

Impact of Selected Metals and Vitamins (C, B₆, B₉) on Oxidative Damage in Human Erythrocytes

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ABSTRACT

This study evaluated the toxic effects of selected heavy metals on human erythrocytes *ex vivo*, focusing on both direct toxicity and oxidative damage. The results indicated that the toxicity of the tested metals followed the order: K >> Cu >> Ba >> Zn >> Ag. Additionally, the study examined the impact of vitamins B₆, B₉, and C on the percentage of hemolysis in erythrocytes exposed to heavy metals. The findings showed that increasing the concentration of these vitamins reduced the percentage of hemolysis compared to the hemolysis induced by K, Cu, Ba, Ag, and Zn. However, specific combinations, including Vit. C: K, Vit. B₆: Ba, Vit. B₆: K, Vit. B₉: Ba, and Vit. B₉: Ag, led to an increase in hemolysis at higher vitamin concentrations. This suggests that, under certain conditions, excessive levels of vitamins (C, B₆ and B₉) may enhance erythrocyte damage in the presence of these metals. The study also assessed the toxicity of the vitamins themselves, with the following toxicity order: Vit. C >> Vit. B₉ >> Vit. B₆. Both vitamin C and vitamin B₉ increased absorbance due to hemolysis, indicating their stronger hemolytic effects. Among the 15 analyzed metal-vitamin combinations, 13 exhibited a Model Deviation Ratio (MDR) greater than 0.5, indicating an additive toxic effect. In contrast, three combinations showed an antagonistic effect, with the most significant interactions observed in Vit. B₉: Cu (MDR = 0.215), Vit. B₉: K (MDR = 0.48), and Vit. C: K (MDR = 0.4). Overall, this study highlights the complex interactions between heavy metals and vitamins in human erythrocytes and underscores the importance of understanding these relationships to mitigate heavy metal-induced toxicity.

Keywords: Heavy Metals, Hemolysis, MDR, Vitamin C, Vitamin B₆, Vitamin B₉

INTRODUCTION

Heavy metals are naturally occurring elements that exist in varying concentrations across ecosystems, found in both elemental form and various chemical compounds. Some of these metals are volatile, while others attach to fine particles, enabling their transport over long distances [1]. The chemical form of a heavy metal determines its behavior within the food web and influences its toxicity. Human activities have significantly altered the biochemical cycles and natural balance of certain heavy metals, resulting in a range of environmental and biological consequences [2]. One major consequence of heavy metal exposure is oxidative damage, which plays a critical role in various physiological and pathological processes. Due to its significance, oxidative damage has been extensively studied, with erythrocytes frequently used as a model system [3]. Oxidative stress in erythrocytes affects overall cell integrity, including hemoglobin and membrane structures, making them ideal for studying oxidative mechanisms and their potential implications [4].

Oxidative Stress and Hemolysis

Several hypotheses have been proposed to explain the mechanism of erythrocyte hemolysis under oxidative stress, both *in vivo* and *in vitro*. Hemoglobin is considered the primary target of oxidative damage when exposed to certain agents [5]. However, under specific oxidative conditions, the erythrocyte membrane becomes the primary site of injury, ultimately leading to hemolysis. A key process involved in oxidative damage is the oxidative denaturation of hemoglobin, which occurs when increased intracellular oxidant levels disrupt its structure [6]. This results in the formation of intracellular aggregates of denatured hemoglobin, known as Heinz bodies, which are directly associated with the development of hemolytic anemia. While many chemical compounds can induce hemoglobin oxidation, the precise

molecular mechanisms underlying this process remain unclear. Although vitamins are generally considered nontoxic, excessive intake can lead to adverse effects, including oxidative stress [7].

Methods for Assessing Oxidative Damage

Various techniques have been developed to evaluate the antioxidant activity of different compounds [8]. These include pulse radiolysis and methods that assess the inhibition of oxidation in chemical and biological systems [9]. Among these techniques, hemolysis assays have been widely used to measure oxidative damage caused by free radicals [10]. Previous studies have demonstrated that ascorbic acid (Vitamin C) can induce erythrocyte hemolysis through oxidative mechanisms, a phenomenon referred to as oxidative hemolysis [11].

Objective of the Study

The current study is to examine the hemolytic impact of specific heavy metals and water-soluble vitamins, including pyridoxine (B₆), folic acid (B₉), and ascorbic acid (C), when supplied at relatively high concentrations, in light of the possible oxidative effects of these chemicals. Gaining knowledge about these substances' oxidative effects on erythrocytes could help us better understand how they contribute to hemolysis and illnesses linked to oxidative stress.

MATERIALS AND METHODS

Collection and Preparation of Blood Samples

Five milliliters of whole blood were drawn from the antecubital vein of seventy healthy donors, ages 18 to 35, for each trial. To avoid coagulation, the samples were immediately put in EDTA tubes. They

were then centrifuged for ten minutes at 2500 xg. Every technique was carried out strictly in accordance with institutions policies regarding the safety of biohazards and research involving human subjects. None of the donors had taken any medications in the week before blood collection in the week before blood collection in order to guarantee the study's correctness.

