Redox status in erythrocyte of Northeast Indians suffering from chronic liver disease (CLD)

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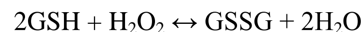
Abstract
Chronic liver disease is a hepato-necrotic disease persisting for more than six months. The objective of the study was to assess the redox process in chronic liver disease (CLD). The Redox process has been recognized as a significant regulatory system in various biological processes like cell proliferation, signal transduction and gene expression etc. Glutathione plays a crucial role by acting as a regulatory molecule in the immune system. Here a study has been conducted on 57 indoor patients suffering from CLD and 57 healthy controls to observe the redox potential in the erythrocyte by estimating reduced glutathione and L-cysteine levels. Liver cirrhosis causes a decreased level of glutathione in plasma and hepatic cells due to impaired uptake of L-Cysteine by RBC. Therefore we conclude that L-cysteine uptake by RBC is impaired in CLD which causes the vicious circle of oxidative stress.

Keywords: CLD, Redox process, Liver cirrhosis, Reduced glutathione, L-cysteine, RBC.

Introduction
Chronic liver disease is a variety of diseases occurring due to liver cell dysfunction and is defined as hepato–necrotic inflammation (detectable by biochemical or histological methods) continuing for more than 6 months. The disease is self-limiting but usually progresses to fibrosis and subsequent architectural destruction with regenerating nodules leading to cirrhosis.[1]

Multiple factors have been found responsible for the genesis of the disease. Oxidative stress is considered the most common feature of any type of CLD. Oxidative stress is a pathological state due to disturbance in the balance between oxidants (ROS) and antioxidants in the body. Oxidative stress results from the shifting of the balance between pro-oxidants and anti-oxidants. There are innumerable number of agents generating oxidative stress. Oxygen under aerobic condition is reduced and the electronic configuration is changed and generates ROS, which includes primary oxidants like superoxide radical, H_2O_2 and hydroxyl radical. Secondary oxidants like hydro peroxide and peroxy radicals of bio-molecules are also produced.[2] Redox processes have been implicated in various biological processes including signal transduction, gene expression and cell proliferation, several molecules have been identified as redox regulators in cell activation.[3] Glutathione is the most investigated molecule among them, it is evident that GSH plays an important role in regulating human's immunity by antibody responses and lymphocyte proliferation.[4,5]

Deficiency of reduced glutathione is one of the most important antioxidants observed to be deficient in CLD. Reduced glutathione (GSH) is a tri-peptide, composed of glutamate, cysteine and glycine. It is the key antioxidant and a protective molecule which can work both in and outside the cell. It has multiple functions in disease prevention and detoxification of chemicals and drugs and its deficiency is associated with increased risk of toxicity and diseases. In healthy cells and tissues more than 90% of the total GSH pool is in reduced form (GSH) and less than 10% exists in the disqualified form (GSSG). An increased GSSG to GSH ratio is considered indicative of oxidative stress.[6]



$$2GSH + H_2O_2 \leftrightarrow GSSG + 2H_2O$$

It works synergistically with other antioxidants and utilizes scavengers, other free radicals and oxygen, and thereby prevents oxidative stress. There are only a few studies in the medical literature which relate to the role of antioxidant particularly GSH is CLD. In 1996 *Baro baro*[7] *and colleagues* from Italy reported the levels of GSH in liver, blood and lymphocyte and observed that GSH quantity was significantly reduced in CLD. *De. Maria*[8] *and co-investigators* at *Oklahoma Medical Research Centre* also confirmed this observation.

