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Electrophoretic Analysis of Erythrocyte Proteins in Neonatal Jaundice

[1]Dr. Mathew John, [2]Ms. Aghimol Varghese, [3]Ms. Evelyn Maria, [4]Ms. Sinju. R
Scientist B Jubilee Centre for Medical research (JCMR) Jubilee Mission Medical College & Research Institute
Project Trainee Jubilee Centre for Medical research (JCMR) Jubilee Mission Medical College & Research Institute
Research assistant Jubilee Centre for Medical research (JCMR)
Tutor, Department of Biochemistry Jubilee Mission Medical College & Research Institute
Corresponding Author: Dr. Mathew John

Abstract: *Neonatal jaundice is an alarming situation manifested by hyperbilirubinemia in early infancy. In the present study we analysed the proteins using electrophoretogram in erythrocyte lysate of neonatal jaundice in comparison with normal control. From the elctrophoretogram we observed there was a decrease in band intensity corresponding to catalase and pyruvate kinase monomers. Besides, there were other proteins such as peroxiredoxin and haemoglobin showed marginal decrease at their respective bands in neonatal jaundice hemolysates in comparison to control and the band in the region 260 KDa showed higher band intensity in neonatal jaundice sample compared to control in the electrophoretogram which could be spectrin. The catalase activity by spectrophotometry also observed there was lowered catalase activity in both hemolysate and plasma of neonatal jaundice compared to control.*

Key words: *Neonatal jaundice, catalase, erythrocyte lysate, electrophoretogram*

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I. Introduction

Physiological jaundice is a common condition encountered in most of neonatal population, which can be lethal when persisted resulted in severe damage to systemic functions [1, 2]. Neonatal jaundice was best predicted by first day serum bilirubin (adjusted odds ratio of 3.1 mg/dL with Confidence interval 2.4 to 4.1) and by a change in serum bilirubin from the first to second day of life (2.4 mg/dL) with a confidence interval 1.9 to 3.0. The regression model so adopted based on maternal physiology, ethnicity, social and demographic factors, blood type, life styles and medications and neonatal conditions such as Apgar score and hematocrit had a sensitivity and specificity above 80 % and a false positive rate of 80 % and a false negative rate of 1.1 % in predicting neonatal jaundice [3]. In neonates, the common major cause of jaundice includes red blood cell compatibility and hepatic immaturity along with minor factors such as hypothyroidism and galactosemia [4, 5]. The red blood cell incompatibility reported in neonatal jaundice is strongly associated with glucose 6 phosphate dehydrogenase [6], an important enzyme in the pentose phosphate pathway. The jaundice due to red blood cell incompatibility usually appeared 24 hours postnatal. The jaundice due to hepatic immaturity is normally identified as physiological jaundice and is reported in 60% of normal full term infants. Although neonatal jaundice is harmless, newborns should be routinely monitored for bilirubin levels which in its sustained increasing levels lead to kernicterus [7-9]. Physiological jaundice in healthy term newborns is characterised by an average of total serum bilirubin level 5 to 6 mg/dL on the 3rd to 4th day of life and then declining over the first week after birth. Birth is a sudden state of change to newborns where they are exposed to oxygen resulting in oxidative stress [10, 11]. The present study had focussed on the importance of erythrocyte proteins and its alterations pertained to neonatal jaundice condition.

II. Methodology

Total bilirubin and direct bilirubin estimation of samples were done according to di azo method in the autoanalyser of the central lab facility in the institute. The haematological parameters also were done in hemo autoanalyser of the central lab facility. All the experiments were carried out according to institutional ethical guidelines and approval from the Institutional research committee.

Study design

Group 1 – Normal control neonatal samples (Erythrocyte hemolysate and plasma) where total bilirubin levels 48 hours postnatally at a mean concentration ≤ 10 mg/dL

Group 2 – Neonatal jaundice samples (erythrocyte hemolysate and plasma) where total bilirubin levels tend to be sustained and elevated 48 hours postnatally at a mean concentration ≥ 15 mg/dL.