After centrifugation, the packed erythrocytes were carefully cleaned of any remaining plasma and other impurities using a saline solution three times. The plasma, buffy coat, and upper cell layer were then carefully removed.

Ex Vivo Hemolysis Assay

Experimental Procedure

In each experiment, 0.5 ml of a metal or vitamin solution containing Cu, Zn, Ag, Ba, K, Vitamin B₉, Vitamin B₆ or Vitamin C at varying concentration was mixed individually with 1 ml of erythrocyte solution in a plain tube.

After that, the samples were incubation, they were vortexed and centrifuged for two minutes at 2500xg. To evaluate hemolysis, the supernatant's absorbance was measured spectrophotometrically at 570 nm.

Solution for Control

In order to guarantee precise measurement, the following control samples were made: 1. Zero hemolysis (blank): 1ml of erythrocytes solution was mixed with 0.5 ml of phosphate buffer solution. 2. Complete hemolysis (100% hemolysis): 1ml of erythrocyte solution was mixed with 0.5 ml of Tween 20.

Calculating Hemolysis and Other Experiments

The following formula was used to calculate the hemolysis percentage:

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

In subsequent tests, erythrocytes were exposed to 0.5 ml of the most hemolytic concentration of a single metal along with 0.5ml of different

vitamin B₆, vitamin B₉, vitamin C concentration. This made it possible to assess how they could affect hemolysis.

Determination the Combination Effect of Heavy Metals

Determination of Combination Effect To assess the type of interaction—whether antagonistic, additive, or synergistic—an effect addition model was utilized, based on the Model Deviation Ratio (MDR) approach developed by Cedergreen [12]. The effect of individual metals or vitamins was compared to the effect of their corresponding mixtures. The following formula was used to determine the MDR:

$$\text{MDR} = O_v / E_v$$

Where E_v is the predicted effect value and O_v is the observed impact value (% hemolysis). If the MDR is between 0.5 and 2, it indicates an additive effect; if it is greater than 2, it suggests antagonism; and if it is less than 0.5, it indicates synergism. Cedergreen's method of removing false positives and concentrating on combinations where the amplitude of the interaction has the potential to be quantitatively significant served as the foundation for these criteria [12].

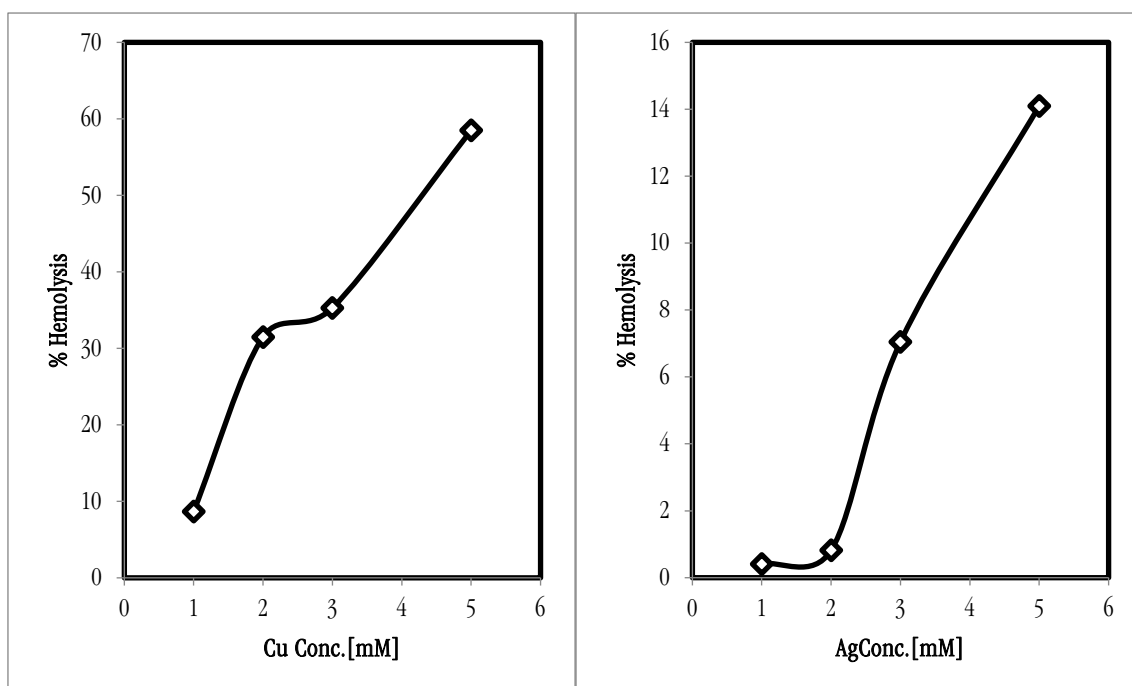
Data Management and Statistical Analysis

Because of the large sample size for analyzing groups, exposure data for a particular mineral is calculated as the average of four independent trials, and for vitamin C, vitamin B₆, vitamin B₉ and vitamins and minerals complexes, four separate tests are employed, each of which yields a single data point. Each data point in the results, which are displayed as the mean $\pm$ standard error of the mean (SEM), is the average of the four trials.