GSH synthesis, L-cysteine influx and oxidative stress in human erythrocytes: Red blood cells (RBCs) are unique, highly specialized and one of the most abundant cells in human beings. Their primary function is transportation of the respiratory gases, O_2 and CO_2, between lungs and tissues. They are equipped with effective anti-oxidative systems. Several scavengers provide antioxidant protection to RBCs against ROS.[9,10] ROS are neutralized by endogenous antioxidants such as reduced glutathione (GSH), alpha-tocopherol, vitamin-C, and other antioxidant enzyme systems. However, a condition of oxidative stress develops when the critical balance between oxidants and antioxidants is disrupted due to depletion of antioxidants and or excess accumulation of ROS.[11] RBCs are highly susceptible to oxidative damage due to the high cell concentration of oxygen and hemoglobin, a powerful promoter of the oxidative process.[12]

Human erythrocyte has all the enzymes necessary for synthesis of reduced glutathione (GSH), but unlike other cells, cannot uptake GSH. It can efflux oxidized glutathione (GSSG) by an energy dependent process. Therefore, the level of GSH in erythrocytes is greatly influenced by the

rate of its synthesis. The rate of synthesis of GSH is dependent on the availability of L-cysteine. On the other hand the availability of L-cysteine is dependent on the rate of L-cysteine influx.

A number of research publications have been reported regarding this, but their associations with L-Cysteine uptake by cells is lacking or not clear. L-Cysteine, one of the most important components of reduced glutathione, is required to be taken by RBC to synthesize glutathione. Its deficiency may lead to the deficiency of reduced glutathione and may be one of the important contributors or enhancers of the disease. With this background the present work has been conducted. Here the reduced glutathione concentration of RBC along with its uptake level of L-Cysteine has been analyzed in case of CLD and compared with control subjects.

Materials and Methods

This is a case-control study conducted on patients suffering from CLD (as diagnosed by standard protocol), admitted in the Department of Medicine, MGM Medical College and LSK Hospital, Kishanganj, Bihar, India from December 2013 to September 2015. Patients were heterogeneous and emigrated from different districts and states especially the northeastern region of India. The study was approved by the institutional ethical committee and proper informed consent was taken from every patient after explaining the procedure in detail before their inclusion in the study. All patients included in this study underwent a detailed clinical evaluation.

Inclusion criteria

Patients that were included for this study, were suffering from hepatitis for more than six months.

Exclusion criteria

Patients suffering from Hyperlipidemia, Hypertriglyceridemia, Hepatocellular carcinoma, Anemia and Obesity were not taken for this study.

Tools & Technique

Sample Collection: Blood sample was collected in three types of vacutainers from patients admitted in the indoor patient ward, Department of Medicine, MGM Medical College & LSK Hospital, Kishanganj, Bihar, India.

1. ***Clot Blood Sample*:** Where the vacutainer had no anticoagulant and the sample was used for LFT, KFT, Lipid Profile tests.
2. ***Fluoride Vacutainer*:** Blood samples were collected for fasting blood glucose estimation.
3. ***EDTA Vacutainer*:** Here the sample was collected for performing L-Cysteine uptake and Glutathione estimation test.

Total 57 subjects were taken, 57 cases suffering from CLD and 57 normal individuals for control. A proper history was taken and blood samples were collected aseptically for performing the following tests:

1. 3ml blood for Liver Function Test (LFT), Kidney Function Test (KFT), Lipid Profile & Random Blood Glucose – performed by 'SELECTRA Pro' fully automated clinical chemistry analyzer (MERK).
2. 0.4ml whole blood for estimation of L-cysteine influx as per Yeildtz's method – performed by UV-VIS Spectrophotometer (SYSTRONICS).
3. 0.2ml whole blood for estimation of reduced glutathione (GSH) as per 'Dacie and Lewis Practical Haematology' Book – performed by UV-VIS Spectrophotometer (SYSTRONICS).

Estimation methods of different biochemical parameters

Glucose: Photometric determination of glucose based on GOD-POD method; *Bilirubin (total & direct):* Malloy – Evelyn modified method (End Point); *AST/SGOT:* IFCC method without pyridoxal phosphate (P-5-P), Kinetic UV. *ALP (Alkaline Phosphatase):* Based on DGKC and SCE Method; *Serum Total Protein estimation:* Biuret reaction method; *Urea estimation:* Enzymatic – UV. Cinetique;[13] *Creatinine estimation:* Jaffe's Colorimetric Method – Kinetic;[14] *Total cholesterol estimation:* Direct Enzymatic colorimetric, Liquid;[15] *Triglyceride estimation:* Enzymatic colorimetric test – GPO PAP;[16] *HDL estimation:* Direct Enzymatic colorimetric, Liquid.

