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Explanation of the Decrease in Alkaline phosphatase (ALP) Activity in Hemolysed Blood Samples from the Clinical Point of View: *In vitro* study

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Abstract

Hemolysis is still the most common reason for rejecting samples, while reobtaining a new sample is an important problem. The aim of this study was to explain the decrease in the activity of Alkaline phosphatase (ALP) enzyme after hemolysis of blood samples and whether conversion of zinc and magnesium ions to inhibit alkaline phosphatase activity after they released from red blood cells. Twenty healthy volunteers were enrolled in the study. Four hemolysis levels were constituted according to hemoglobin concentrations (0.02, 0.27 ,0.75 and 3.34 g/L). Non–hemolysed samples was obtained from each volunteer and considered as control. Hemolysis was achieved by mechanical trauma. Alkaline phosphatase activity and the concentrations of zinc and magnesium ions were measured in the hemolysed and non-hemolysed samples. Ten non-hemolysed serum samples (Hb concentration was < 0.02 g/L) were divided into two groups samples A and B. ALP activity was measured in these samples. In vitro study was carried out including the addition 68.3 mg/dL of magnesium chloride to group A and 5.1 µg/dL of zinc chloride to group B. ALP activity was measured in the sera of the two groups. The significant decrease (p<0.001) in ALP activity was observed at moderate (13.2 ± 7.2IU/L), and severe hemolysis (5.5± 2.3IU/L) compared with that in non hemolysed samples. In these levels of hemolysis the concentrations of Zn^{+2}ions (5.1 ±1.1µg/dL) and Mg^{+2}ions (68.3± 8.6mg/dL) were significantly increased (p<0.01) compared with their concentrations in non-hemolysed samples. Alkaline phosphatase activity was inversely proportional with the increase in the hemoglobin concentrations in the hemolysed samples. A significant decrease (p<0.005) in the activity of ALP was observed after the addition of 68.3.0 mg/dl of magnesium chloride to group A. There was no significant decrease (p>0.1) in activity of ALP in the samples of group B. The findings of this study indicate that blood cell hemolysis reduces the activity of ALP which is directly proportional to the level of hemolysis. Released Mg^{+2} ions were found to inhibit ALP activity in the blood hemolysed samples.

Keywords: Alkaline phosphatase activity, hemolysed blood samples, Mg^{+2} Ions, *in vitro* study.

1. Introduction

Hemolysis is the most common preanalytical source of error in clinical laboratories and responsible for nearly 60% of rejected samples (Plebani *et al*., 1997; Bonini *et al*., 2002). Blood hemolysis may occur *in vivo* or *in vitro*. The ratio of *in vivo* hemolysis is only 3.2% of all the hemolyzed specimens (Carraro *et al*., 2000). In vitro hemolysis occurs more often and it is caused by improper sample drawing, handling or centrifugation. Especially hardly collected samples, or stored and/or transported, have increased risks for hemolysis.

Most of the hemolyzed samples are being rejected on pre-analysis stage according to the visual detection of serum interferences, even if the requested tests may not be interfered with hemolysis. Besides, according to the reports, visual assessment of sample hemolysis showed little agreement with the actual concentration of hemoglobin interferent (Hinckley, 1997; Plebani, 2007; Simundic *et al*., 2009). Conversely, even if the hemolysis is not visible, there is also a discharge of the cell constituents into serum or plasma (Thomas, 2010). So invisible hemolysis is an important cause of false results and has to be detected before the investigation procedure.

Alkaline phosphatase enzyme has an important diagnostic value in liver diseases and bone diseases. The effect of hemolysis on the activity of ALP is less understood. Some studies (Yucel and Dalva, 1992; Lippi *et al*., 2006) revealed a decrease in the activity of Alkaline phosphatase in hemolysed blood samples; other studies (Grafmeyer *et al*., 1995**;** Arise *et al***.,** 2008) have not found any change in the activity of ALP in hemolysed blood samples.

Alkaline phosphatase is a metallo-enzyme that is activated by magnesium and zinc ions (Mehmet *et al*., 2011). Hemolysis causes a release of intracellular ions in the serum among these are the magnesium and zinc ions which are usually found in large concentration in hemolysed samples. The present study aims, therefore, at

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investigating the possible inhibitory role of Zn^{+2} and Mg^{+2} ions on the activity of ALP in hemolysed blood samples.

2. Materials and Methods

2.1. Preparation of Blood Samples

All reagents, Medical Kits and were purchased from Agencies Med. Lab. Trading Com. (L.L.C) Amman /Jordan. Twenty healthy volunteers (23.0± 4.0 years old) were tested for their fitness and health by measuring their levels of ALP before they are enrolled in the study. Venous blood samples were collected from twenty healthy volunteers. Hb concentration was measured to make sure that these sample were free from hemolysis.