RESULTS AND DISCUSSION

Heavy metal toxicity to erythrocytes

Erythrocytes were subjected to increasing concentration of each metal in order to determine the concentration at which hemolysis occurs. The degree of hemolysis was assessed following a 2- hour incubation period, as illustrated in figure 1.



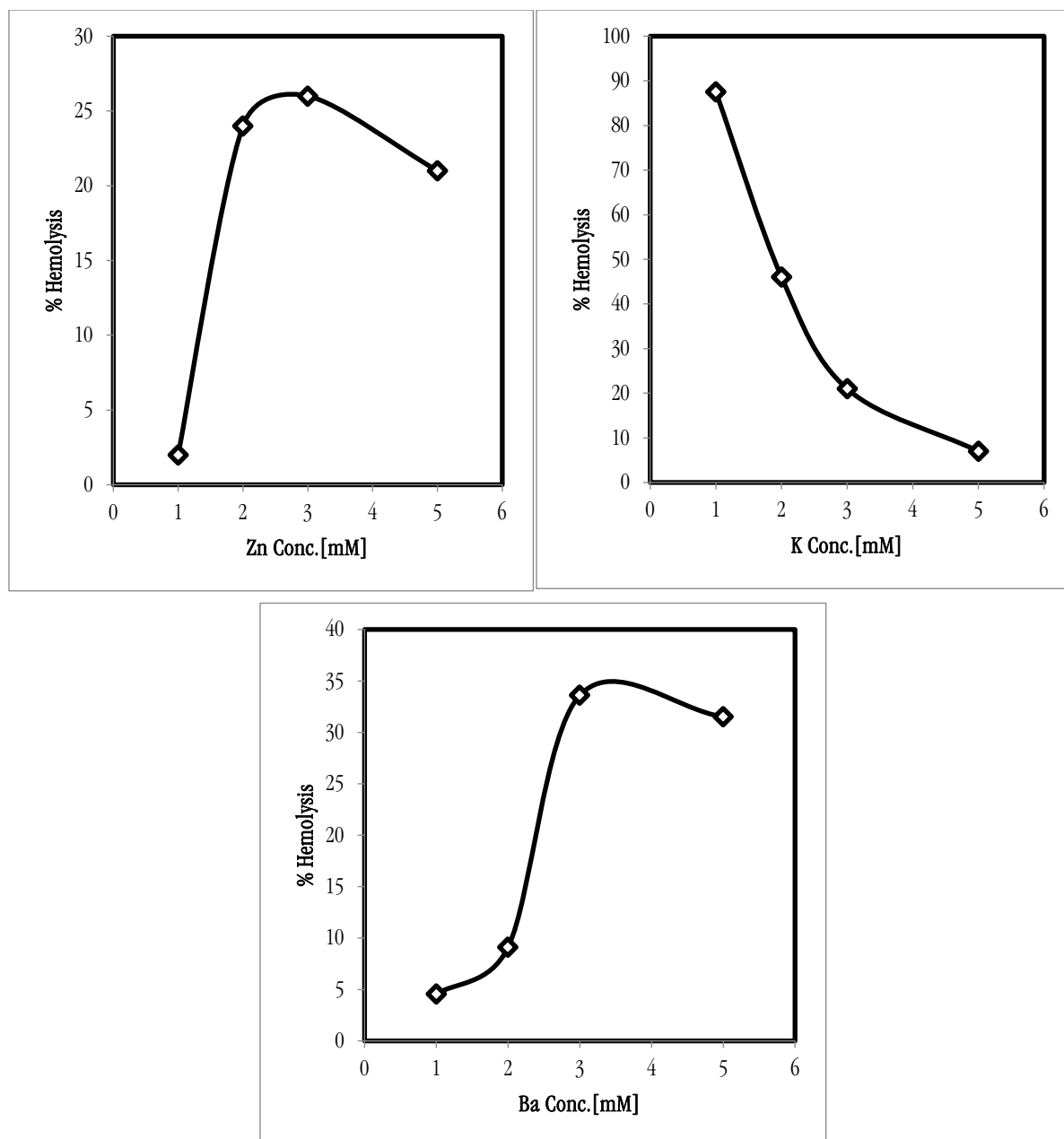


Figure 1: The Impact of Rising Cu, Ag, Zn, Ba, And K Concentrations on the Induction of Hemolysis in Human Erythrocytes was Examined.

Heavy metal toxicity to erythrocytes using unique function formulas, the percentage of hemolysis caused by each metal's greatest breakdown levels was determined. The findings were as follows: Potassium (87.55%) > Copper (58.5%) > Barium (33.6%) > Zinc (26%) > Silver (14.1%). Potassium and copper were the most poisonous metals examined, whereas zinc, barium, and silver had comparable levels of toxicity. Figure 4 depicts the average percentage of hemolysis generated by each metal, vitamin, and their mixture at their determined concentration.