Estimation of reduced glutathione[17]

Reagents

(a)Lysing solution: Disodium EDTA 1g/L; *(b)Precipitating Reagent*: 1.67g Metaphosphoric acid (sticks), 0.2g Disodium EDTA and 30g NaCl dissolved in 100 ml distilled water. For rapid solubility reagents are added to boiling water and the volume is made up after cooling. This precipitating reagent is stable for at least 3 weeks at 4°C; *(c)Sodium hydrogen phosphate (300 mmol/L)*: 107.4g/L $Na_2HPO_4.12H_2O$ or 53.4g/L $Na_2HPO_4.2H_2O$ or anhydrous 4.6g/L Na_2HPO_4; *(d)Sodium citrate (34 mmol/L)*: 1g of sodium citrate (trisodium salt) in 100 ml of distilled water, pH 8.0. *(e)DTNB Reagent*: Dissolve 20mg of DTNB in 100 ml of sodium citrate buffer (pH-8); *(d)Glutathione Standard:* For standard curve construction, dilutions are made from 1.62 mmol/L (50 mg/dl), the stock solution of GSH. As GSH oxidizes slowly in solution, stock solution should be prepared freshly with degassed (boiled) water or saline for each run.

Method

0.2 ml of EDTA blood dissolved to 1.8 ml of lysing solution and allowed to stand at room temperature (not more than 5 minutes) for lysis to be completed. Add 3 ml of precipitating solution, mix well and allow to stand for a further 5 minutes. After mixing properly, filter using a single-thickness Whatman No. 42 filter paper. Add 1 ml of the clear filtrate to 4 ml of freshly prepared Na_2HPO_4 solution. Measure the optical density at 412 nm (A1). Then add 0.5 ml of DTNB reagent and mix thoroughly. The color develops rapidly and begins to fade after about 10 min so the second reading should be within this period. Measure

the optical density at 412 nm (A2) and calculate the change in absorbance (ΔA^{412}). A reagent blank is made using saline or plasma instead of whole blood.

If assays are carried out frequently, it is not mandatory to construct the standard curves each time. However, a standard curve is essential initially to calibrate the instrument and should be done regularly to check the suitability and validity of the reagents. Suitable dilutions of GSH are obtained by substituting 5, 10, 20 and 40 ml of the GSH stock solution (make up to 0.2 ml with lysing solution) for the blood in the reaction.

Calculation of GSH Concentration: Concentration of GSH in the cuvette sample (GSH_c) is given by the following mathematical expression: $\Delta A^{412} \times E1/\varepsilon \times 5.5$ µmol

Where ε = extinction coefficient and E1 = derived correction factor.

The concentration of GSH in the whole blood sample is as follows:

$$\frac{GSH_c \times 5}{0.2}\ \mu\text{mol/ml}$$

The unit is often expressed in terms of mg/dl of red cells. The molecular weight of GSH is 307. Thus GSH in mg/dl packed red cells is given by the following.

$$\frac{GSH_c \times 5}{0.2} \times \frac{1}{PCV} \times 370 \times 100$$

Estimation of L-cysteine uptake by RBC:[18-20]

Reagents

(1)Phosphate Buffer Saline glucose (pH- 7.4) –10 mg of glucose. prepared by mixing equal volume of 150 mM sodium phosphate buffer, pH 7.4 and 0.9 %, NaCl) containing 10 mM D-glucose; *(2)10 mg/dl cysteine (L-Cysteine); (3) Tris EDTA buffer (pH 8.9); (4)DTNB*

Method

2 test tubes were taken, one for test (T) and another for control (C). 400µL of PBS-glucose buffer was taken in C-tube and 400µl of L-Cysteine was taken in T-tube. 400µL of EDTA blood sample was added to each tube (C and T). Both the test tubes were incubated at 37˚C for 1 hour and the following protocol was followed:

