Section: Miscellaneous

Original Research Article

EVALUATION OF RED CELL MEMBRANE COMPOSITION AND METABOLIC ASPECTS IN THE CONTEXT OF OXIDATIVE STRESS IN CORONARY ARTERY DISEASE

P. V. Rajini[1], Dadala Soundarya Mahanthi[2], Tadi Anil Kumar[3], T. Arun Manas[4], Anvesh Buddha[5]

[1]*Associate Professor, Department of Biochemistry, Government Medical College, Paderu, Andhra Pradesh, India.*
[2]*Assistant Professor, Department of Biochemistry, Government Medical College, Paderu, Andhra Pradesh, India.*
[3]*Consultant, Department of Anesthesiology, RK Hospital, Visakhapatnam, Andhra Pradesh, India.*
[4]*Post Graduate, Department of Orthopedics, GSL Medical College, Rajahmundry, Andhra Pradesh, India.*
[5]*MBBS, Department of Orthopedics, GSL Medical College, Rajahmundry, Andhra Pradesh, India.*

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Corresponding Author:
Dr. P. V. Rajini,
Associate Professor, Department of Biochemistry, Government Medical College, Paderu, Andhra Pradesh, India.
Email: rajinitallapudivsp@gmail.com

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ABSTRACT

Background: The role of oxidative stress in cardiovascular disease processes, such as atherogenesis, ischemic-perfusion injury and cardiac remodelling, has been increasingly recognized in the past few decades. Currently, an increasing number of studies suggest that level of oxidative stress markers in body fluids correlate with atherosclerotic disease activity this finding may lead to novel clinical approaches in patients with coronary artery disease. Assessment of oxidative stress markers could modify risk stratification and treatment of patients with suspected coronary artery disease or myocardial infarction.[1]
ROS are highly reactive chemical species containing oxygen, controlled by both enzymatic and non-enzymatic antioxidant defence systems. In the heart ROS play an important role in cell homeostasis, by Modulating cell proliferation, differentiation, and excitation - contraction coupling.[2] Oxidative stress occurs ROS production exceeds the buffering capacity of the antioxidant defence systems, leading to cellular and molecular abnormalities, ultimately resulting in cardiac dysfunction.[2] Formation of atherosclerotic plaques is the major cause of coronary artery disease. The acute form of CAD is more susceptible to oxidative D, damage, suggesting the use of Antioxidant therapy may be warranted to ameliorate oxidative stress in this condition.[3] The aim of this study is to estimate the free and esterified fractions of total cholesterol of erythrocyte membrane (CEM) protein and sialic acid and phospholipid content of RBC membrane along with the measurement of the oxidative stress and anti-oxidant status in RBC and serum Malondialdehyde level, Glutathione per oxidase activity, Catalase activity, superoxide dismutase activity and Ceruloplasmin levels and interleukin (IL- 8) estimation.
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Materials and Methods: • Case Control Study was conducted on 100 cases and 100 healthy persons. • RBC membrane protein assay. • Estimation of esterified and free cholesterol in RBC membrane preparations is by KIT method, ELISA is used to estimate the Total phospholipid, Sialic acid, Interleukin -8, MDA, Catalase activity, Human glutathione peroxidase and Ceruloplasmin activity.
Results: In the present study, RBC and WBC counts, hemoglobin, hematocrit, mean corpuscular volume (MCV), and RDW levels will be measured and especially, RDW will be focused more than others. RDW represents the variability in RBC volume distribution and can be considered as an index of heterogeneity in the size of circulating erythrocytes. Therefore, it is expected to

obtain higher RDW levels in cases of coronary artery disease, especially with a higher mortality. As Coronary artery disease is associated with a sort of narrowing of artery with an overlying obstruction symptomatology interleukin-8(IL-8) is expected to rise.

Conclusion: ROS represent important second messengers within the heart, since they are involved in multiple physiological processes including differentiation, proliferation, and excitation-contraction coupling. However, when the production of ROS exceeds the buffering capacity of the antioxidant defence systems in the heart, oxidative stress arises, resulting in cardiac dysfunction, ischemia reperfusion injury, hypertrophy, cell death, and heart failure.

Keywords: Coronary Artery Disease, Red blood cells, Cholesterol of erythrocyte membrane, Risk factors, Case control study.

INTRODUCTION

The elucidation of structural composition and of basic metabolic pathways in living organisms has been a prerequisite to understanding of many diseases. Clinical science has derived many important benefits from studies on metabolisms. One of these is understanding of mechanisms underlying coronary artery disease (CAD) and associated risk factors. One of the many risk factors for cardiovascular diseases appears to be oxidative stress. Erythrocytes are the cells that are aimed at the delivery of oxygen and nitric oxide (NO) to the periphery and carbon dioxide to the lungs. Also they exert a scavenging action towards reactive oxygen and nitrogen species. Their deformability is essential for their circulation, specially in small blood vessels and this is an important prerequisite for such vascular antioxidant function. If the redox status exceeds the counteracting status of micro environment the oxidatively modified red cells increases its aggregability and adhesiveness to the endothelium and other blood cells, thus contributing to vascular damage.[4] To survive the rigors of circulations, RBCs are equipped with extraordinary properties as, specialized flexible spectrin –based membrane skeleton, a very efficient anti oxidant machinery and thirdly, mechanisms of repair and eventually remove damaged proteins and lipids.[5] RBCs being devoid of protein synthesis counteract these alternations by scavenging devices, as prooxidant bullets, or as signaling mediators providing long distance information. Thus RBCs sensing microenvironment found tissues, could be considered as reporter cells for the antioxidant status of the whole organism.[4]