Heavy metals pose serious health hazards, and their toxicity is still a major concern. This study looked at the harmful effects of copper (Cu), potassium (K), zinc (Zn), barium (Ba), and silver (Ag) on erythrocytes in vitro. The data demonstrated that exposure to various doses of these metal salts resulted in increased hemolysis. Erythrocytes exposed to these metals experienced mechanical damage, biochemical disturbances, and oxidative stress, which contributed to lipid

peroxidation of the cell membrane and oxidation of hemoglobin (from Fe^{2+} to Fe^{3+}), ultimately impairing the biological function of hemoglobin [13].

Among the metals, Copper was identified as the most harmful. The addition of Copper to erythrocytes significantly increased the hemolysis rate. Additionally, erythrocytes exposed to Copper changed color from red to brown, further indicating toxicity. Copper acts as a cofactor in the Fenton reaction, generating hydroxyl radicals ($\text{OH}^{\bullet}$), which induce lipid peroxidation and decrease glutathione levels, although it does not directly affect antioxidant enzymes [14, 15].

In contrast, increasing the concentration of Potassium reduced osmotic fragility, with hemolysis decreasing to 7%. Zinc, Barium, and Silver exhibited similar toxicity levels. Zinc interacted with both hemoglobin and erythrocyte membranes, and although it increased osmotic stability in vivo, it induced hemolysis in vitro. This suggests that Zinc may have therapeutic potential for conditions such as sickle

cell anemia. Barium, however, did not induce hemolysis at higher concentrations, and its cytotoxicity decreased at elevated levels [16]. Silver was found to be the least cytotoxic, likely due to the aggregation of Silver ions, which reduced the bioavailability of free Ag⁺ ions [17].

Hemolytic Effect of Water-Soluble Vitamins on Erythrocytes

The percentage of hemolysis induced by the highest decomposition levels of each vitamin was calculated as follows: Vitamin C (20.8%) > Vitamin B₉ (20.75%) > Vitamin B₆ (13.5%). The hemolytic effects of water-soluble vitamins B₆, B₉, and C on erythrocytes were studied spectrophotometrically at relatively high concentrations [9]. The oxidation mechanism of hemoglobin was consistent across all three vitamins. Vitamin C and Vitamin B₉ were

identified as the most potent hemolytic agents, while Vitamin B₆ exhibited the weakest effect.

The kinetics of hemoglobin breakdown in erythrocytes exposed to varying concentrations of ascorbic acid (2, 3, 4, and 5 mM) or folic acid (2, 3, 4, and 5 mM) is shown in Figure 2. The absorbance increased with higher concentrations, indicating a greater degree of hemolysis (hemoglobin breakdown). These results suggest that the degree of hemolysis is directly correlated with the concentration of either ascorbic acid or folic acid, with higher concentrations leading to more significant hemolysis. The data also demonstrated that erythrocyte numbers decreased rapidly at higher concentrations, confirming that hemolysis increases as the concentration of these vitamins rises [11, 18].