The tubes were taken out from incubator and centrifuged at 5000 rpm for 10 minutes, the supernatant discarded, washed with PBS-glucose saline (equal amount) for 3 times at 5000 rpm for 10 minutes. Again supernatant was discarded. 800µL of double distilled water and 320µL of 100 % TCA was added and again the tubes were centrifuged at 5000 rpm for 10 minutes. The supernatant was taken only 50µl and to this 950µl of DTNB and 950µl of Tris-EDTA buffer was added and optical density was measured at 412nm. The concentration of L-cysteine was calculated using a standard curve, rate expressed as microgram per hour. Influx rate was calculated by subtracting control from the test.

Table 1: Distribution of reduced glutathione (GSH) among control and case

Table Analyzed	**Data 1**
Column A	control (GSH)
vs	vs
Column B	case (GSH)
Unpaired t test	
P value	**P<0.0001**
One- or two-tailed P value?	Two-tailed
t, df	t=12.08 df=112
Mean ± SEM of column A	70.05 ± 1.107 N=57
Mean ± SEM of column B	39.77 ± 2.250 N=57
Difference between means	30.28 ± 2.507
95% confidence interval	25.31 to 35.25
R squared	0.5656
F test to compare variances	
F,DFn, Dfd	4.129, 56, 56
P value	**P<0.0001**

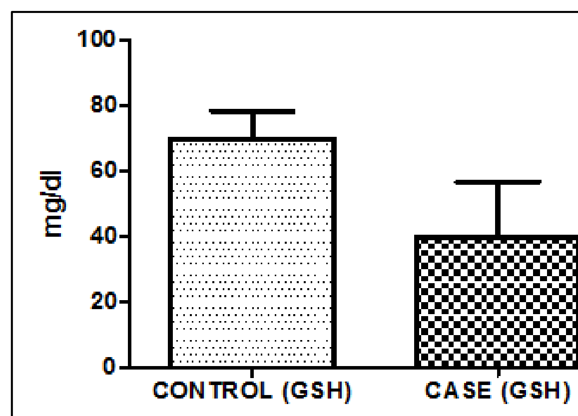


Figure 1: Distribution of reduced glutathione (GSH) among control and case

Table 2: Distribution of L-cysteine uptake by RBC among control and case

Table Analyzed	**Data 2**
Column A	control (L-Cys)
vs	vs
Column B	case (L-Cys)
Unpaired t test	
P value	**P<0.0001**
One- or two-tailed P value?	Two-tailed
t, df	t=14.57 df=112
Mean ± SEM of column A	645.9 ± 14.24 N=57
Mean ± SEM of column B	301.5 ± 18.87 N=57
Difference between means	344.4 ± 23.64
95% confidence interval	297.5 to 391.3
R squared	0.6546

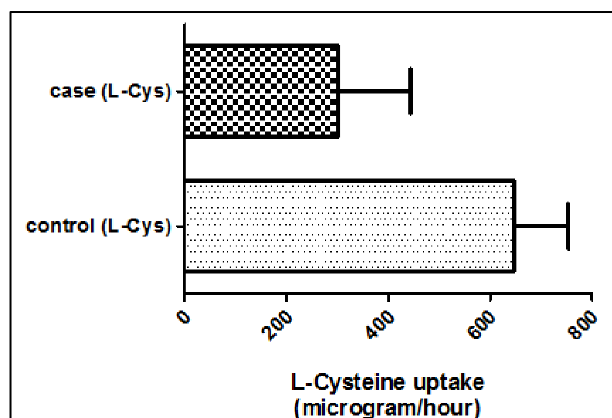


Fig. 2: Distribution of L-cysteine uptake by RBC among control and case

Results & Analysis

Oxidative stress increases with the advanced stage of CLD along with altered redox status; this may be predicted by measurement of in-vitro L-Cysteine influx in erythrocytes.

