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The Effect of Zinc, Magnesium, and Copper Ions on the Activity of Alkaline Phosphatase Enzyme and Ceruloplasmin Protein in Hemolyzed Blood Samples

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Hemolysis is a prevalent source of discrepancies in blood sampling. This study, involving 68 volunteers, systematically assessed the impact of hemolysis on Alkaline phosphatase (ALP) and Ceruloplasmin (Cp) levels, as well as the interplay of Zinc (Zn), Magnesium (Mg), and Copper (Cu) ions. Blood samples (n=68) were subjected to mechanical trauma to produce varying degrees of hemolysis - mild, moderate, and severe. After hemolysis occurred, the ALP activities were measured and recorded as 74.94±15.98 IU/L, 63.47±18.51 IU/L, and 50.29±17.23 IU/L respectively, against 89.47±18.13 IU/L in non-hemolyzed samples. Cp levels were found to decrease in a manner dependent on the degree of hemolysis to 38.53±5.14 mg/dL, 36.29±4.85 mg/dL, and 34.12±5.08 mg/dL from an initial 41.06±6.13 mg/dL in non-hemolyzed samples. The ion concentrations (Mg, Zn, Cu) in hemolyzed blood samples exhibited a direct correlation with the extent of hemolysis. When specific concentrations of these ions equivalent to those measured during severe blood hemolysis were introduced to non-hemolyzed samples, the activity of the ALP enzyme was notably reduced ($p<0.001$). The concentration of Cp decreased upon the addition of Mg and Zn alone. These findings highlight the crucial role of hemolysis and metal ions in influencing the level of ALP and Cp.

Keywords: Alkaline phosphatase; Ceruloplasmin; Copper; Hemolysis; Magnesium; Zinc.

Hemolysis, the rupture of red blood cells, remains a consistent challenge in modern diagnostic procedures.[1] Numerous intracellular constituents, including important electrolytes like Zn, Mg, and Cu, are released into the plasma during such occurrences.[2] The disruption that

these electrolytes bring about in both the activity of (ALP and the concentration of Cp has been an area of sustained scientific curiosity. A previous and contemporary study,[3] have often underlined ALP's diagnostic significance for liver and bone disorders, with an emphasis on its reliance on Zn and Mg as vital cofactors. However, there is conflicting evidence in the literature regarding the impact of hemolysis on ALP activity, The effect of hemolysis on ALP activity, however, have sparked considerable debate. A previous report demonstrated a decrease in Alkaline phosphatase activity in hemolysed samples.[4] Some studies observed no significant effect and concluded that low ALP levels in adults may indicate a deficiency in magnesium and zinc ions.[5,6] Ceruloplasmin (Cp) is a copper-rich protein found in the blood that stores and transports copper throughout the body. It is well-known for its function in Wilson disease development and regulation of iron metabolism and is regarded as a valuable diagnostic tool, aiding in the detection of liver disorders such as hepatitis and liver fibrosis.[7] Very few studies have investigated the potential effect of hemolysis on the activity of Cp. One study found a significant decrease in the concentration of Cp following hemolysis.[8] Other findings reported that deficiency of copper level may decrease the level of ceruloplasmin.[9] Yet, the implications of hemolysis on its activity have only been sporadically addressed in the literature.[10] Several studies have underscored the profound effects of hemolysis on various enzyme activities, indicating that the phenomenon often leads to clinical misinterpretations and potential misdiagnoses.[11–13]

One of the most significant issues in handling blood samples is thought to be the hemolysis of red blood cells. Numerous laboratory test results are impacted by this issue. This study focused on the tests (ALP and Cp) that caused a great lot of disagreement due to the distinct variations in how blood hemolysis affected them. Furthermore, the true cause of this is unknown. A significant finding in this study is the identification of the potential cause for the apparent effect of hemolysis on ALP. This investigation has concluded that understanding the cause of this impact is necessary to address the issue of hemolysis's impact on these tests. This study situates itself within this broader academic conversation, seeking to reconcile historical and current findings on the interactions between zinc, magnesium, and copper ions and enzyme like ALP and Cp protein in non-hemolyzed samples. Our goal in investigating the complex interplay of hemolysis and its effects on enzyme function is to provide a detailed perspective that will serve as the foundation for informed clinical analysis and diagnosis.