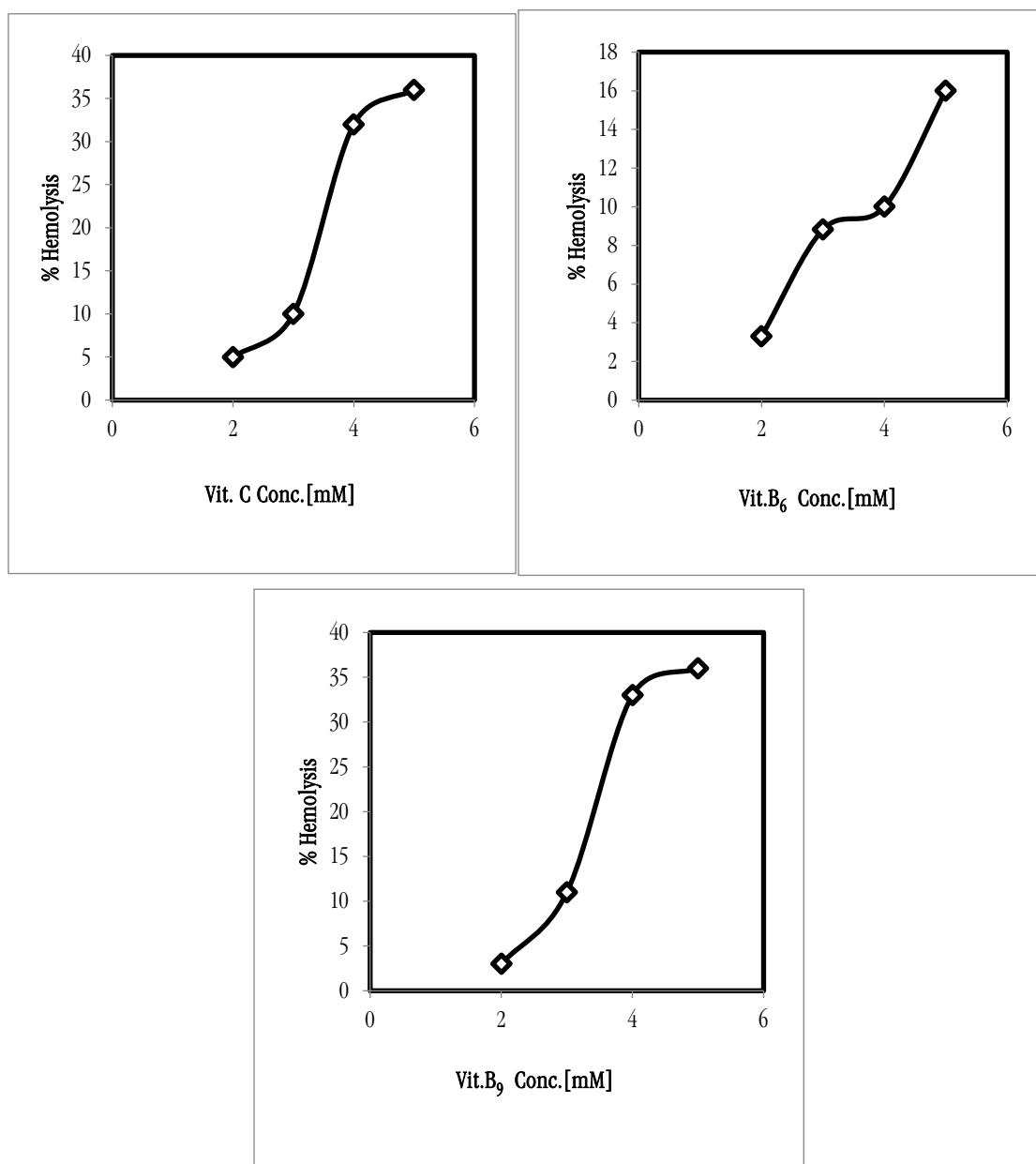
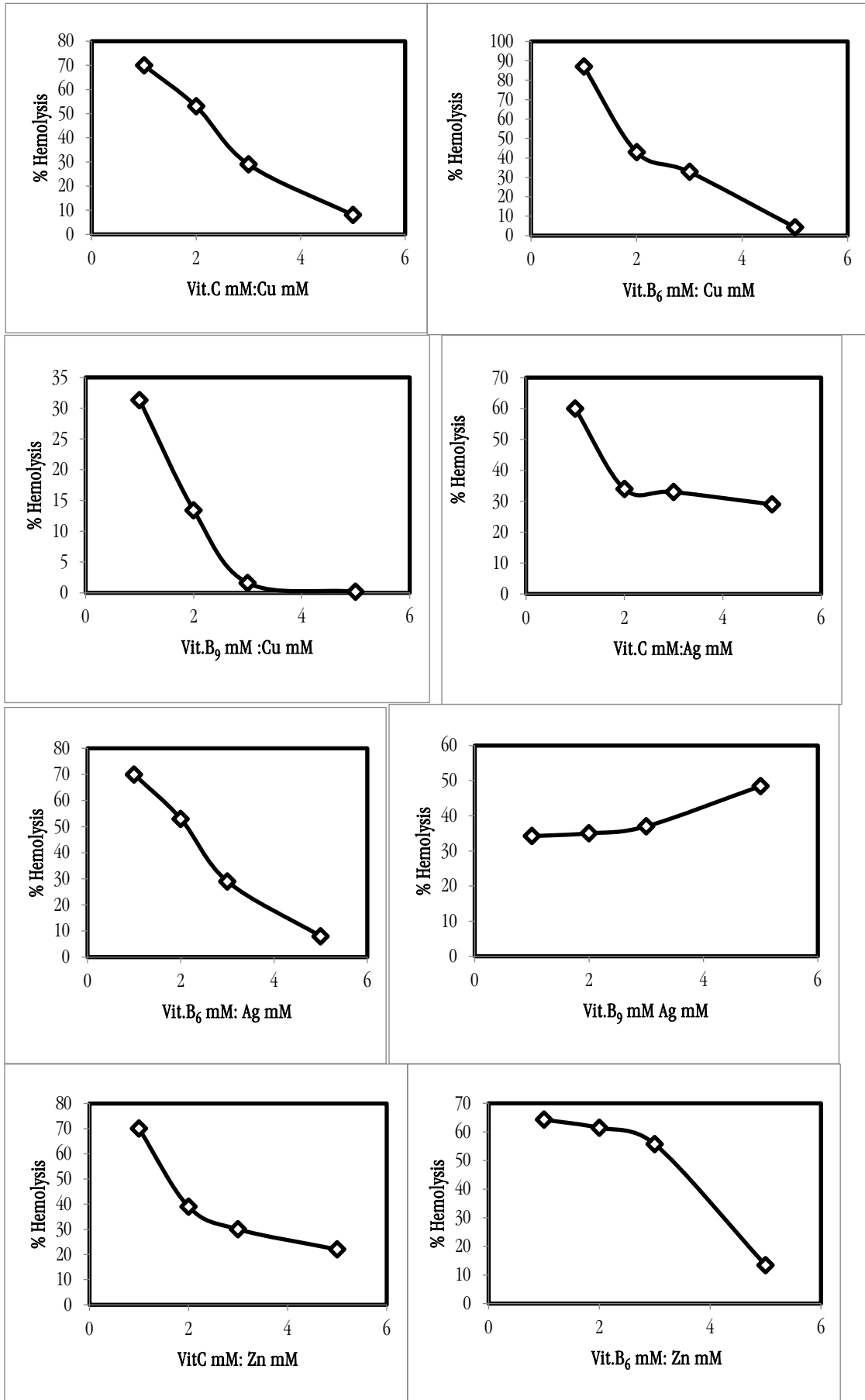


FIGURE 2: The Effect of Increasing Concentrations of Vitamin C, Vitamin B₆ And Vitamin B₉ on the Induction of Hemolysis in Human Erythrocytes was Investigated.