Discussion

Chronic liver disease (CLD) in clinical content is a disease process of the liver that involves a process of progressive destruction and regeneration of liver parenchyma leading to fibrosis and cirrhosis. It consists of a wide range of liver pathologies which include inflammation, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. There are numerous reports of oxidative stress in this condition.[21] A deficiency of GSH and antioxidant parameters or an increase in toxic free radical species may contribute to the progression to this liver disease. Liver is the principal site of GSH synthesis in the body and level is reflected in blood and RBC.

Glutathione is the most abundant low molecular weight thiol and GSH/ GSSG is the major redox couple in the animal cell. Synthesis of GSH from glutamic acid, cysteine and glycine is done by two enzymes (1) γ-glutamylcysteine synthetase, (2) glutathione synthetase, and this synthesis is dependent on the availability of L-cysteine in the cell. L-Cysteine is received by the cell from extracellular space through a specific amino acid transporter.

In the present study GSH and L-Cysteine uptake have been estimated in CLD cases in RBC. CLD is diagnosed according to criteria given in Harrison. In this study it is observed that both GSH and L-Cysteine uptake was significantly low in CLD cases in respect to control. So GSH synthesis may be affected or its utilization may be increased due to excess generation of ROS or both, and may lead to this low GSH concentration. In this condition it is also observed here, that GSH concentration is positively correlated to L- Cysteine uptake. So decrease in GSH concentration may be due to proportionate fall of L-Cysteine concentration within the cell as observed by the L-Cysteine uptake pattern. It is also observed that SGOT, SGPT are negatively correlated with reduced GSH and L-Cysteine uptake. So the degree of fall of GSH and L-Cysteine uptake is dependent upon degree of liver damage. So fall of GSH might be due to the decrease in cellular L-Cysteine concentration.[22]

In the laboratory, RBC is a reliable model for the study of oxidative stress. There are certain red blood cell biomarkers that can be used as measures of oxidative stress. MDA is a product of lipid peroxidation, GSH level and GSH/GSSG ratio reflects redox state of the cell, enzymes like glutathione peroxidase, catalase, superoxide dismutase (SOD), etc., reflects capacity of the cell fighting against oxidative stress.[23]

There are studies indicating that the level of GSH decreases in erythrocytes along with a decrease in L-cysteine influx (availability of L-cysteine is a rate-limiting factor in GSH synthesis in erythrocytes) in cases associated with increased oxidative stress – like ageing, type 2 diabetes mellitus etc. In a study by Rizvi *et al*, 2008,[24] L-cysteine influx was measured in human erythrocytes by suspending cells in a solution containing 10mM L-cysteine, a significant decline in the influx of L-cysteine in erythrocytes during aging in humans was observed. The decrease in L-cysteine influx correlates with the decrease in the antioxidant potential of plasma measured in terms of FRAP (Ferric Reducing Ability of Plasma) during aging. The conclusion was that a decreased influx of L-cysteine could be an important factor contributing to the development of oxidative stress in human erythrocytes during aging. Erythrocyte oxidative stress has been implicated in the pathogenesis of diabetes mellitus and the deficiency of antioxidant defense by the glutathione (GSH) pathway. This is thought to be one of the factors responsible for the development of complications in diabetes. In another study by Rizvi *et al.* 2010,[25] it was found that the L-cysteine influx in erythrocytes from type 2 diabetic patients was significantly lower compared to age-matched controls.

From this observation it may be concluded that the disease process somehow affected the cysteine transport system of the cell irrespective of the cause of the disease. The disease which is either of viral or non-viral causes does not always lead to the progression of the CLD state. Probably those groups of patients, by some unknown mechanism, damage the L-Cysteine transport system and are the poor victims of the disease, but this requires extensive research work in this field.

Conclusion

GSH concentration of RBC is significantly low in cases of CLD with respect to control. L-Cysteine uptake by RBC was also significantly low in patients of CLD with respect to control. Both changes are proportionately correlated to the degree of damage to the hepatic cells.

Conflict of interest

None.

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