MATERIALS AND METHODS

Preparation of blood samples

Venous blood samples were collected from sixty-eight healthy individuals (n=68). Hemoglobin (Hb) levels were measured to ensure homolysis-free blood samples. Moreover, three additional blood samples were withdrawn from each volunteer and subjected to mechanical trauma by centrifugation 2, 4, and 8 times to achieve mildly, moderately, and severely hemolyzed blood samples respectively. The serum was separated from the blood sample by using the Electra BL - 12 Tube Lab Centrifuge (ElectraMed, USA) at 3500 rpm for 5 minutes.

Determination of Hemoglobin (Hb) concentration

The Hb levels in all blood samples were measured using a Gen5 Multimode plate reader (Biotek, USA) with 10 mg/100 mL Na_2CO_3 solution as a reagent. The absorbance was measured at 415, 450, and 700 nm for both hemolysed and non-hemolysed samples. The total serum hemoglobin was calculated using the following formula[14]:

$$Hb = 154.7 \times (A425) - 130.7 \times (A450) - 123.9 \times (A700) \quad ...(1)$$

Determination of Alkaline Phosphatase (ALP) Activity

The activity of ALP in the blood samples was measured using a previously established procedure.[15] Briefly, 20 mL of serum was gently mixed with 1 ml of working reagent containing ALP substrate. The mixture was placed in an incubator (Sanyo Electrical Co., Ltd, Japan) at 37 °C for 3 minutes and the absorbances were measured every minute at 405 nm using Multimode plate reader (Gen5, Biotek, USA). The ALP activity was calculated according to the following formula[15]:

(U/L)=(ΔA/min)x 2764

...(2)

Determination of Ceruloplasmin (Cp) Activity

Cp activity was measured according to the procedure described previously.[16] Under the optimal pH conditions and by the addition of polyethylene glycol, the anti-ceruloplasmin antiserum reacts with its corresponding antigen to induce turbidity. The degree of formed turbidity reflects the concentration of Cp in the sample.[16] The absorbance was measured at 340 nm using a Multimode plate reader (Gen5 ,Biotek, USA).

Determination of Zn^{+2} concentration in hemolyzed and non-hemolyzed blood samples

The concentration of Zn+2 ions in blood samples was measured according to a procedure described previously.[17] A total of 400 mL of work reagent was transferred to the appropriate reagent bottle. Then 250 iL of serum, distilled water, or standard were added to glass test tubes. The mixtures were left at room temperature for 10 minutes and the absorbance was read at 578 nm on a biochemistry analyzer (HumaStar 200, human company, REF16895. Germany). The concentration of Zn^{+2} was determined from a standard curve prepared from zinc standard concentrations.

Determination of Mg^{+2} concentration in hemolyzed and non-hemolyzed blood samples

Mg^{+2} levels were measured according to the method previously described.[18] 50 iL of serum, standard and distilled water was mixed with 1 mL of working reagent. The mixture was incubated for 10 minutes at room temperature and the absorbance was measured at 546 nm using a biochemistry analyzer (HumaStar 200, human company, REF16895. Germany).

Determination of Cu^{+2} concentration in hemolyzed and non-hemolyzed blood samples

The concentration of Cu^{+2} in plasma samples was determined using copper-colorimetric method.[17] Briefly, 12 uL of distilled water, standard, and plasma samples were mixed with 240 uL of working solution. The mixture was incubated at room temperature for 10 minutes. The absorbance was then measured at 578 nm by a Multimode plate reader (Gen5, Biotek, USA). The concentration of Cu^{+2} in samples was calculated from a standard curve prepared from copper standard concentration.

