Zagazig J. Pharm. Sci., December, 2011
Vol. 20, No. 2, pp. 19-23

ISSN 1110-5089

IN VITRO STUDY TO EXPLAIN THE DECREASE IN ALKALINE PHOSPHATASE (ALP) ACTIVITY IN HEMOLYSED BLOOD SAMPLES FROM THE CLINICAL POINT OF VIEW.

Husni S Farah , Ali A Al- Atoom , Gaber M G Shehab
Department of Medical Biochemistry, College of Medicine, Taif University, KSA

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 enzyme after hemolysis of blood samples and the possible conversion of zinc and Magnesium ions to inhibitors of 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 were 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. 10 non-hemolysed serum samples (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 through addition of magnesium chloride (68.3 mg/dL) to group A and Zinc chloride (5.1 µg/dL) to group B. ALP activity was measured in the sera of the two groups. The significant decrease ($p<0.001$) in ALP activity (13.2 ± 7.2 IU/L) was at moderate and at severe hemolysis (5.5 ± 2.3 IU/L) as compared with that in non hemolysed samples. In these levels of hemolysis the concentrations of Zn^{+2} ions (5.1 ±1.1 µg/dL) and Mg^{-2} ions (68.3 ± 8.6 mg/dL) were significantly increased ($p<0.01$) compared with their concentrations in non-hemolysed samples (2.75 ± 0.82 ; 17.66 ± 2.3µg/ml) respectively. ALP 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 from 93.7± 10.2 to 47.4 ±10.7 IU/L was observed after the addition of 68.3.0 mg/dL of magnesium chloride to group. There was no significant decrease ($p>0.1$) in activity of ALP in the samples of group B to which 5.1 µg/dL of Zinc chloride was added.

INTRODUCTION

Hemolysis is the most common preanalytical source of error in clinical laboratories and responsible for nearly 60% of rejected samples.[1,2] It may occur both *in vivo* and *in vitro*. The ratio of *in vivo* hemolysis is only 3.2% of all the hemolyzed specimens.[3] *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.[4-6] Conversely, even if the hemolysis is not visible, there is also a discharge of the cell constituents into serum or plasma.[7] 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 [8,9] revealed a decrease in the activity of Alkaline phosphatase in hemolysed blood samples, other studies have not found any change in the activity of ALP in hemolysed blood samples.[10,11] ALP is a metallo-enzyme that is activated by magnesium and zinc ions.[12] Hemolysis causes a release of intracellular ions in the serum among these are the magnesium and zinc ions which will be found in large concentration in hemolysed samples. The present study aims to investigate the possible inhibitory role of Zn^{+2} and Mg^{+2} ions on the activity of ALP in hemolysed blood samples.

EXPERIMENTAL

Materials

All Reagents, Medical Kits and hard wares were purchased from Agencies Med. Lab. Trading Com. (L.L.C, Amman, Jordan).

Methods

Preparation of blood samples

Twenty healthy male 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 those volunteers. Hemoglobin concentration was measured to make sure that these samples were free from hemolysis.

To study the effect of in vitro hemolysis, procedure of Mehmet *et al.* [12] 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 hemolysed samples. They were all centrifuged at 1000 x g for 15 minutes. Sera were collected.

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.[13] Absorbances were measured at 415, 450 and 700 nm for non-hemolysed and hemolysed. Free hemoglobin in the hemolysed samples was taken as indicator of hemolysis levels.[7] Total serum hemoglobin was calculated according to the formula of: Hb = 154.7 × (A415) – 130.7 × (A450) – 123.9 × (A700). The Reference ranges from 0 - 0.1 g/L for serum free Hb.