The Effect of Vitamins (C, B₆ And B₉) on % Hemolysis of Erythrocytes When Compared with Heavy Metal The effect of vitamins (C, B₆, and B₉) on the percentage of hemolysis in erythrocytes was evaluated in comparison with heavy metals. Erythrocytes were exposed

to increasing concentrations of vitamins (C, B₆, and B₉) along with the concentrations of Cu, K, Zn, Ba, or Ag that caused higher percentages of hemolysis. After 2 hours of incubation, the extent of hemolysis was measured, as shown in Figure 3.



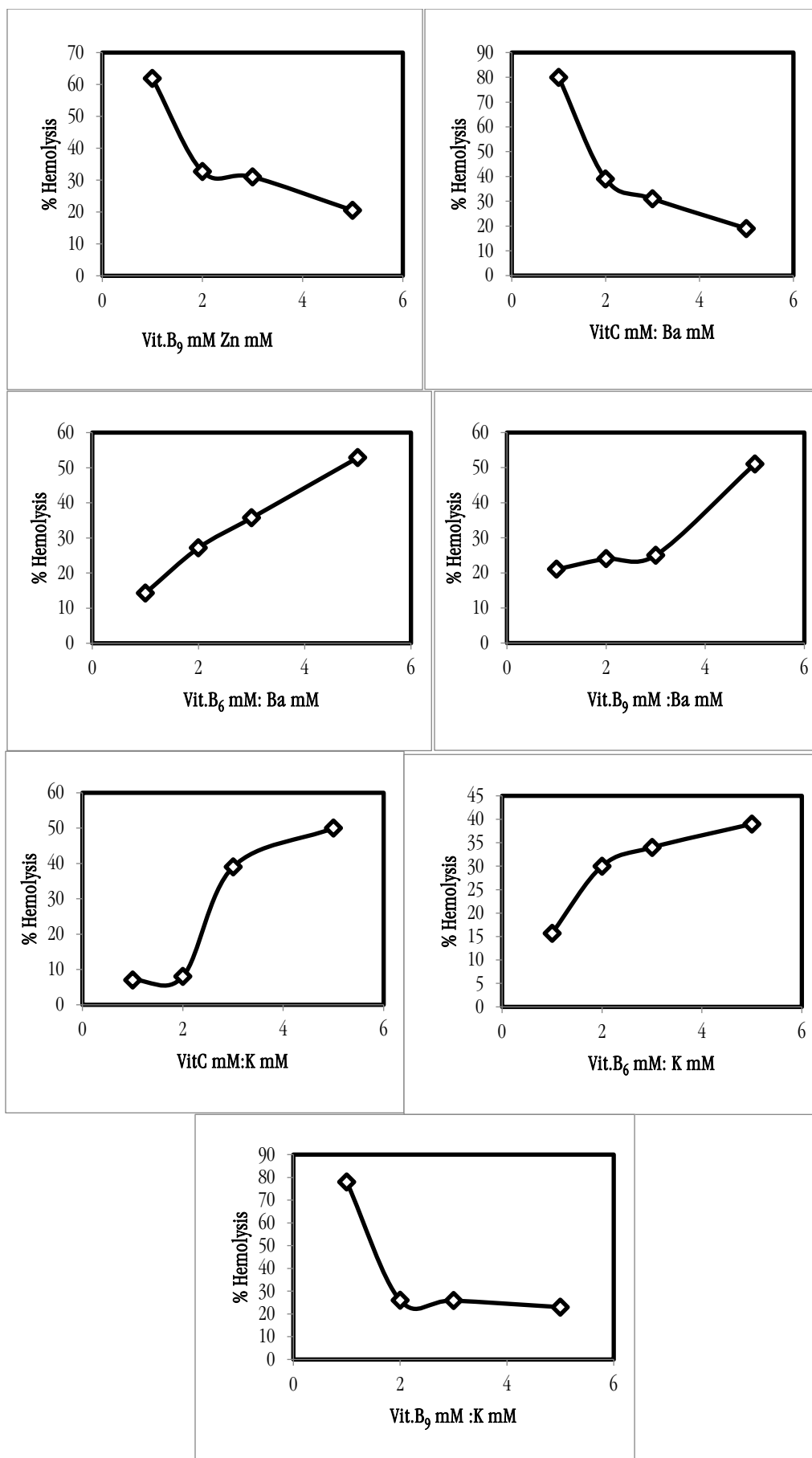


FIGURE 3: The Effect to increase the Concentration of (Vit. C, Vit B₆ or Vit. B₉) when Compared with Heavy Metal to Induce Hemolysis in Human

The study found that exposing erythrocytes to varying amounts of vitamins C, B₆, and B₉, together with metals including Cu, Ba, Ag, K, and Zn, reduced hemolysis rates. However, as vitamin concentrations climbed, their protective effect diminished in comparison to the inhibitory effects of metals.