ALP Activity and the Level of Cp in non Hemolysed non hemolysed Blood Samples to Which Mg, Zinc and Cu are Added

Concentrations equivalent to those found in severely hemolyzed blood samples, namely 3.3 mg/dl for magnesium, 181.35 µg/dl for zinc, and 182.79 µg/dl for copper, were prepared. To non-hemolyzed blood samples, 1ml of these concentrations was added separately. After incubation for 10 minutes, ALP activity and the level of ceruloplasmin protein were measured following the same procedure as described earlier.[4,9]

Statistical analysis

Data were represented as mean ± standard deviation (SD). One-way ANOVA was used to analyse the mean difference. Tukey Honestly Significant Difference test was used for all post-hoc analyses to determine which group differed significantly. The two-tailed p-value of 0.05 or lower was interpreted as statistically significant. Statistical analyses were performed using IBM SPSS statistical software version 26.0. The images were created using a GraphPad Prism version 9.2.0.

RESULTS

The findings showed that the moderately hemolyzed samples (160.41±56.5 µg/dL) and severely hemolyzed samples (181.35±58.9 µg/dL) had significantly higher Zn+2 levels than the non-hemolyzed samples (117.00±45.3 µg/dL). On the other hand, mild hemolysis did not significantly alter Zn+2 levels (Figure 1).

In parallel with these results, hemolysis significantly affected the serum levels of Mg^{+2} and Cu^{+2} compared to the non-hemolyzed samples. This was indicated by increased Mg^{+2} and Cu^{+2} concentrations in a hemolysis-dependent manner (figure 2,3).

In contrast to Zn^{+2}, mild hemolysis induced a significant increase in the Mg^{+2} (2.36±0.16 mg/dl) and Cu^{+2} (168.08±15.54µg/dl) serum concentrations compared to non-hemolyzed samples (2.16±0.30 mg/dl ; 168.08±15.54 µg/dl ; $p<0.01$ respectively) (Figure 2,3). The concentrations of hemoglobin in hemolyzed blood samples significantly increased in mild (0.24±0.03 g/l), moderate (0.77±0.068 g/l)

and severe hemolysis (3.24±0.1185 g/l) (P<0.001) compared to nonhemolysed samples (0.12±0.007 g/l)(Figure 4).

The activity of ALP and ceruloplasmin was measured in hemolyzed blood samples (mild, moderate, and severe) and compared to non-hemolyzed samples. The activity of ALP was significantly decreased after hemolysis in a hemolysis-dependent manner. Specifically, mild, moderate, and severe hemolysis induced ALP activities of 74.94±15.98 IU/L, 63.47±18.51 IU/L, and 50.29±17.23 IU/L respectively, compared to 89.47±18.13 IU/L in non-hemolyzed samples (Figure 5).

Hemolysis significantly reduced serum ceruloplasmin levels, with mild, moderate, and severe hemolysis resulting in 38.53±5.14 mg/dL, 36.29±4.85 mg/dL, and 34.12±5.08 mg/dL,

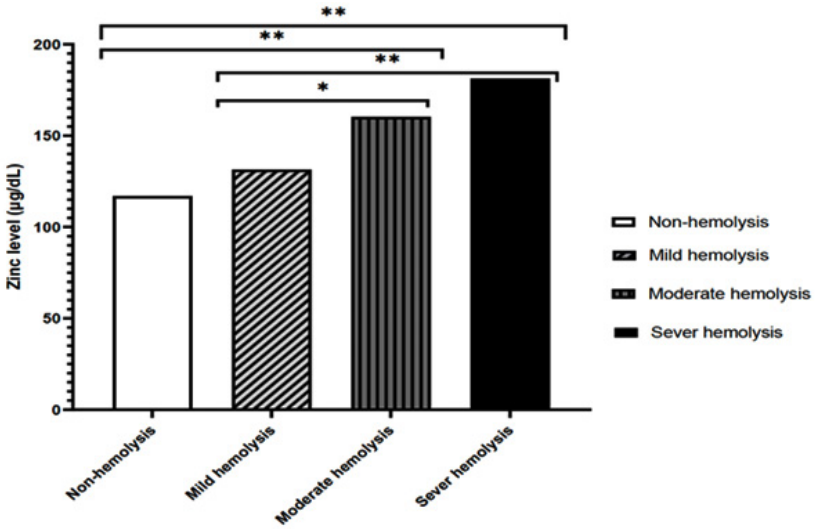

Fig. 1. Levels of serum Zn^{+2} in the samples with varying degrees of hemolysis p** < 0.01, p* < 0.05