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.[14] 50µl 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) = Δ Abs./min. x 2187

Normal level of ALP = 40-140 IU/L

Determination of Zinc (Zn^{+2})and Magnesium (Mg^{+2}) ions in non-hemolysed and hemolysed samples

1. Determination of Zn^{+2} ions

The determination of Zn^{+2} ions was based on the procedure of Knoell *et al.* [15] Transfer 200 µL standard, sample and sample blank (200 µL Sample + 8 µL EDTA) to appropriately labeled tubes. Add 800 µL working reagent and tap tightly to mix. Incubate 30 minutes and read the absorbance at 425 nm. The concentration of Zn^{+2} in the samples was determined from a standard curve prepared for Zinc standard concentrations (Zinc standard curve is not available in this study). Normal zinc ion concentration (µg/dL) = 45.2 ± 4.4

2. Determination of Mg^{+2} ions

Procedure of Whang [16] was used for the determination of Mg^{+2} ions. Twenty five µL diluted standard and samples were transferred to appropriately labeled tubes and 1000 µL working reagent were added and vortexed to mix. After incubation for 2 minutes, they were transferred to cuvet and read at 500nm. Fifty 50 µL EDTA solution were added, mixed well, incubated for 2 minutes and read at 500 nm.

Calculation

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

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

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

Determination of Alkaline phosphatase activity in non hemolysed blood samples to which specific amounts of Zinc and Magnesium are added.

Ten non-hemolysed serum samples (free Hb concentration was < 0.02 g/L) were divided into 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 of 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.

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

Zagazig J. Pharm. Sci., December, 2011
Vol. 20, No. 2, pp. 19-23 ISSN 1110-5089

Table (1): 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)[b] (mg/dL)	Zn^{+2} (±SD)[b] (µg/dL)	ALP activity(IU/L) (±SD)[b]
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)

[b]Mean ± SD ; *p< 0.01; †p < 0.01

The decrease in the activity of ALP was inversely proportional with the increase in the hemoglobin concentrations in the hemolysed samples (Figure 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. 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±10.7 IU/L was recorded after the addition of 68.3.0 mg/dL of magnesium chloride to group A and the activity of ALP in group B showed non-significant decrease (85.1 ± 8.6 IU/L) versus 77.2 ± 12.3 IU/L (Table 2).

Table (2): 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 $MgCl_2$ Group A(±SD)[b]	After addition of $ZnCl_2$ Group B(±SD)[b]
	Group A(±SD)[b]	group B (±SD)[b]		
ALP activity (IU/L)	93.7* (10.2)	85.1 (8.6)	47.4*(10.7)	77.2 (12.3)

