INTERNATIONAL JOURNAL OF PHARMACEUTICAL, CHEMICAL AND BIOLOGICAL SCIENCES

Available online at www.ijpcbs.com

Research Article

SERUM LACTATE DEHYDROGENASE AND ADENOSINE DEAMINASE AS PREDICTORS OF HEMOLYSIS IN CHILDREN WITH HEMOLYTIC ANEMIA

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ABSTRACT
Tissue-specific increase in normal adenosine deaminase (ADA) is associated with hereditary hemolytic anemia. Lactate dehydrogenase (LDH) is clinical marker of intravascular hemolysis. Both are associated with pulmonary hypertension in hemolytic anemia. To validate the relevance of ADA and LDH as early predictors of hemolysis by ascertaining their dose response with severity of anemia. ADA was estimated by Colorimetric Method of GIUSTI and GULANI based on Bertholet Reaction while LDH was estimated enzymatically. Complete hemogram was done in Beckman coulter. ADA and LDH were significantly higher in all types of anemia but only ADA was significantly raised in hemolytic anemia ($p < 0.001$). Both correlated significantly with hemoglobin percentage but only ADA correlated significantly with reticulocyte count. ADA was better index of the severity of hemolytic anemia.

Keywords: ADA, LDH, Hemolytic anemia.

INTRODUCTION

Adenosine deaminase (ADA) is a purine catabolic enzyme that catalyzes the irreversible deamination of adenosine to inosine and 2′-deoxyadenosine to 2′-deoxyinosine[1].

Abnormalities of adenosine deaminase have been reported in association with immune dysfunction, acute leukemia, and hereditary hemolytic anemia[1,2]. The ADA gene is expressed in all tissues, and thus may be categorized as a “housekeeping” gene. However, the level of expression varies by more than 1,000-fold in different tissues and developmental states, with the highest level of expression in cortical thymocytes and in T lymphoblasts[3,4].

In contrast to immature T cells, red blood cells (RBCs) normally have low amounts of ADA activity. Mutations in the ADA gene that result in decreased or absent activity have no effect on the function or longevity of the RBC; however, the tissue-specific overproduction of ADA in RBCs causes hemolytic anemia. RBCs from affected individuals have 40- to 70-fold increased levels of ADA activity, leading to the increased catabolism of adenosine and adenosine tri phosphate (ATP) depletion, while ADA activities in leukocytes and

fibroblasts are normal. Metabolic studies with the patient's erythrocytes show that low ATP concentration in these cells (64% of comparably reticulocyte-rich blood) is due both to a diminished synthesis of adenylic nucleotides from adenosine, and to an excessive catabolism of AMP. The resultant depletion of ATP deprives erythrocytes of their major energy source and leads to loss of membrane integrity and premature destruction. The specific molecular defect underlying the tissue-specific enzyme over expression has not been elucidated. From linkage analysis it has been determined that the mutation causing RBC-specific ADA overproduction lies within or near the ADA locus[1,2,3,4,5,6,7].

LDH isoforms 1 & 2 which catalyse the conversion of pyruvate to lactate are increased in intravascular hemolysis and to some extent in extravascular hemolysis. LDH has long been considered a useful clinical marker of intravascular hemolysis.[8,9] In addition, LDH elevation may serve as an indirect marker of a hemolysis-endothelial dysfunction syndrome[8,9,10].

Furthermore, recent advances indicate that both ADA and LDH are strongly elevated in the vascular complications seen in hemolytic anemia the most notable of which is pulmonary hypertension. Spurts of ADA and LDH correspond to incidences of vascular crisis in these patients[11,12,13]. In this study we have tried to ascertain if there is a dose response between ADA and LDH and the severity of anemia, in an attempt to establish it as an early predictor of hemolysis. We have also attempted to find whether there is a correlation between LDH and ADA with other parameters like hemoglobin percentage and reticulocyte count.

MATERIALS AND METHODS

Patient population

The patient population consisted of 91 children in the age group of 0 to 10 years. These 91 children were divided into three groups. The first group consisted of 30 diagnosed cases of hemolytic anemia .The second group comprised of 30 children who were diagnosed as having anemia due to causes other than hemolytic anemia. Children of both the first and second groups were admitted as inpatients in the department of Paediatrics of JSS Medical College and Hospital, Mysore. The third group consisted of 31 age and sex matched healthy controls. Informed consent was taken from all the patients and their parents, in accordance with the 1964 Declaration of Helsinki and the research protocol was approved by the institutional ethical committee.