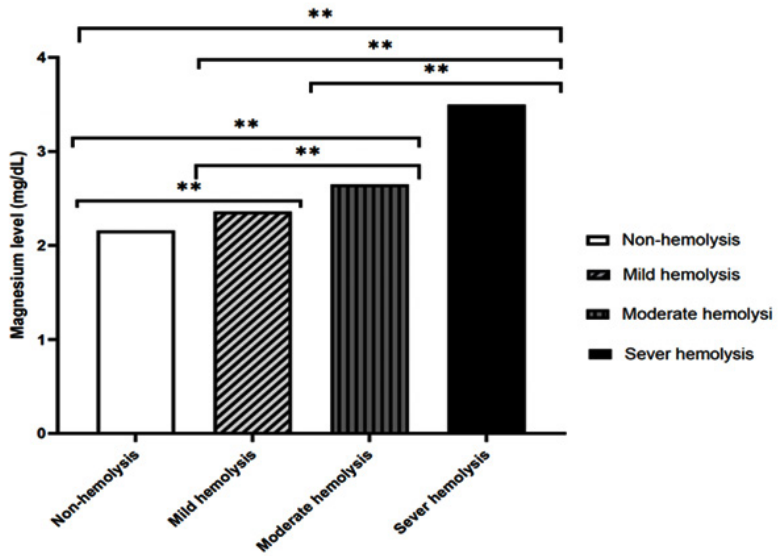

Fig. 2. Levels of serum magnesium (Mg^{+2}) in samples with varying degrees of hemolysis (P** < 0.01)

respectively, compared to 41.06±6.13 mg/dL in non-hemolyzed samples (Figure 6).

When non-hemolyzed blood samples were treated with 0.9 mg/mL of zin chloride, 0.0175 mg/mL of magnesium chloride, and 1.17 mg/mL of copper chloride, the analysis revealed that the activity of ALP was significantly suppressed following treatment with Zn^{+2}, Mg^{+2}, and Cu^{+2} compared to blood samples treated with distilled water as control (Figure 7).

