

Development and clinical validation of antioxidants based dried blood spots assay - Status as early biochemical marker for rare genetic disorders in Neonates.

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Abstract

Introduction: The present study is to investigate the total antioxidant activity (TAC) in suspected neonates of inborn errors of metabolism with high risk, moderate risk and low risk. Dried blood spots have potential use in remote health applications for individual and population diagnosis and can enable epidemiological surveillance for known and unknown diseases. Dried blood spot sampling is most common method for collection, storing, transporting and analyzing a variety of human body fluids.

Materials and methods: After performing, while newborn screening is early period, the balance residual samples were used for specific secondary research studies with patient consent. Here we are using the secondary studies of DBS for total antioxidant activity as one of the biomarkers to find out the disease condition. TAC was evaluated in 449 risk subjects and 531 control neonates including high, moderate, low risk and healthy individuals. The risk variables were included birth weight, APGAR score, gestational age and previous complications of pregnancy. DBS -TAC method, is to elute sample by using phosphate buffered saline. After sample preparation, TAC by using FRAP assay (ferric reducing antioxidant power assay) were estimated by among four groups with risk indicators.

Results: Statistical comparisons and correlations at 5% level of significance were determined. The mean TAC concentration was significantly elevated in high risk group. The mean TAC for low, moderate and high-risk group (Mean \pm SD values were 954.8 ± 132.2 $\mu\text{mol/L}$, 700.4 ± 100.6 $\mu\text{mol/L}$ and 510.4 ± 110 $\mu\text{mol/L}$ respectively) patients were significantly reduced compared with control group (Mean \pm S.D. valued was 1017.3 ± 122.2 $\mu\text{mol/L}$) ($p < 0.05$). Likely the SOD, Vit C, Vit E and MDA also were estimated.

Discussion: DBS-TAC can be used as early biochemical marker for oxidative stress in high risk for IEM (Inborn errors of metabolism) which may result in reduced tissue damage by free radicals and help to monitor and optimize antioxidant therapy in such high risk neonates.

Conclusion: FRAP assay is presented as a novel method of assessing total antioxidant capacity and is considered as a useful indicator of the body's antioxidant status to counteract the oxidative damage due to ROS. The advantage of the FRAP assay is in being fast, easy to handle, with highly reproducible results. A significantly lower DBS TAC concentration in the IEM high risk patients compared to controls reflects a lower total antioxidant capacity. Thus, TAC may be useful as an early marker of oxidative stress to monitor and optimize antioxidant therapy as an adjunct in the management of IEM patients.

Keywords: Antioxidant therapy, Lipid peroxidation, Metabolic disorders, Newborn Screening, Superoxide mutase, Vitamin C, Vitamin E.

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Introduction

Dried blood sampling and newborn screening aims at the earliest possible recognition of disorders to prevent the most serious consequences by timely intervention screening is not a confirmatory diagnosis and requires further investigations. New-born screening is a simple blood test to identify neonates who may have one of 29 primary treatable rare inherited disorders. A new-born baby can look healthy but one of these rare disorders. The most common disorders are congenital hypothyroidism (CH), cystic fibrosis (CFTR), medium chain acyl coA deficiency (MCAD), maple syrup urine disease (MSUD), congenital adrenal hyperplasia (CAH), biotinidase deficiency (BIOT), phenylketonuria (PKU), galactosemia (GALT) and glucose-6-phosphatase deficiency (G-6-PD).

These diseases will be included in the first case, because the annual birth rate is 21.76 births /1000 population and in Delhi alone, nearly 900 births takes place every day; considering this figure there may be one or two babies born Delhi alone with a metabolic defect each day [1]. One in every 1,000 newborns has inborn errors of metabolism (IEM) or other disorders that can be detected at birth by screening (**Table 1**). Inborn errors of metabolism, often referred to as congenital metabolic diseases or inherited metabolic disorders are rare genetic (inherited) disorders in which the body cannot properly turn food into energy. The disorders are usually caused by defects in specific proteins (enzymes) that help break down (metabolize) parts of food [2].

In the present study, we investigated total antioxidant activity as early biochemical marker of oxidative damage in dried blood spots samples from IEM subjects with high, moderate and low risk to measure of lipid peroxidation and super dioxides mutase, a measure of protein oxidation. We also determined DBS- total antioxidant activity in these samples. Subsets of IEM patients showed significantly higher levels of the damage markers with total antioxidant activity.