Eligibility criteria included children between the age group of 0-10 years who were diagnosed with hemolytic anemia (including hemoglobinopathies and erythro-enzymopathies) and anemias other than hemolytic anemia.

All patients provided medical histories, blood samples, underwent physical examination and baseline laboratory and radiological investigations.

All children diagnosed with Pernicious anemia, Rheumatoid arthritis, Tuberculosis, Immunological, Liver and renal disorders and concomitant co-morbidity were excluded from the study.

Methods

Venous blood (3.5 ml) was collected and transferred into sterile EDTA /Heparin tubes. The tubes were gently rotated to mix the contents and centrifuged at 2000 x g for 20 minutes at 4^{0}C and the supernatant was discarded. The pellet containing RBCs were washed thrice with ice cold 0.85 % NaCl and centrifuged at 200 x g for 10 minutes at 4^{0}C. The final pellet was taken up in 4.0 ml chilled water, left in cold for 1 hour for hemolysis and then centrifuged at 200 x g for 20 minutes. The supernatant thus obtained was used for analysis. ADA Estimation was done by Colorimetric Method of GIUSTI and GULANI based on Bertholet Reaction. Serum LDH was estimated by enzymatic method. Complete hemogram was done in Beckman coulter.

Statistical Analysis

The comparison of medians was done by Kruskal-Wallis test and Mann-Whitney test. Correlation was done using Spearman correlations. Results were considered significant if p value was less than or equal to 0.05. Statistical analysis was done using SPSS 11.5 software.

RESULTS

The medians of both ADA and LDH were significantly higher in children with both types of anemia as compared to controls (Table 1) but of the two erythrocytic enzymes only ADA was found to be significantly raised in hemolytic anemia group when compared to anemia of other causes ($p < 0.001$) (Figures 1 and 2). These data confirm the marked elevation of erythrocyte ADA in hemolytic anemia. Correlation analysis was performed between these two erythrocytic enzymes and Hb% and reticulocyte count. ADA and LDH both correlated significantly with Hb% but only ADA and not LDH correlated significantly

with reticulocyte count also ($p < 0.001$; here difference in r value was significant with p value 0.003, Z-score 2.274) (Figures 3 and 4). These data document that although both ADA and LDH could be considered as candidate of biomarkers of hemolysis, ADA was the better index of the severity of anemia.

DISCUSSION

Disorders of red blood cell enzymes, membranes, and haemoglobin cause hereditary hemolytic anemias. Kinetic and electrophoretic studies have shown that most erythro-enzymopathies are due to the production of a mutant enzyme caused by point mutations. An exception is hemolytic anemia secondary to increased adenosine deaminase (ADA) activity. Red cell ADA activity increases in affected individuals. The basic abnormality appears to result from overproduction of catalytically and immunologically normal enzyme due to abnormal translational efficiency[7].

Such an increase could result either from overproduction of the enzyme or from increased stability of the protein in erythrocytes. Hirschhorn has shown that ADA activity in normal RBCs is highly stable with a half-life of about 231days [16]. Since the average RBC life span is 120 days, it is impossible to attribute a 70-to100-fold increase in ADA activity to a structural gene mutation that increases the protein stability[1].

The degree of secondary structure of the 5′ non-coding region of the mRNA appears to be inversely proportional to the translational efficiency of the mRNA. The 5′ non-coding region of ADA mRNA is comprised of 80% guanosine and cytosine residues [17] with considerable potential for formation of stable secondary structures. Mutations in this region and consequent loss of secondary structure might result in increased ribosomal binding to and /or scanning of the mRNA. Point mutations flanking the initiation codon have also been shown to alter translational efficiency [7,17,18].

The mechanism underlying the tissue specificity of this defect is not yet clear. Inhibitors of translation have been demonstrated in erythroid cells[19], and one potential mechanism for specific increased translational efficiency of ADA mRNA in red cell is a mutation that prevents the binding of an inhibitor. It is also possible that tissue specific splicing of a primary RNA transcript may give rise to minor differences in the 5′ non-coding regions of ADA mRNAs that thus affect translational efficiency[1].