[b]Mean ±SD; p*<0.005

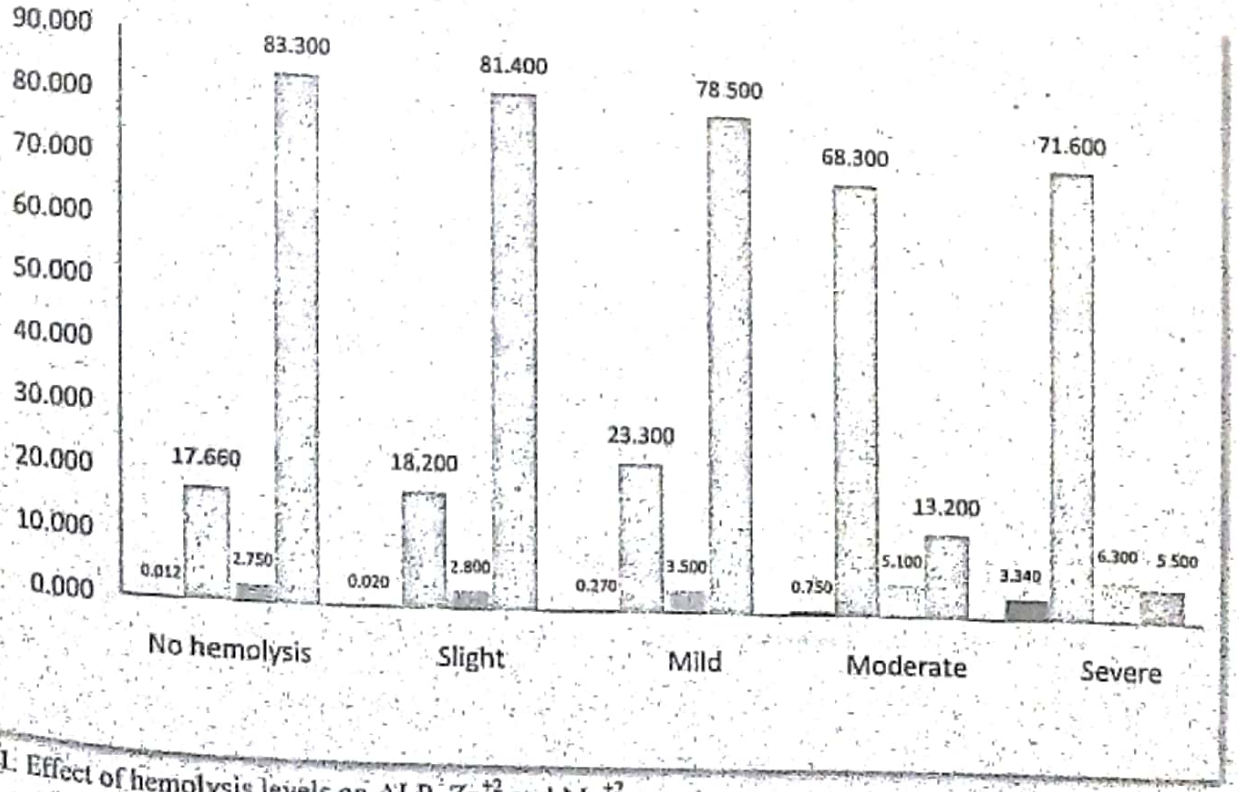

Fig. 1. Effect of hemolysis levels on ALP, Zn^{+2} and Mg^{+2}

DISCUSSION

Most studies [1, 3, 5, 6, 8] have unanimously agreed on the effect of hemolysis on the activity of ALP. Some studies [5,6] 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 [3,8] attributed this such 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 expected in the current study hemolysis significantly decreased the activity of ALP and the data suggest a progressive inhibition of ALP when exposed to increasing level of hemolysis. This method of cell lysis was chosen because blood transferring into a tube by pushing forcely 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, as a result, 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 these hemolysis levels. 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 another 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 result was not observed in the ALP activity in non-hemolysed sample to which $Zncl_2$ was added. From the data presented in table 2, it is very obvious that elevated level of Mg^{+2} ions play an inhibitory effect on the ALP activity. This such suggestion seems to be reasonable since ALP is a metallo-enzyme which depends on Mg^{+2} and Zn^{+2} ions as cofactors [11] and as shown 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 proceeding the measurement of ALP. Slight and mild hemolysis as the concentration of Hb is ≤ 0.27 g/L would 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.

In conclusion, the current study gave an explanation for the decrease in the activity of ALP after blood hemolysis where the other studies failed to do so. Mg^{+2} ion is known as a cofactor of ALP , released Large concentration of Mg^{+2} due to hemolysis inhibits The activity of ALP which confirms the feedback inhibition of Mg^{+2} on ALP activity.

ACKNOWLEDGEMENT

We wish to thank Taif University represented by the Deanship of higher studies and scientific research for their financial support. Special thank to our colleagues in the Medical Biochemistry Department, College of Medicine for their technical support and valuable discussions.