Study Design

The study has been approved by the institutional human ethical committee approval from Madras Medical College, Chennai: Approval No. 32042018 and obtained consent form from parents for the participation of this research study. Under aseptic conditions with precautions about dried blood spots sampling was collected from suspected IEM neonates and from healthy controls. All neonates aged (0-28) days after birth, normal newborns and including low birth weight neonates and that admitted to Neonatal Intensive Care Unit (NICU), Institute of obstetrics and Gynaecology and Govt. hospital for Women and Children, will be systematically

enrolled into the study. The neonatal heel prick or Guthrie test is a screening test done on newborns. It consists of making a pinprick puncture in one heel of the newborn and soaking the blood into pre-printed collection cards known as Guthrie cards. Heel prick capillary blood collected on S&S 903 filter paper, air dried and transported along with ice pack in cold storage.

Methods and Materials

The study was conducted at Department of Biochemistry, Regenix Super speciality laboratories pvt ltd, Chennai. The study included 980 neonates aged under 28 days of life of either sex (201 male babies with mean age of 11 days, 223 female babies with mean age of 9 days and 25 ambiguous genitalia babies with mean age of 6 days) were suspected as inherited metabolic disorders confirmed by specific assays like BIORAD-Quantase neonatal screening, amino acid and acyl carnitine profiling by Tandem mass spectrometry, before the commencement of therapy and 531 neonates, age and sex matched healthy controls. The demographics of this study population shows risk factors associated with maternal complications type of delivery, familial conditions, mother's history, drugs usage, gestational age, number of births, and number of previous siblings death (**Figure 1**). IEM suspected neonates were further classified according to the ACMG recommended panel criteria. All subjects underwent an initial screening that included a detailed history, signs, symptoms, clinical and laboratory findings of neonates were eligible if they had symptomatic conditions. Exclusion criteria were as follows as: signs of severe infection, severe vomiting, and congenital anomalies.

Modified Method of Total Antioxidant activity - Ferric reducing power assay by using dried blood spots

Elution Tactics

Phosphate Buffered with Saline (10X): 800 ml of distilled water; 8 g of NaCl; 200 mg of KCl; 1.44 g of Na_2HPO_4 ; 240 mg of KH_2PO_4 , pH-7.4 and add distilled water upto 1L. For sample preparation, simply punch out single 3 mm disc with dried blood spots in a 1.5 ml eppendorf, Add 200 μl of phosphate buffered saline then centrifuge with 12,000 rpm spin column for 2 to 5 minutes. The eluted sample is ready to use. The standard method for the total antioxidant activity was estimated by FRAP method by Benzie.F.F. and J.J.Strain. Total antioxidant capacity was measured by FRAP assay according to the method of Benzie.F.F. and J.J.Strain [3]. At low pH, when a ferric tripyridyltriazine (Fe III-TPTZ)

Table 1. Burden of Genetic and Metabolic Diseases in India: Recent Studies (2015).

Disorder	Incidence	Births/year
Congenital Malformation	1:50	678,000
Down's Syndrome	1:800	34,000
Metabolic Disorders	1:1200	22,500
Beta-Thalassemia and Sickle cell Disease	1:1700	16,700
Congenital Hypothyroidism	1:2500	10,900
Duchenne muscular dystrophy	1:10000	2,700
Spinal Muscular atrophy	1:10000	2,700

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complex is reduced to the ferrous (Fe II) form, an intense blue colour with an absorption maximum at 593 nm develops. Antioxidant power convert ferric to ferrous tripyridiazine complex. Ferric reducing ability of plasma (FRAP) values are obtained by comparing the absorbance change at 593 nm in mixture(test), with those containing ferrous ion in known concentration (Table 2).

Procedure

Acetate buffer (pH 3.6): 3.1 gm sodium acetate was dissolved in 500 ml distilled water and to this 16 ml of glacial acetic acid was added. pH of the solution was adjusted up to 3.6 by 1% Na₂CO₃ and finally diluted with distilled water to 1000 ml. Ferric Chloride (20M/I): 324 mg. of FeCl₃.6H₂O was dissolved in distilled water and diluted to 100 ml. Ferrozine (10 mM): 49 mg Ferrozine was dissolved in 10 ml of 0.04 M HCL. Standard (1mmol/L): 278 mg Iron(II) sulfate heptahydrate (FeSO₄.7H₂O) was dissolved in 1000 ml distilled water.