Statistics

The sample size for catalase assay was 8 numbers per group and for electrophoretic study the three samples each of neonatal hemolysate and controls were independently pooled and analysed. The statistical analysis was done using Microsoft excel and values were expressed as Mean ± SD and P value less than 0.05 considered as significant. For comparison between the groups students-t-test was used.

Erythrocyte lysate preparation

RBC lysis was performed according to the protocol by Dodge et.al [12] with appropriate modifications. Briefly, samples were firstly centrifuged at 1000 g at 4°C for 10 minutes to eliminate plasma and buffy coat. A density gradient separation was performed to remove other blood cells. Erythrocytes washed 3 times with Phosphate buffered saline (PBS) along with 15 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4 containing 0.1 mM Phenyl methane sulphonyl fluoride (PMSF). At each step of separation upper red blood cell layer was removed.

Red blood cells were diluted to a 1:3 ratio with hypotonic lysis buffer (5 mM phosphate buffer, pH 8.0 containing 1 mM EDTA and 0.5 mM PMSF containing 1X protease inhibitor cocktail (PIC) and left in ice for 30 minutes. After freezing, red cells were incubated at 37°C. At the end of lysis step, after a centrifugation of 18,000 g for 10 minutes at 4°C the clear supernatant was collected and stored in deep freezer till further analysis. Each samples prepared in this manner was subjected to protein quantitation by Bicinchoninic acid (BCA) method according to kit protocol (Thermo Fisher). For SDS-PAGE equal amount of protein was loaded to each lane. The sample was mixed with Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl). The mixture was heated in boiling water bath and loaded onto SDS-PAGE. Staining was done with Coomassie brilliant blue – R (CBB-R) followed by destaining with glacial acetic acid methanol mix in distilled water.

Catalase assay

The erythrocyte lysate and plasma samples were subjected to catalase assay according to a protocol by Hadwan et.al. [13] Briefly, 50 µL plasma samples and erythrocyte lysates, were mixed with 500 µL hydrogen peroxide [H_2O_2] (65 µmol/mL in 60 mmol/L sodium phosphate buffer, PH 7.4). The samples vortexed and incubated at 37°C for 3 minutes. This was followed by addition of 2000 µL 32.4 mM ammonium molybdate. Absorbance of the yellow complex of ammonium molybdate and H_2O_2 was measured at 374 nm against appropriate blank.

Catalase activity KU = 2.303/t* Log[S°/S]*Vt/Vs

S° = absorbance of the standard

S = absorbance of sample

Vt = total volume of reagents in test tube

Vs = volume of sample

III. Results

Comparative electrophoretogram of erythrocyte lysates of neonatal jaundice and control

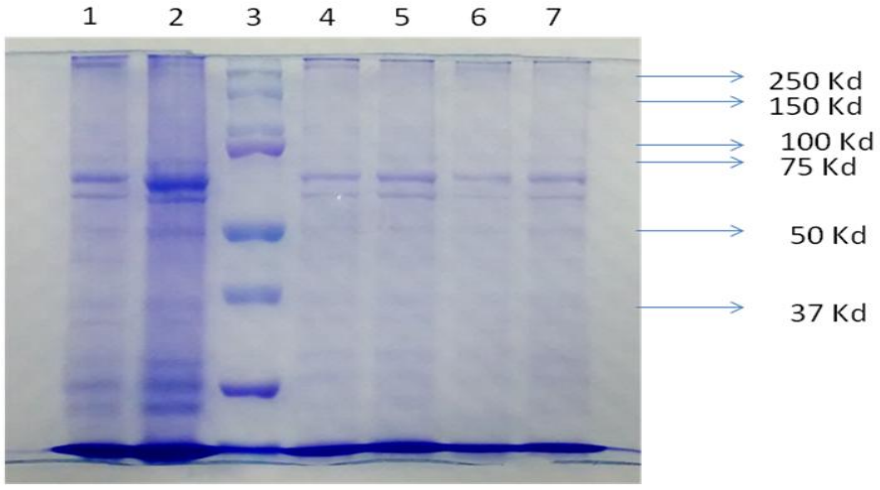

Figure – 1

Lane 1 – Neonatal jaundice samples loaded with 300 µg protein
Lane 2 – Normal control samples loaded with 300 µg protein
Lane 3 – Dual stained precision marker (250 Kda – 15 Kda)
Lane 4 - Neonatal jaundice samples loaded with 150 µg protein
Lane 5 - Normal control samples loaded with 150 µg protein
Lane 6 - Neonatal jaundice samples loaded with 75 µg protein
Lane 7 - Normal control samples loaded with 75 µg protein