To study the effect of *in vitro* hemolysis, procedure of Mehmet *et al*., (2011) was used where four samples were drawn from the healthy volunteers through the needles of 5 mL syringe (1.5 inch, 21 gauge) speedily for 2, 4, 6 and 8 times respectively to lyse the cells by mechanical trauma to obtain slightly, mildly, moderately and severely hemolyzed samples. They were all centrifuged at 1000 x g for 15 minutes and sera were collected.

2.2. Determination of Hemoglobin (Hb) Concentration

Free Hb of all samples were measured spectrophotometrically (Shimadzu Corporation; Kyoto, Japan) with Na_2CO_3 solution (10 mg/100 mL) as a reagent (Fairbanks and Klee, 1999). Absorbencies were measured at 415, 450 and 700 nm for non-hemolysed and hemolysed blood samples.

Total serum hemoglobin was calculated according to the formula:

Hb = 154.7 × (A425) – 130.7 × (A450) – 123.9 × (A700) (9). The Reference range was from 0 - 0.1 g/L for serum free Hb.

2.3. Determination of Alkaline Phosphatase (ALP) Activity Non-Hemolysed and Hemolysed Samples

The activity of ALP was determined according to the procedure of Matsushita and Komoda (2011). 50 µl of the each serum sample was mixed with 1ml of the reagent of alkaline phosphatase enzyme. The mixture was incubated for 1minute.The absorbance of the each sample was measured for 3 consecutive minutes at 550 nm. The activity of ALP (IU/L) was calculated according to the following formula:

(IU/L) = ΔAbs./min. x 2187

Normal level of ALP = 40 - 140 IU/L.

2.4. Determination Zn^{+2} ions in non-Hemolysed and Hemolysed Samples

The determination of Zn^{+2} ions was based on the procedure of Knoell *et al* (2009). 200µL standard, sample and sample blank (200 µL Sample + 8 µL EDTA) were transferred to appropriately labeled tubes. 800µL working reagent were added, mixed and Incubated for 30 min and the light absorbance were read at 425 nm. Concentration of Zn in the samples was determined from a standard curve prepared for Zinc standard concentrations (data are not shown).Normal zinc ion concentration (µg/dL) = 45.2 ± 4.4.

2.5. Determination of Mg^{+2} ions

Procedure of Whang (1987) was used for the determination of Mg^{+2} ions**.** 25 µL diluted standard and samples were transferred to appropriately labeled tubes.1000 µL working reagent was added and mixed. After incubation for, the absorbance was read at 500nm. 50 µL EDTA solution was added, mixed well, incubated for 2 minutes and read at 500 nm.

Absorbance of Mg^{+2}(Abs. $_{Mg+2}$) and Absorbance of Mg blank (Abs. $_{Mg+2blank}$) are Absorbance values at 500 nm of the standard(2 mg/dL) before and after the addition of EDTA.

$$\text{Concentration of } Mg^{+2} = \frac{\text{Abs. }_{\text{Sample}} - \text{Abs. }_{\text{Blank}}}{\text{(mg/dl) Abs. }_{\text{Mg}} - \text{ Abs. }_{\text{Mg Blank}}}$$

Normal Magnesium ion concentration (mg/dL) = 1.77 ± 0.02

2.6. Alkaline Phosphatase Activity in non Hemolysed Blood Samples to Which Zinc and Magnesium are Added

Ten non-hemolysed serum samples (free Hb concentration was < 0.02 g/L) were divided in to two groups samples (named as Group A and B). ALP activity was measured in these samples. In vitro study was carried out including the addition 68.3 mg/dL of magnesium chloride to group A and 5.1 µg/dL of Zinc chloride to group B. All samples were incubated for 10 minutes. ALP activity was measured again in the sera of the two groups.

3. Results

As seen in table 1, Alkaline phosphatase activity was significantly decreased in hemolysed samples compared with that in non hemolysed samples (83.3 ±10.6 IU/L). The significant decrease ($p<0.001$) in the activity of the enzyme was observed at moderate (13.2 ± 7.2IU/L), and severe hemolysis (5.5±2.3IU/L) compared with that in non hemolysed samples (83.3 ±10.6 IU/L). The activity of ALP at slight hemolysis (81.4±18.4 IU/L) did not show any significant decrease ($p<0.5$), however. In these level of hemolysis, the concentrations of Zn^{+2} (5.1± 1.1µg/dL) and Mg^{+2} (68.3± 8.6mg/dL) were significantly increased ($p<0.01$) compared with their concentrations in non-hemolysed samples (2.75 ± 0.82µg/dL and 17.66 ± 2.3mg/dL, respectively) as observed in table 1.

The decrease in the activity of ALP was inversely proportional with the increase in the hemoglobin concentrations in the hemolysed samples (Table 1).

Significant decrease of ALP activity started at moderate hemolysis where the concentrations of Mg^{+2} and Zn^{+2} ions were 68.3 ± 8.6 mg/dl and 5.1± 1.1 µg/dl, respectively.