Like FRAP assay, estimation of lipid peroxidation by MDA, Vit E, Vit C and SOD enzyme levels were carried out on DBS samples were measured by UV-Spectrophotometry (Chemito). Vitamin E was estimated by Baker and Frank method [4] and vitamin C by 2, 4 – DNPH method [5]. SOD enzyme activity was determined with the direct spectrophotometric method employing KO₂ as previously described by Marklund, with some modifications [6,7]. Lipid peroxidation was measured

by MDA estimation according to the colorimetric method of Satoh.k [8]. Lipoproteins are precipitated from the specimen by adding trichloroacetic acid. 0.05 M sulphuric acid and 0.67% thiobarbituric acid (TBA) in 2 M sodium sulphate are added to this precipitate and the coupling of lipid peroxide with TBA is carried out by heating in a boiling water bath for 30 minutes. The resulting chromogen is extracted in n-butanol, which is measured at 530 nm.

Statistical Analysis

Each result was expressed as mean ± standard deviation. The statistical significance of the data was determined by Student's t-test and one way ANOVA test at 5% level of significance (Table 3). Pearson's correlation coefficient was determined at 5% level of significance. Statistical analysis was done using SPSS software version 21.0.

Results

There was a significantly increased levels of MDA in the high risk patients comparing to moderate, low risk and control groups (p<0.05). They were significantly decrease levels of Vitamin E, Vitamin C, SOD enzyme activity levels, along with the TAC in the positive patients (significantly decreased in IEM high risk positive patients compared with the control groups(p<0.05). Statistically significant negative correlations found between TAC & MDA in high and moderate risk

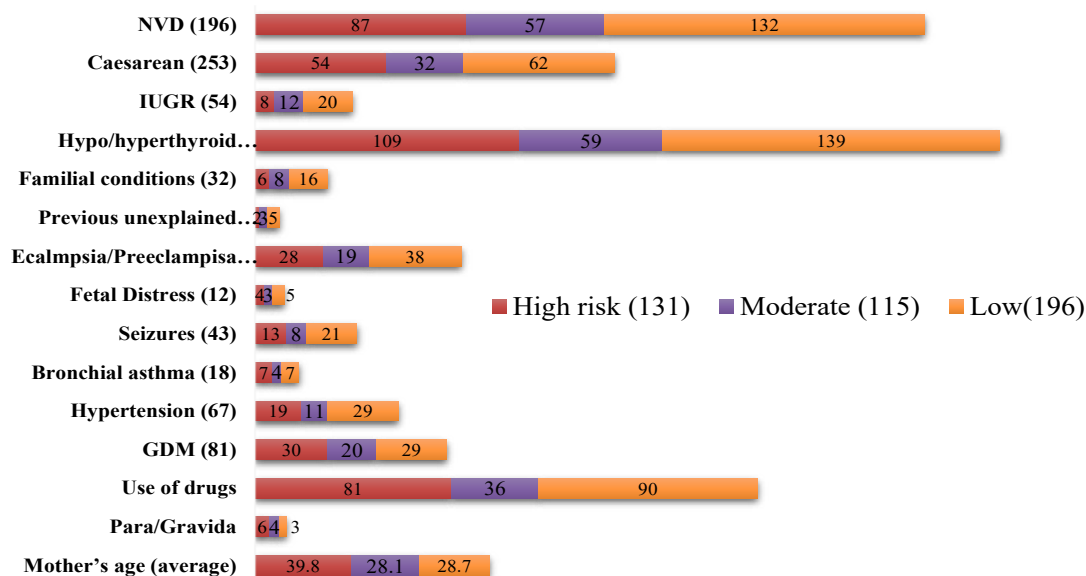


Figure 1. Demographics features of the study population: Maternal complications with Low/Moderate/ High risk neonates.

Table 2. New Assay method for DBS-TAC (dried blood spots- total antioxidant activity by FRAP assay).

Reagent	Test	Control	Standard	Blank
Acetate Buffer	500 ul	550 ul	500 ul	500 ul
Fecl3 6H20	50 ul	---	50 uL	50 ul
Ferrozine	50 ul	50 ul	50 ul	50 ul
Sample	100ul	---	---	---
Std (stock)	---	---	100 ul	--
D/W	---	100 ul	---	---
Mixed well and incubated at 37 C for 30 min				
Sample	----	100 ul	----	----
Incubated at 37 C for 10 min and read at 593 nm by UV -Spectrophotometry.				

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Table 3. Comparison of oxidants and antioxidants profile in suspected cases of IEM.