The electrophoretogram in the figure 1 depicted the comparative analysis of erythrocyte lysate proteins between neonatal jaundice and normal neonatal samples. Based on related studies on red blood cell proteomics, the two prominent bands appeared on the gel between 50 KDa and 75 KDa could be Pyruvate kinase (58 KDa) and Catalase (60 KDa) in its monomeric forms. The electrophoretogram showed that band intensity of both pyruvate kinase and catalase were decreased in neonatal jaundice samples compared to normal controls. The same trend was observed in other lanes with lesser protein loaded concentrations corresponding to jaundice and control samples. On the top portion of the gel, it was observed that there was a presence of a band in the region of 260 KDa which could be spectrin. The band intensity corresponded to this region appeared to be increased marginally in neonatal jaundice sample compared to control. [Comparison between lanes 1 & 2 (300 µg protein) and lanes 4 & 5 (150 µg protein)], these variations were visible distinctly.

There were some other bands visible in the lower portion of the gel. Among which the bands in the 15 KDa region could be that of hemoglobin (both α and β chain). The band intensity tends to be decreased in neonatal jaundice samples in comparison with control. The region corresponded to 25 KDa region could be peroxiredoxin (21 KDa) where the band intensity was decreased in jaundice samples. This was visible better in lanes 1 & 2 where higher protein was loaded respective of jaundice and control samples.

Catalase activity in erythrocytes and plasma of neonatal jaundice and control

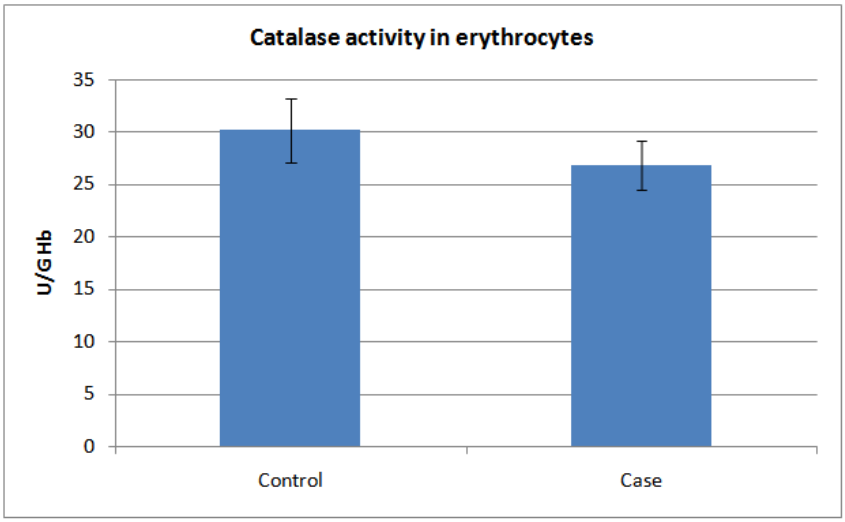

Figure – 2A

Figure – 2A was a graphical representation of Catalase activity in hemolysates of neonatal jaundice and normal control. It was observed that catalase activity was lowered in neonatal jaundice compared to control. The unit of catalase activity was represented as U/gram of haemoglobin (U/g Hb) and the results were expressed as mean ± SD with sample size n = 8. The result was tabulated in table -1 with p value < 0.05 considered as significant.