Recent advances indicate that both ADA and LDH are strongly elevated in the vascular complications seen in hemolytic anemias, the most notable of which is pulmonary hypertension. Spurts of ADA and LDH correspond with incidences of vascular crisis in these patients[11,12,13].

Hemolysis-associated pulmonary hypertension (HA-PH) is a serious clinical complication of various hemolytic disorders, and pulmonary hypertension (PH) is considered the greatest risk factor for death in patients with a hemolytic disorder. It is now well established that hemolysis causes the release of soluble hemoglobin and arginase from injured erythrocytes into plasma. This leads to nitric oxide (NO) deficiency, oxidative stress and a state of endothelial dysfunction that is associated with clinical development of pH[9].

Serum LDH represents a convenient biomarker for the pathologic accumulation of hemoglobin and arginase in blood plasma, with consequent impaired NO bioavailability. The red cell membrane normally serves as a physical and diffusion barrier that segregates erythrocyte proteins from plasma and endothelium. Intravascular hemolysis disrupts this protective compartmentalization. This allows 2 sets of pathological biochemical reactions to occur. The first involves the stoichiometric inactivation of NO by cell free plasma hemoglobin with consequent impairment of NO-dependent blood flow. The second involves the release of erythrocyte arginase, which converts plasma L-arginine to ornithine, resulting in depletion of plasma L-arginine, the required substrate for NO production by NO synthase, with associated pulmonary hypertension. In addition, LDH correlates with endothelial derived soluble adhesion molecules, considered markers of endothelial activation that are normally repressed by NO[9,10,11,12]. LDH elevation is also associated with low transcutaneous oxygen saturation[9].

Newer evidences indicate that in addition to the NO-arginase pathway, the adenosine deaminase-adenosine pathway plays a significant role in HA-PH and that modulation of this pathway may offer protective/therapeutic effects in HA-PH[13].Preliminary data suggest that in HA-PH adenosine deaminase (ADA) is released from injured erythrocytes into plasma and that metabolic conversion of adenosine (ADO) to inosine by ADA reduces extracellular ADO levels. Adenosine, mainly via activation of adenosine A(2A) receptors, mediates a number of biological

responses that may reduce hemolysis-induced vasculopathy and the risk of PH. Hypoxia is the strongest stimulus for ADO synthesis, and this increased ADO production counteracts some of the tissue/vascular injury caused by hypoxia itself. Unfortunately, under hypoxic conditions (anemia, vasoconstriction, and vaso-occlusion) in HA-PH, this "ADO negative-feedback" is abolished and the vascular protective effects of ADO are severely diminished by ADA released from injured erythrocytes [13,14]. Our study suggests that both ADA and LDH may be useful biomarkers in children with both types of anemia as compared to controls (Table 1) but of the two erythrocytic enzymes only mean ADA was found to be significantly raised in hemolytic anaemia group when compared to anemia of other causes ($p<0.001$). These data confirm the marked elevation of erythrocyte ADA in hemolytic anemia. Correlation analysis was performed between these two erythrocytic enzymes and Hb% and reticulocyte count. ADA and LDH both correlated significantly with Hb% but only ADA correlated significantly with reticulocyte count also ($p <0.00$, here difference in r value was significant with p value 0.003, Z-score 2.274). These data document that although both ADA and LDH could be considered as candidate of biomarkers of hemolysis, ADA was better index of the severity of anaemia.

However, further preclinical and clinical investigation with larger sample size to conclusively establish which among the two is the biomarker of hemolysis in children with hemolytic anaemia.