Previous study has shown that micronutrients from fruits, such as vitamins A, C, B₂, folic acid, and phenolic compounds, can protect against heavy metal- induced toxicity by reducing lipid peroxidation. Combining vitamin C, B₆ and B₉ with higher quantities of Ba, K, or Ag resulted in increased hemolysis. Vitamins at high concentration produced a higher percentage of hemolysis than metals such as Ba, Ag, and K.

This implies that vitamins C, B₆, and B₉ may stimulate hemolysis at high concentrations by interacting with erythrocytes, causing lipid peroxidation of the membrane, and oxidizing hemoglobin (Fe²⁺ to Fe³⁺), which results in cell rupture [11, 19]. This effect was especially noticeable in the samples where Vitamin C was combined with K, Vitamin B₆ with Ba and K, and Vitamin B₉ with Ba and Ag. At high concentrations, these combinations produced a higher percentage of hemolysis, outstriking the inhibitory effects of Ba, Ag, and K alone. At lower doses, however, vitamins (C, B₆, and B₉) act as antioxidants, reducing oxidative stress and inhibiting hemolysis, especially when paired with metals such as K, Ag, and Ba [13, 20].

Antagonistic, Synergistic, and Additive Hemolytic Effects of Metals and Vitamins

A total of 15 metal-vitamin combinations were analyzed, including Vit.B₆: Ag, Vit.B₆: Zn, Vit.B₆: Ba, Vit.B₆: Cu, Vit. B₉: Ag, Vit.B₉: Zn, Vit.B₉: Ba, Vit.B₉: Cu, Vit.B₉: K, Vit.C: Ag, Vit.C: Zn, Vit. C: Ba, Vit. C: Cu and Vit. C: K. Statistical comparisons between these combinations and individual metals or vitamins revealed significant differences. The percentage of hemolysis induced by the metal-vitamin mixtures was notably different from that caused by the individual vitamins alone. However, in the cases of K and Cu, no significant difference in hemolysis was observed between the individual metals and their corresponding metal-vitamin combinations. While statistically significant differences were observed between single and combined exposures to metals and vitamins, these differences do not necessarily imply a direct biological relationship. Moreover, statistical analysis alone cannot determine whether these variations result from synergistic, antagonistic, or additive interactions. To overcome this limitation, the Model Deviation Ratio (MDR) analysis was employed. Originally developed to evaluate drug and insecticide interactions, this method was applied in the present study to assess the combined effects of metals and vitamins [13]. The MDR analysis helped determine whether these mixtures exhibited synergistic, antagonistic, or additive effects, with the findings summarized in figure 4.