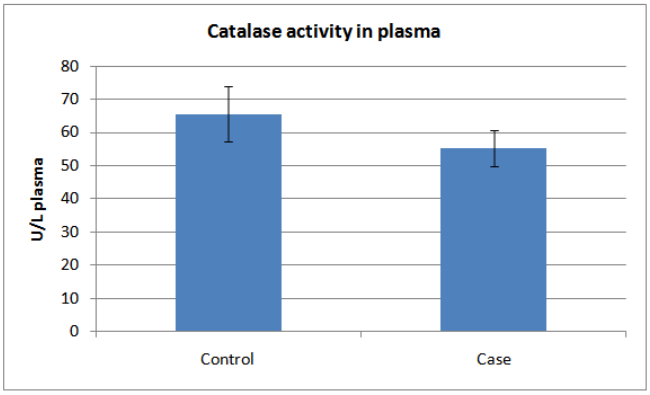

Figure – 2B

Figure – 2B was a graphical representation of Catalase activity in plasma of neonatal jaundice and normal control. It was observed that catalase activity was lowered in neonatal jaundice compared to control. The unit of catalase activity was represented as U/litre of plasma (U/L) and the results were expressed as mean ± SD with sample size n = 8.

Table -1
Comparison of Catalase activity in Hemolysate and Plasma between Neonatal jaundice (Case) and Control

Sample	`Catalase activity		p value	Significance
	Control	Case		
Hemolysate (U/G Hb)	30.12±3.06	26.82±2.37	0.030	p < 0.05 Significant
Plasma (U/L plasma)	65.5±8.34	55.25±5.54	0.011	p < 0.05 Significant

The table – 1 is a compilation of result of catalase activity in hemolysate and plasma of neonatal jaundice cases and control. We observed a significant decrease in catalase activity of both neonatal jaundice hemolysate and in plasma compared to control, $P<0.05$.

IV. Discussion

In the present study, we analysed the electrophoretic pattern of proteins in neonatal jaundice and compared the band intensity of the proteins with normal control. The catalase monomer with molecular weight in the region between 55 KDa and 60 KDa were visible in the electrophoretogram and found that the band intensity in the region corresponding to catalase was lowered in neonatal jaundice samples compared to control. The same trend in catalase activity was observed in hemolysate samples of both neonatal jaundice and control loaded at their respective lower concentrations. We also observed a band in the region between 60 KDa and 65 KDa which could be pyruvate kinase and found that band intensity corresponding to pyruvate kinase was lowered in neonatal jaundice compared to control. This might lead to decreased glycolysis in red blood cells in neonatal jaundice. Henceforth, it can be inferred that the neutralisation of H_2O_2, a potential pro oxidant was decreased in neonatal jaundice due to lowered catalase activity and due to possibly lowered pyruvate kinase response there could be attenuated glycolysis. It was also previously reported that pyruvate kinase activity tends to be lowered in haemolytic anemia and jaundice in adults and also in infant stage jaundice [6, 14]. The similar trend was observed in our elctrophoratogram as visualised in the band intensity corresponding to pyruvate kinase. It was also observed that other lower molecular weight proteins such as haemoglobin alpha and beta chains and peroxiredoxin was lowered in neonatal jaundice as observed in the band intensity corresponding to their molecular weights. Further, it was also noteworthy that the region corresponding to 260 KDa region which could be spectrin had marginally higher band intensity in neonatal jaundice hemolysates in comparison to control. The lowered activity of both catalase and pyruvate kinase implies that both redox mechanism and glycolysis could be compromised in neonatal jaundice. It was also observed that the catalase activity in both erythrocytes and plasma of neonatal jaundice was reduced significantly compared to control. This further reinstated that catalase, one of the major antioxidant defense mechanism was attenuated in neonatal jaundice. Therefore, the present study put forward an overview of one of the mechanisms contributing to pathophysiology of neonatal jaundice.

V. Summary

The present work summarised that catalase activity in both plasma and erythrocyte lysate was lowered in neonatal jaundice in comparison to normal control. We observed the same trend in electrophoretogram as well, as it was observed that the band region corresponding to catalase had lesser intensity in neonatal jaundice when compared to control.

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Conflict of Interest: The authors declared no conflict of interest.

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