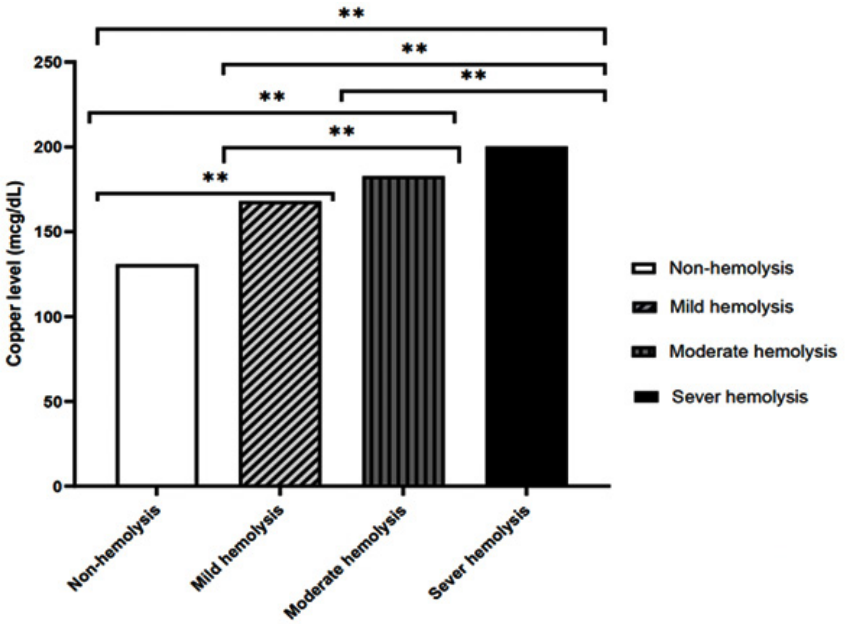

Fig. 3. Levels of serum copper (Cu^{+2}) in 68 blood samples with varying degrees of hemolysis (P** < 0.01, P* < 0.05)

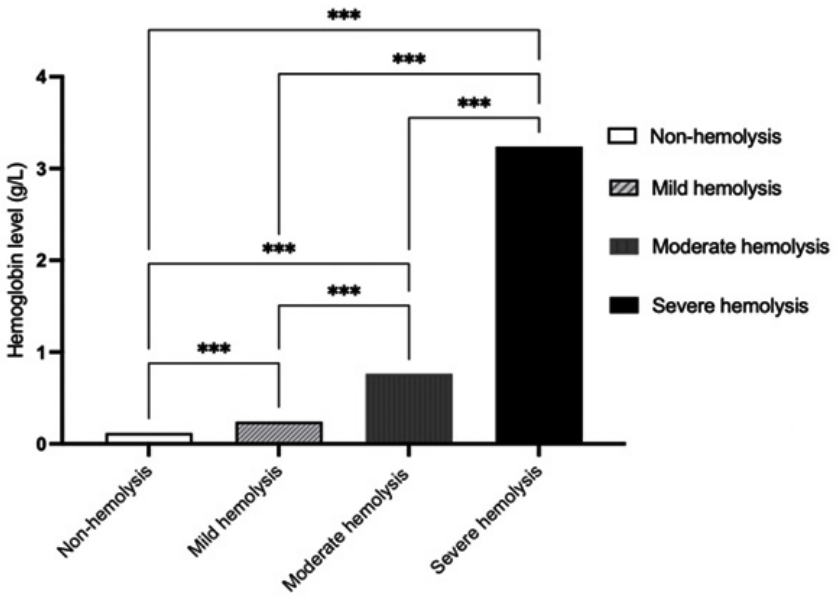

Fig. 4. Levels of hemoglobin in 68 blood samples with varying degrees of hemolysis P ***< 0.001)

In contrast, while Zn^{+2} and Mg^{+2} treatment significantly reduced Cp activity, Cu^{+2} had no significant effect on Cp activity (Figure 8)**.**

DISCUSSION

The reduction in ALP and ceruloplasmin activities coincided with a massive and significant increase in Hb levels following hemolysis compared to the non-hemolyzed samples (Figure 4). Hemolysis is a major source of sampling errors in diagnostic procedures due to the leakage of intracellular ions as well as hemoglobin into the extracellular matrix of blood.[18] Improper handling during sample collection is a major cause of in vitro hemolysis. The presence of hemolysis