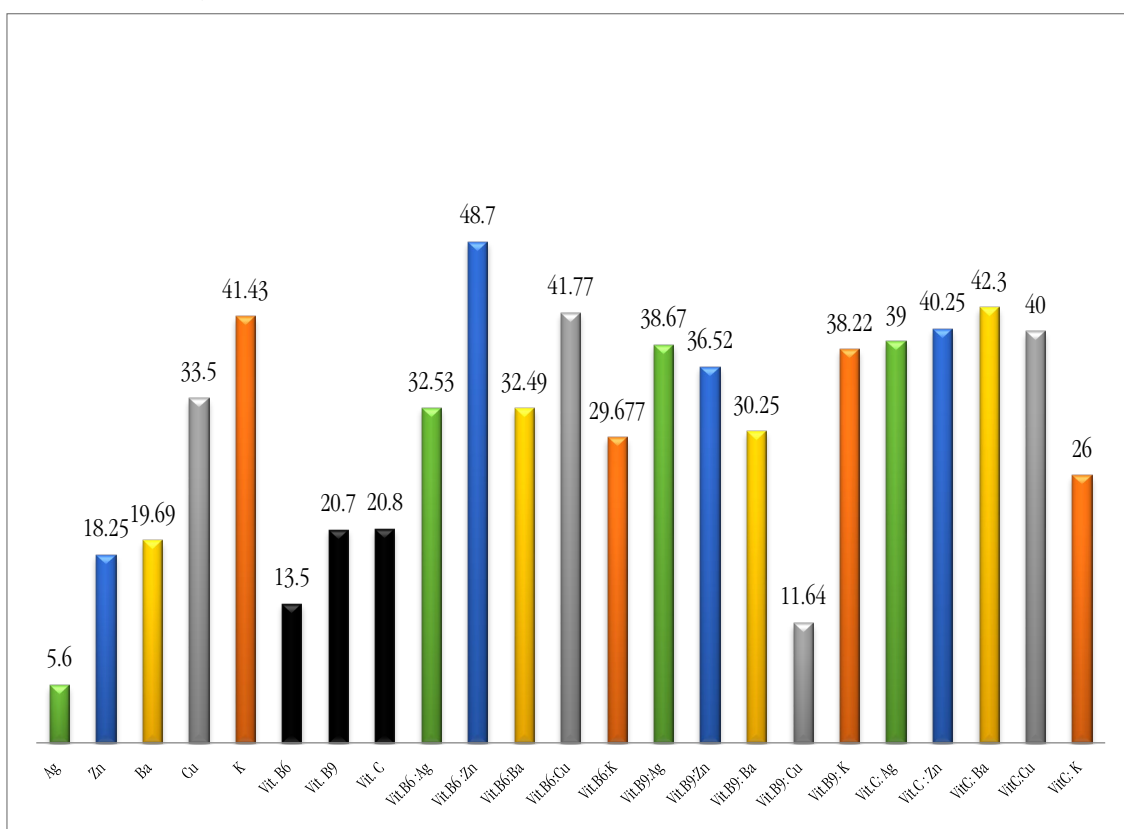


Figure 4: Average Percentage of Hemolysis Caused by Each Heavy Metal, Vitamin, and their Mixtures in Human Erythrocytes Studied.

To determine the interaction type, the % hemolysis of individual metals and vitamins was first summed: K + Vit. B₉ = 41.43 + 38.22 = 79.65. The observed hemolysis value for the K and Vit. B₉ combination was then divided by the expected additive value: MDR = 38.22 / 79.65 = 0.47. Since the calculated MDR value is less than 0.5, this indicates an antagonistic interaction. If the MDR value had been greater than 2, it would have suggested a synergistic interaction. Among the 15

analyzed metal-vitamin combinations, 13 exhibited a Model Deviation Ratio (MDR) greater than 0.5, indicating an additive toxic effect. However, three combinations demonstrated an antagonistic effect, with the strongest observed in the Vit B₉: Cu, Vit B₉: K and Vit.C: K combinations, which had MDR values of 0.215, 0.48 and 0.4, respectively. The detailed MDR values for all combinations are presented in Table 1.

Table 1: Synergistic, Antagonistic, and Additive Effects of Metal and Vitamin Mixtures Based on MDR Calculation.

Metals And Vitamins	Syn	Ant	Add
Vit.B ₆ : Ag	-	-	1.7
Vit. B ₆ : Zn	-	-	1.53
Vit. B ₆ : Ba	-	-	0.97
Vit. B ₆ : Cu	-	-	0.888
Vit. B ₆ : K	-	-	0.54
Vit.B ₉ : Ag	-	-	1.46
Vit. B ₉ : Zn	-	-	0.93
Vit. B ₉ : Ba	-	-	0.74
Vit.B ₉ : Cu	-	0.215	-
Vit.B ₉ : K	-	0.48	-
Vit.C: Ag	-	-	1.47
Vit.C: Zn	-	-	1.03
Vitc: Ba	-	-	1.043
Vitc: Cu	-	-	0.736
Vitc: K	-	0.4	-

Conclusion

Conclusions the key findings of this study can be summarized as follows:

1. This study demonstrated that exposure to various heavy metals, including Cu, Zn, Ba, K, and Ag, at different concentrations, significantly increased the percentage of hemolysis in human erythrocytes. These results indicate that these metals may damage erythrocyte membranes, ultimately leading to cell lysis.

2. The hemolytic effects of water-soluble vitamins (B₉, B₆, and C) on erythrocytes were assessed ex vivo using spectrophotometric analysis at relatively high concentrations. Among the tested vitamins, vitamin C exhibited the strongest hemolytic effect, while vitamin B₆ showed the weakest.

3. Vitamins B₆, B₉, and C displayed a protective effect against heavy metal-induced hemolysis. However, at higher concentrations, the reduction in hemolysis was less pronounced compared to the inhibitory effects of heavy metals alone. This suggests that these vitamins may help counteract the toxic effects of heavy metals on erythrocyte membranes, possibly by preventing lipid peroxidation and hemoglobin oxidation.

4. At high concentrations, specific vitamin-metal combinations, including Vit. C with K, Vit. B₆ with Ba and K, and Vit. B₉ with Ba and

Ag, resulted in an increased hemolysis percentage, surpassing the inhibitory effect of Ba, Ag, and K alone. These findings suggest that excessive levels of these vitamins, particularly in the presence of certain heavy metals, may intensify erythrocyte damage rather than provide protection.

5. Among the 15 analyzed metal-vitamin combinations, 13 exhibited a Model Deviation Ratio (MDR) greater than 0.5, indicating an additive toxic effect. However, three combinations demonstrated an antagonistic interaction, with the most significant effects observed in Vit. B₉: Cu (MDR = 0.4941), Vit. B₉: K (MDR = 0.4832) and Vit.C: K (MDR=0.4). The detailed MDR values for all combinations are presented in Table 1.

6. The findings of this study support previous research suggesting that micronutrients derived from fruits, such as vitamins B₆, C and B₉, offer protective effects against heavy metal-induced toxicity by preventing lipid peroxidation.

7. The objectives of this study were successfully addressed by analyzing the effects of different heavy metal concentrations on erythrocyte hemolysis and investigating the role of vitamins B₆, B₉, and C in modulating this process. The results highlight the importance of understanding the interactions between heavy metals and antioxidants, offering valuable insights into potential strategies for mitigating heavy metal-induced toxicity.

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