.08	72.12	68.58
0.2	65.45	69.41	67.53	65.12
0.3	59.03	61.59	61.09	56.36
0.4	48.89	59.68	52.42	44.86
0.5	42.54	52.23	36.32	40.27

Table 6: The inhibition of % hemolysis induced by combination heavy metals using vitamin C and / or vitamin

Cd:Vit. C (mol/L)	% hemolysis	Reduced %Hemolysis
0.1	1.55%	70.45%
0.2	6.43%	65.57%
0.3	10.57%	61.43%
0.4	14.42%	57.58%
0.5	18.22%	53.78%
Cd: Vit. B2 (mol/L)	% hemolysis	Reduced %Hemolysis
0.1	15.41%	56.59%
0.2	17.85%	54.15%
0.3	21.98%	50.02%
0.4	21.78%	50.22%
0.5	33.57%	38.43%
Pb: Vit. C (mol/L)	% hemolysis	Reduced %Hemolysis
0.1	25.87%	62.93%
0.2	29.93%	58.87%
0.3	35.67%	53.13%
0.4	43.78%	45.02%
0.5	48.49%	40.31%
Pb: Vit. B2 (mol/L)	% hemolysis	Reduced %Hemolysis
0.1	19.91%	68.89%
0.2	23.74%	65.06%
0.3	31.36%	57.44%
0.4	37.35%	51.45%
0.5	51.00%	37.80%
Cd+Pb: Vit. C (mol/L)	% hemolysis	Reduced %Hemolysis
0.1	15.81%	81.09%
0.2	31.45%	65.45%
0.3	37.87%	59.03%
0.4	48.01%	48.89%
0.5	54.36%	42.54%
Cd+Pb: Vit. B2 (mol/L)	% hemolysis	Reduced %Hemolysis
0.1	9.82%	87.08%
0.2	27.49%	69.41%
0.3	35.31%	61.59%
0.4	37.22%	59.68%
0.5	44.67%	52.23%

Table 7: The effect of vitamins (C and B2) on % hemolysis of Group I erythrocytes treated with Cd, Pb and Cd: Pb (100mM)

Cd: Vit. C (mol/L)	% hemolysis	Reduced %Hemolysis
0.1	5.71%	66.29%
0.2	10.70%	61.30%
0.3	15.53%	56.47%
0.4	20.56%	51.44%
0.5	24.22%	47.78%
Cd: Vit. B2 (mol/L)	% hemolysis	Reduced %Hemolysis
0.1	14.27%	57.73%
0.2	21.39%	50.61%
0.3	29.06%	42.94%
0.4	28.73%	43.27%
0.5	29%	43%
Pb: Vit. C (mol/L)	% hemolysis	Reduced %Hemolysis
0.1	21%	67.80%
0.2	28.39%	60.41%
0.3	33.79%	55.01%
0.4	40.25%	48.55%
0.5	35.19%	53.61%
Pb: Vit. B2 (mol/L)	% hemolysis	Reduced %Hemolysis
0.1	23.31%	65.49%
0.2	30.31%	58.49%
0.3	38.70%	50.10%
0.4	46.50%	42.30%
0.5	51.84%	37.96%
Cd+Pb: Vit. C (mol/L)	% hemolysis	Reduced %Hemolysis
0.1	24.78%	72.12%
0.2	29.37%	67.53%
0.3	35.81%	61.09%
0.4	44.48%	52.42%
0.5	60.58%	36.32%
Cd+Pb: Vit. B2 (mol/L)	% hemolysis	Reduced %Hemolysis
0.1	28.32%	68.58%
0.2	31.78%	65.12%
0.3	40.54%	56.36%
0.4	52.04%	44.86%
0.5	56.63%	40.27%

Table 8: The effect of vitamins C and B2 on % hemolysis of Group II erythrocytes treated with Cd (100mM)

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