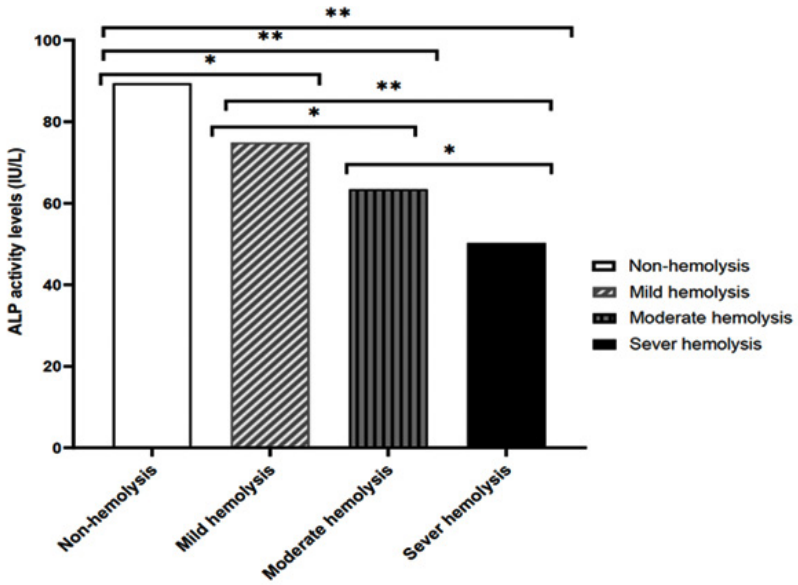

Fig. 5. Levels of alkaline phosphatase (ALP) in 68 blood samples with varying degrees of hemolysis P** < 0.01, P* < 0.05

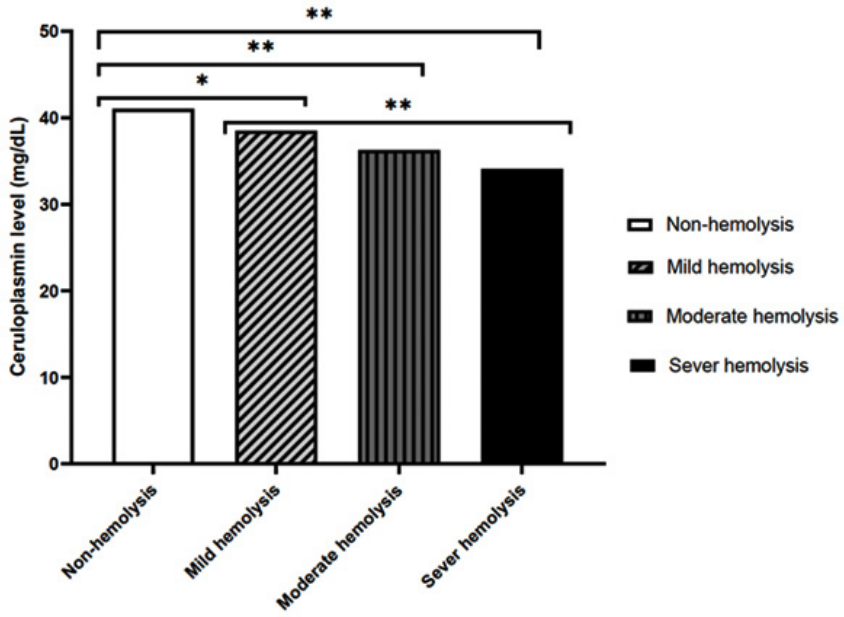

Fig. 6. Levels of ceruloplasmin (Cp) blood samples with varying degrees of hemolysis P **< 0.01, P* < 0.05