Antioxidant status	High Risk	Moderate Risk	Low Risk	Controls	P value
TAC(FRAP) (μ mol/L)	510.4 \pm 110.1	700.4 \pm 100.6	954.8 \pm 132.2	1017.3 \pm 122.2	p<0.05
SOD (units/ml)	3.4 \pm 0.4	4.1 \pm 0.4	5.7 \pm 1.1	7.7 \pm 0.9	p<0.05
Vit C (mg/dl)	3.6 \pm 0.2	5.2 \pm 0.2	5.3 \pm 0.4	6.3 \pm 0.2	p<0.05
Vit E (mg/L)	7.4 \pm 0.6	8.2 \pm 0.6	9.9 \pm 0.7	11.2 \pm 0.8	p<0.05
MDA (mmol/L)	2.8 \pm 0.7	2.1 \pm 0.5	1.3 \pm 0.1	0.9 \pm 0.1	p<0.05

Table 4. Correlations between oxidants and antioxidants in Suspected IEM cases.

Groups (MDA& TAC)	r-value	p value
High Risk	-0.015	p<0.05
Moderate Risk	-0.075	p<0.05
Low Risk	0.136	p<0.05
Controls	0.402	p<0.05

Note: *r* -value describes Negative and positive correlations between MDA and TAC activity.

groups, and positive correlations between low risk and control groups, suggested of oxidative stress. Our results clearly shows that severe oxidative stress in the IEM positive cases is compared with moderate and low risk category and increases significantly with the progression of disease, (i.e) the risk of IEM represents as high- risk positive patients for the specific diseases.

Discussion

The inborn errors of metabolism involve three inter-related and often overlapping factors as accumulation of toxic metabolite, deficiency of essential metabolites, and impaired functions of mitochondria. Oxidative stress often results has been proposed as a prime cause of the cellular injury that causes in the IEM. We quantified total antioxidant activity as early biochemical marker in various IEM to test their potential utility as clinically relevant marker. Likely SOD levels, Vit E, Vit C, MDA levels have been employed to measure oxidative and antioxidants stress in vivo using dried blood spots sampling as a secondary use of residual blood spots after it used for the neonatal screening.

In this study the biomarkers, taken as a whole, elevated markers of oxidative damage and depleted antioxidants defenses were seen in most of the IEM diagnostic group we assessed. In aminoaciduria's, the ketogenic acids have been showing to include oxidative models. Previous studies on Urea cycle disorders under treatment found elevated levels of oxidative damage were seen in subjects with endocrine metabolic disorders like CH and CAH. Decrease antioxidant activity was marked in organic acidurias[9-12]. The summary of the oxidative stress, where elevated levels in high risk patients compared with control groups. DBS-TAC can be used as a early biochemical marker for oxidative stress in high risk for IEM (Inborn errors of metabolism) which may result in reduced tissue damage by free radicals and help to monitor and optimize antioxidant therapy in such high risk neonates (Table 4).

The risk of IEM is inversely proportional to TAC in neonates. Under normal conditions, the ROS produced in the course of metabolism are contained by the natural antioxidant system which consists of a series of antioxidant enzymes such as

superoxide dismutase (SOD), catalase, glutathione peroxidase as well as numerous endogenous and dietary antioxidant compounds that are capable of reacting with and inactivating ROS thereby protects the functional and structural molecules against ROS-mediated tissue damage. MDA is a three carbon, low molecular weight aldehyde that can be produced from free radical attack on polyunsaturated fatty acids of biological membranes [13,14].

The determination of MDA is used for monitoring lipid peroxidation in biological samples. TAC reflects the increased formation of ROS and lipid peroxidation with progressive symptoms to evaluate. Vitamin E, a potent chain breaking lipid soluble antioxidant, reacts with lipid peroxy radicals eventually terminating the peroxidation chain reaction and thereby reducing oxidative damage. Vitamin C represents the major water-soluble antioxidant in the human body. SOD is an endogenous antioxidant that catalyzes the dismutation of the superoxide anion radical [15-19]. There are conflicting reports in the values of antioxidant vitamins (vitamin E and vitamin C) and SOD enzyme activity among IEM high risk cases in various stages in the literature. In our study, vitamin E, vitamin C, SOD and TAC levels are decreased in high risk cases.

Conclusion

FRAP assay is presented as a novel method of assessing total antioxidant capacity and is considered as a useful indicator of the body's antioxidant status to counteract the oxidative damage due to ROS. The advantage of the FRAP assay is in being fast, easy to handle, with highly reproducible results. A significantly lower DBS TAC concentration in the IEM high risk patients compared to controls reflects a lower total antioxidant capacity. Thus, TAC may be useful as an early marker of oxidative stress to monitor and optimize antioxidant therapy as an adjunct in the management of IEM patients.

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