Table1. The activity of Alkaline phosphatase(ALP), the concentrations of Zn+2 and Mg+2 and the Hemoglobin (Hb) concentrations in blood samples hemolysed to different levels.

Levels of hemolysis	Hb concentration (g/L)	Mg^{+2} (±SD)[a] (mg/dL)	Zn^{+2} (±SD)[a] (µg/dL)	ALP activity(IU/L) (± SD)[a]
No hemolysis	0.012	17.66† (2.3)	2.75 (0.82)	83.3•(10.6)
Slight	0.02	18.2 (1.8)	2.8 (0.31)	81.4• (18.4)
Mild	0.27	23.3 (5.3)	3.5 (1.2)	78.5•(9.5)
Moderate	0.75	68.3† (8.6)	5.1 (1.1)	13.2*(7.2)
Severe	3.34	71.6 (11.3)	6.3 (2.3)	5.5*(2.3)

a Mean +SD,*p< 0.01; •p> 0.1 ; †p < 0.01

Slight and mild hemolysis did not affect the ALP activity where the concentration of Hb was 0.02 and 0.27 respectively (Table 1).A significant decrease (p<0.005) in the activity of ALP from 93.7± 10.2 to 47.4 ±10.7IU/L was recorded after the addition of 68.3.0 mg/dl of magnesium chloride to group A (Table 2).The activity of ALP in group B (85.1± 8.6IU/L), before the addition of 5.1 µg/dl of Zinc chloride, was not significantly decreased (p>0.1)with that activity (77.2± 12.3 IU/L) after the addition (Table 2).

Table2. Activity of ALP in non hemolysed serum samples before and after the addition of $MgCl_2$ and $ZnCl_2$.

	Before addition of $MgCl_2$; $ZnCl_2$		After addition of $MgCl_2$	After addition of $ZnCl_2$
	Group A(±SD)	group B (±SD)[a]	Group A(±SD)[a]	Group B(±SD)[a]
ALP activity (IU/L)	93.7• (10.2)	85.1*(8.6)	47.4•(10.7)	77.2*(12.3)

[a] Mean ±SD, p•<0.005; p*>0.1

4. Discussion

Most studies (Carraro *et al*., 2000; Bonini *et al*., 2002; Lippi *et al*., 2006; Plebani, 2007; Simundic *et al*., 2009) have unanimously agreed on the effect of hemolysis on the activity of ALP. Some studies (Plebani, 2007; Simundic *et al*., 2009) attributed the significant decrease in the ALP activity to the dilution factor as a possible effect where the leakage of intracellular components into the surrounding fluid especially in severe hemolysis may cause decreased ALP activity. Other studies (Carraro *et al*., 2000; Lippi *et al*., 2006) attributed such a decrease to the direct impact of some of the contents of blood cells on the activity of ALP without specifying the nature of these contents.

As found in the current study hemolysis significantly decreased the activity of ALP and the data suggests a progressive inhibition of ALP when exposed to increasing level of hemolysis. This method of cell lyses was chosen because blood transferring into a tube by pushing forcedly down on the syringe plunger is analogous to the mechanical disruption of erythrocytes that frequently occurs during blood collection. In this method, there is no standardization way of the force applied while transferring the blood by syringe. Besides, every patient's fragility of red blood cell is different, so free Hb concentrations of all samples were not correlated with the force.

The significant decrease in ALP activity started at moderate hemolysis where the concentrations of Mg^{+2} ions was four times greater than at normal level. The increase in concentration of Zn^{+2} ions was twice greater than the normal at this hemolysis level. It seemed that the effect of the increase in the concentration of Mg^{+2}ions in the hemolysed samples resulted in a significant decrease in ALP activity.

In vitro experiment same concentration of Mg^{+2} ions which was measured at moderate hemolysis was prepared as $MgCl_2$ and was added to non hemolysed sample as a result of this addition there was a significant decrease in the activity of ALP. However this finding was not observed in the ALP activity in non-hemolysed sample to which $ZnCl_2$ was added.

From these observations in table 2, it is very obvious that elevated level of Mg^{+2} ions play an inhibitory effect on the ALP activity. This explanation seems to be reasonable since ALP is a metallo-enzyme which depends on Mg^{+2} and Zn^{+2}ions as cofactors (Arise *et al*., 2008). In the current study, the increase in Mg^{+2} ions resulted in a feedback inhibition on ALP activity.

Hemoglobin concentration is recommended to be measured before carrying out ALP activity. Slight and mild hemolysis as the concentration of Hb is ≤ 0.27 g/L did not affect the ALP activity as manifested in the present study. For ALP measurement, grossly hemolysed samples should be rejected and new samples should be requested. It is recommended to determine free Hb level in serum or plasma, to detect the degree of hemolysis.

5. Conclusion

Inhibition of ALP activity was found due to releasing of large amount of Mg^{+2} during blood hemolysis which confirms the feedback inhibition exerted by the ion on the enzyme action.

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