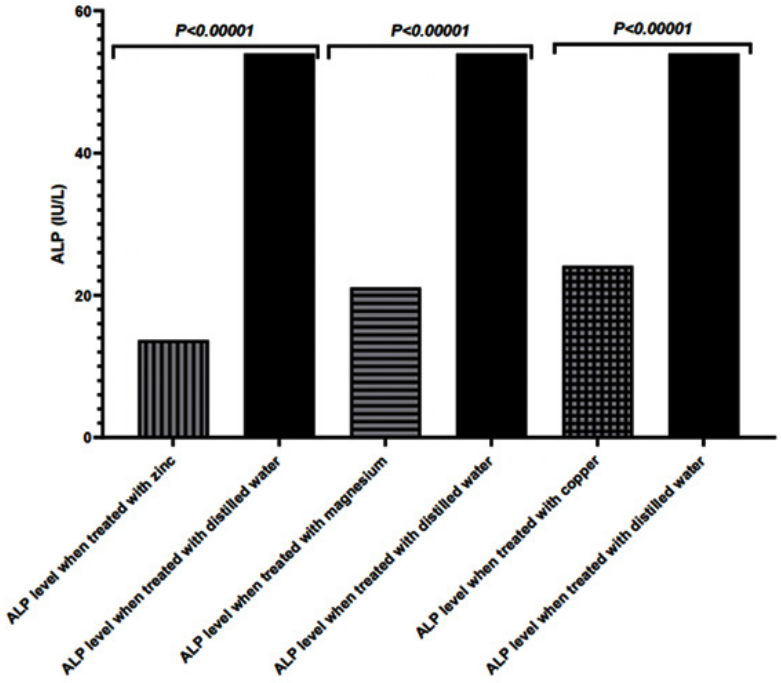

Fig. 7. Level of serum ALP in samples treated with zinc, magnesium and copper compared to samples treated with distilled water

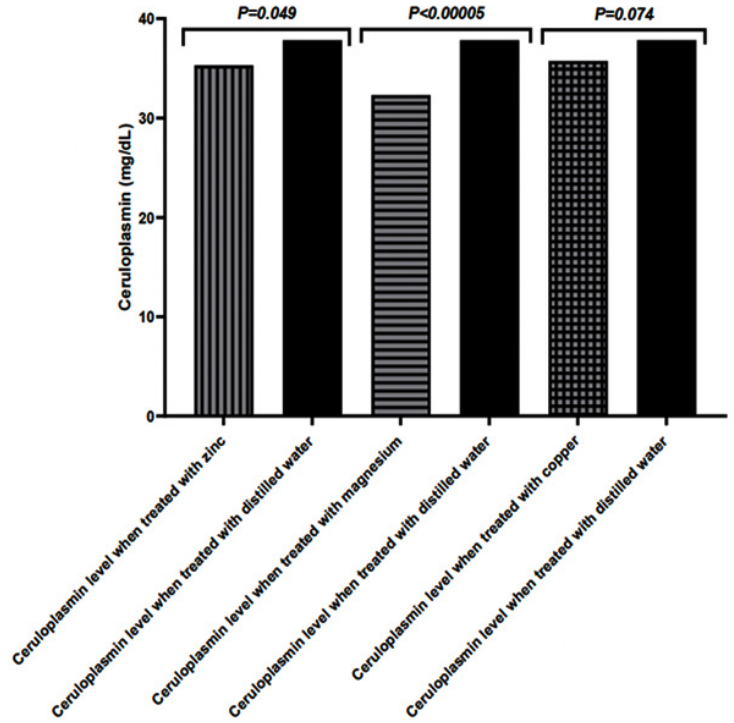

Fig. 8. Levels of serum ceruloplasmin in non-hemolysed samples treated with zinc, magnesium and copper and compared to these treated with distilled water as a control

has raised concerns regarding the reliability of the results. It is imperative to take necessary measures to ensure that the accuracy of the results is not compromised. Failure to do so may lead to incorrect diagnoses, delayed treatments, and potential harm to patients. Therefore, it is crucial to address the issue of hemolysis and take necessary steps to maintain the precision and dependability of the results. A recent study found that hemolysis-induced pseudohyperkalaemia can effectively hide hypokalaemia, a clinically deadly disease. Furthermore, serum sodium levels increased following hemolysis.[19,20] Hemolysis releases hemoglobin into the serum, which can interfere with spectrophotometric assays used to measure numbers of biochemical parameters. Free hemoglobin can cause optical interference, potentially leading to inaccurate readings.[18]