Table 1: Distribution of Hb%, Reticulocyte count, ADA and LDH among cases and controls

	Group 1		Group2		Group3		p value
	Median	ICR	Median	ICR	Median	ICR	
Hb%	6.00	3.00	8.00	2.00	13.00	2.00	p < 0.001
Reticulocyte count (%)	9.00	1.25	0.60	0.30	0.70	0.40	
ADA (U/g Hb)	4.15	2.55	1.96	1.14	1.00	0.10	
LDH (U/L)	965.00	706.25	987.0	413.0	344.0	203.0	

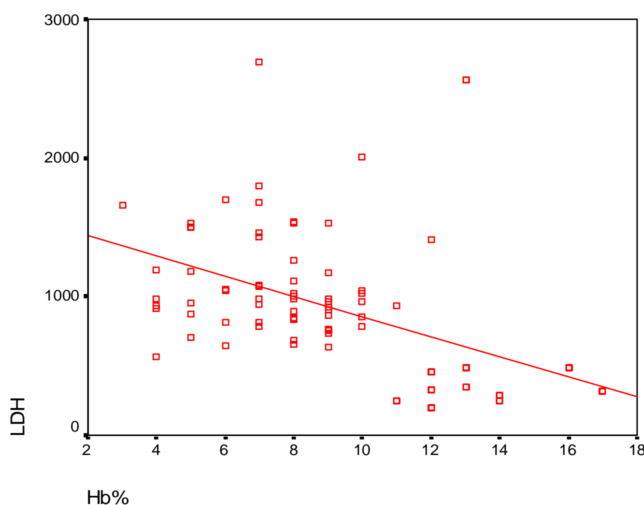

Fig. 1: LDH and Hb% showing negative correlation
(Hb% vs LDH; $r = -0.603$, $p <.001$)

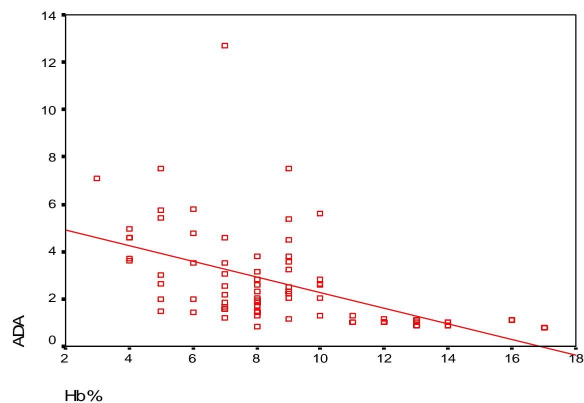

Fig. 2: ADA and Hb% showing negative correlation
(Hb% vs ADA, r = - 0.747, $p < 0.001$)
Difference in r value statistically not significant with p = 0.075 and Z score = -1.78

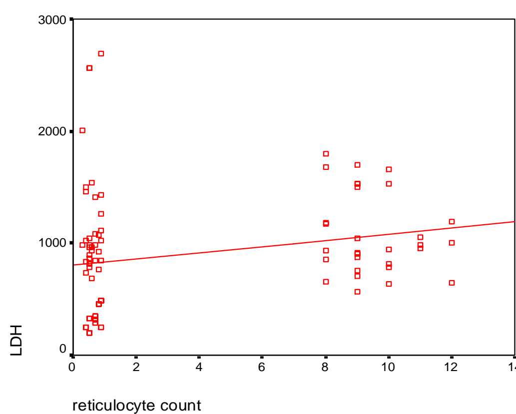

Fig. 3: LDH and reticulocyte count showing positive correlation
(Reticulocyte count vs LDH, r = 0.203, p = 0.054)

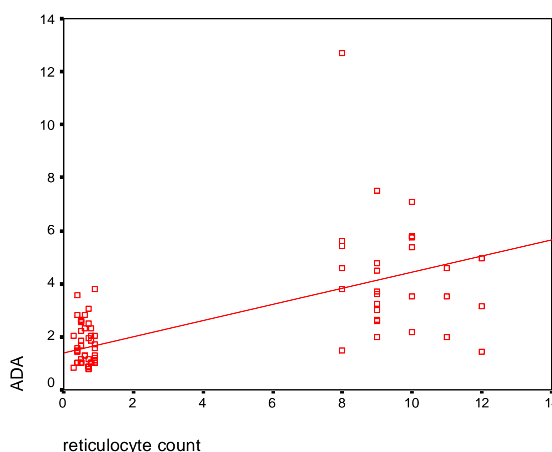

Fig. 4: ADA and reticulocyte count showing positive correlation
(Reticulocyte count vs ADA, r = 0.548, $p < 0.001$)
Difference in r value statistically significant with p = 0.003 and Z score = 2.72

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