REFERENCES

1. Bonini P., Plebani M., Ceriotti F., Rubboli F., **Clin. Chem.**, 48, (2002).
2. Plebani M., Carraro P., **Clin. Chem.**, 43, 1348, (1997)
3. Carraro P., Servidio P., Plebani M., **Clin. Chem.**, 46, 306, (2000).
4. Hinckley C., **Clin. Chem.**, 43, 873 (1997).
5. Plebani M., **Biochem. Med.**, 17, 5, (2007).
6. Simundic A. M., Nikolac N., Ivankovic V., Ferenec-Ruzic D., Magdic B., Kvaternik M., Topic E., **Clin. Chem. Lab. Med.**, 47, 1361, (2009).
7. Thomas L., **EJIFCC**, 13.
8. Lippi G., Salvagno L. G., Montagnana M., Brocco G., Guidi G. C., **Clin. Chem. Lab. Med.**, 44, 311, (2006).
9. Yucel D., Dalva C., **Clin. Chem.**, 38, 575, (1992).
10. Grafmeyer D., Bondon M., Manchon M., Levillain P., **Eur. J. Clin. Chem. Clin. Biochem.**, 33(1), 31, (1995).
11. Arise R. O., Davies F. F., Malomo S. O., **Sci. Res. Essay.**, 3 (10), 488, (2008).
12. Koseoglu M., Hur A., Atay A., Cuhadar S., **Biochemia. Medica.**, 21(1), 79, (2011).
13. Fairbanks V. F., Klee G. G. Tietz Textbook of Clinical Chemistry., 3rd ed. Philadelphia: WB Saunders Company, p.1673, (1999).
14. Matsushita M., Komoda T., **Rinsho. Byori.**, 59 (10), 923, (2011)

Zagazig J. Pharm. Sci., December, 2011
Vol. 20, No. 2, pp. 19-23

ISSN 1110-5089

15. Knoell D. L., Julian Mark W., Bao Shengying, Besecker Beth, Macre Jennifer E., Leikauf George D., DiSilvestro Robert A., Crouser Elliott D., Crit. Care. Med., 37(4), 1380, (2009)

16. Whang R., Magnesium., 6, 1, (1987).

Received:04/10/2011
Accepted:17/11/2011

دراسة مختبرية لتفسير النقص في انزيم الفوسفاتيز القاعدي في عينات الدم المتحلل من وجهة النظر الإكلينيكية

حسني فرح، على العتوم، جابر شهاب
قسم الكيمياء الحيوية الطبية- كلية الطب-جامعة الطائف- الطائف- المملكة العربية السعودية

هدفت هذه الدراسة إلى ايجاد السبب الحقيقي في انخفاض نشاط انزيم الفوسفات القاعدي وربط ذلك بمستوى ارتفاع أيونات المغنسيوم والخارصين ، وامكانية عملها كمثبطات في عينات الدم المتحللة حيث ان عملية تكسير(تحلل) الخلايا الدموية في عينات الدم المسحوبة تؤدي إلى زيادة في نشاط معظم الإنزيمات ماعدا نشاط انزيم الفوسفات القاعدي والذي يظهر انخفاضا في نشاطه حيث لم تتوصل الدراسات السابقة إلى ايجاد تفسير لهذه الظاهرة. تؤدي عملية تكسير الخلايا الدموية إلى انطلاق كميات من الأيونات الخلوية ومنها الأيونات المخصصة لتنشيط فاعلية انزيم الفوسفات القاعدي وهي أيونات المغنسيوم والخارصين. شملت الدراسة سحب عينات الدم من من عشرين متطوع أصحاء، حيث تم قياس نشاط انزيم الفوسفات القاعدي وتركيز أيونات المغنسيوم والخارصين في عينات الدم السليمة والمتحللة إلى درجات مختلفة حسب تركيز الهيموغلوبين في هذه العينات. تم إضافة تراكيز محددة من المغنسيوم والخارصين إلى عينات دم سليمة ثم تم قياس نشاط انزيم الفوسفات القاعدي في هذه العينات. وبينت الدراسة انخفاضا حادا في نشاط انزيم الفوسفات القاعدي وزيادة في تركيز أيونات المغنسيوم والخارصين في عينات الدم المتحللة وان هذا النشاط يتناسب عكسيا مع درجة تحلل الدم في هذه العينات. وتم تسجيل انخفاض نشاط انزيم الفوسفات القاعدي في عينات الدم المضاف لها المغنسيوم. وقد اثبتت الدراسة أيضا أن السبب في انخفاض نشاط انزيم الفوسفات القاعدي يعود إلى ارتفاع تركيز ايون الماغنسيوم في عينات الدم المتحللة حيث يتحول ايون الماغنسيوم وعند إرتفاع تركيزه من منشط لانزيم الفوسفات القاعدي إلى مثبط له.