ALP activity showed a significant decrease ($P<0.01$) upon hemolysis. Specifically, levels dropped to 74.94±15.98 IU/L, 63.47±18.51 IU/L, and 50.29±17.23 IU/L under mild, moderate, and severe hemolysis, respectively, compared to 89.47±18.13 IU/L in non-hemolyzed samples (Figure 5). The findings revealed that severe hemolysis had a greater impact on the activity of ALP than moderate or mild hemolysis. These results align with previous literature. [4] identifying a pronounced reduction in ALP levels following moderate to severe hemolysis.[21] This was in addition to several studies that agreed on the negative impact of hemolysis on ALP activity.[22,23] These studies, however, postulated that the significant reduction in the ALP activity is attributed to the dilution induced by the leakage of intracellular components into the surrounding environment.[15] Conversely, this study demonstrated that the decline in ALP activity following hemolysis is attributed to the direct impact of the released electrolytes on the ALP.[18,24] ALP activity was significantly decreased ($p<0.001$) from (54.01±16.06 IU/L) in distilled water-treated samples to 13.72±1.76 IU/L, 24.19±2.45 IU/L , and 21.09±2.79 IU/L upon the addition of Cu, Mg and Zn respectively (Figure 7). The surge in concentrations of Zn^{+2}, Cu^{+2}, and especially Mg^{+2} is believed to induce negative feedback inhibition on ALP activity. This explanation seems to be reasonable since ALP is a metalloenzyme that depends on Mg^{+2} and Zn^{+2} ions as cofactors. [25-27]

In agreement with our findings, a study found that increasing concentration of Mg^{+2} resulted in the displacement of Zn^{+2} from its binding site on the ALP that eventually led to a lower ALP activity.[28] In parallel, hemolysis had a detrimental impact on the Cp levels indicated by significant reduction in serum Cp levels in which mild, moderate, and severe hemolysis induced 38.53±5.14 mg/dL, 36.29±4.85 mg/dL, and 34.12±5.08 mg/dL of Cp respectively compared to 41.06±6.13 mg/dL in non-hemolyzed samples (Figure 6). While only a few studies have highlighted the adverse effect of hemolysis on Cp activity, some suggest that a deficiency in ceruloplasmin may result in hemolysis without addressing the underlying cause of this condition.[29] The heightened activity of ceruloplasmin following hemolysis might serve as a protective feedback mechanism.[30]

Moreover, a significant reduction in the activity of Cp was observed following the addition of Zn^{+2}, Mg^{+2}, and Cu^{+2} in the tested samples (Figure 8). There are several hypotheses, including one suggesting that erythrocytes' leaked components directly affect Cp activity and another proposing that elevated hemoglobin concentrations cause color interference.[31,32]

The study found that adding magnesium, zinc, and copper concentrations (equal to those released from blood cells upon hemolysis) to non-hemolysed blood samples resulted in a decrease in the activity of the ALP; the activity was more affected by the Zn ion than by the other ions. While Cp was affected by Mg ions more than other ions (Cu, Zn). This demonstrates that the ions produced from the hemolysis of red blood cells are the likely cause of the decrease in ALP enzyme and Cp protein. The cause of hemolysis's impact on the efficiency of and ALP enzyme Cp has not been examined in any previous research.

CONCLUSION

This study conclusively demonstrates that hemolysis of red blood cells significantly alters the levels of key electrolytes, such as Mg, Zn, and Cu, within cells. Furthermore, hemolysis affects two important proteins, ALP and Cp, with ALP being more severely impacted. The study clearly identifies the underlying mechanism behind the pronounced effect of hemolysis on ALP levels.

These findings emphasize the critical need for careful sampling to avoid hemolysis and prevent diagnostic errors. Future research should prioritize developing strategies to minimize hemolysis and further explore the molecular interactions responsible for these alterations.

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Conflict of Interest

The author(s) do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

Before starting blood sample collection, ethical approval was obtained from the scientific committee of the Faculty of Allied Medical Sciences, Al-Ahliyya Amman University, Jordan with the protocol number IRB: AAU/1/7/2021-2022. Informed written consent was taken from each participant before the commencement of the study to explain the benefits and/or any risks related to participation in the study. Furthermore, each participant filled out a health history questionnaire before the start of the study.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Author contributions

Hayder Abbas did all the experimental work, Zainab Zakaraya conceptualized and supervised the article. Husni Farah and Laila AL-Omari wrote the initial draft , Sana Audeh revised the data analysis and manuscript, Mohammad Aladwan did all the statistical analysis., Fouad S. El-shehabi, Safwan M. Al-adwan, Sima Zein and Usamah Sayed have revised subsequent drafts.

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