Compared to other cells, RBCs exhibit high activities of the most important antioxidant enzymes like superoxide dismutase (sod), catalase, glutathione peroxidase, glutathione reductase, and other membrane oxidoreductases to reduce intra and extracellular oxidants.[6] Certain lipid peroxidation products such as malondialdehyde (MDA) may trigger some endogenously formed factors capable of causing the disease.[7] Oxidative stresscauses a plethora of RBC changes like cytoskeleton rearrangement and loss of lipid asymmetry in their membranes and can also modulate certain other blood cell function such as inducing platelet activation.[8] Altered antioxidant status has been observed in asymptomatic hypercholestrolemic subjects. Therefore in the present study the free and esterimic fractions of cholesterol of RBC membrane will be estimated in cardiac cases.[9] Scavenging function of RBC is directed towards immune and endothelial cells, RBCs, through scavenging the free radical species promote t- cell growth and survival and upregulating of cytoprotective proteins.[10] To delineate the cross talk between the vessels and RBCs, in the inflammatory relevance interleukin especially il- 8 was studied, the surface roughness of RBCS or skeletal integrity of membranes, if decreased is shown to be associated with the disease. Red cell membrane width which is a measurement of size variation is an index of heterogenecity.[11] In the present study, it will be assessed whether RDW of RBCS could be a prognostic marker in the selected cardiovascular disorders.

Aims and Objectives

- To estimate the free and esterified fractions of total cholesterol of erythrocyte membrane(CEM), protein and sialic acid and phospholipid content of RBC membrane.
- To measure the oxidative stress and anti oxidant status regarding the disease condition in RBC(hemolysate) and plasma/serum Malondialdehyde level, Glutathione peroxidase activity, Catalase activity, superoxidedismutase activity and Ceruloplasmin levels and interleukin (IL-8) estimation.
- Hematorheological studies of the disease population, compared to controls.
- The hemolysed samples will be analyzed for MDA, GPx, Catalase and ceruloplasmin mostly involved in oxidative matters.
- The Red cell membrane suspension be analyzed for free and esterified cholesterol contents (CEM), Phospholipid content, protein content, sialic acid and interleukin - 8(iL-8) levels in serum, will be estimated.
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Review of Literature

- Kaneda et al,[12] showed that plasma levels of AOPP were significantly higher in patients with coronary artery disease than in those without. AOPP levels correlated with severity score of CAD according to the the Gensini scoring system.
- In contrast, Azumi et al,[13] observed that even when there was no significant difference in angiographic stenosis, the generation of ROS was significantly higher in unstable angina pectoris patients compared with stable angina patients.

ADOPTED METHODOLOGIES

Preparation of RBC pellets

Blood samples were collected in heparinised tubes or EDTA tubes then centrifuge Blood at low speed (1125 x g) to remove plasma. Then pellet cells are washed three times (each time the portion of top layer of RBC removed to minimize contamination of nucleated cells) with phosphate buffered saline(PBS) containing 100 micrometers disodium EDTA. Washed RBC pellets will be frozen on dry ice for further analysis.

RBC MEMBRANE PROTIEN ASSAY

These are isolated from RBC Pellets (stored as frozen on dry ice), after thawing, in the presence of 1mM(Final concentration) of phenyl methyl sulphonyl fluoride(PMSF), a serine protease inhibitor, by adding a stock solution of 100mM PMSF in ethanol, to the cell pellet. Cells in the pool of RBC pellets will be lysed by diluting them, with an addition of 9ml of10mM phosphate buffer, pH 7.4. Hemolysate will be centrifuged at 9000g for 30 mins. Membrane pellets will be resuspended in 10mMphosphate buffer and washed till the supernatant was clear. Membrane pellets will be solubilized by vortexing with 500micro liters of 10mM TRIS-Hcl, PH-7.4 containing 2%SDS.Insoluble debris removed by spinning at 1400g for 3 mins. Now protein assay in clear supernatant will be performed using BCA (Bisinconinic acid) reagent from" Pierce" (company)SDS PAGE: sample with 50 micrograms of RBC Membrane protein will be subjected to SDS-PAGE. Following electrophoresis, gels will be stained with coomassie blue.

Isolation of erythrocyte membrane

1 ml of washed RBC (obtained by resuspending and washing RBC, after plasma separation, twice in 154 mM of Nacl isotonic solution) is hyotonically lysed in 30 volumes of cold hemolysis buffer (ImMTris – Hcl,ImM EDTA,10mM Nacl,Ph-7.2), then mixed, vertexed and allowed to stand for 15 mins,

Membranes will be separated from the hemolysate (i.e. supernatant from hemolysed RBC) by centrifugation at 1500 rpm for 15 mins at 4°C.This step will be repeated 3 times until a pink pale pellet containing hemoglobin free erythrocytes are obtained. Then ghosts will be resuspended in 1ml of PBS buffer and stored at -20°C until further analysis.

Estimation of free and esterified cholesterol in RBC membrane preparations (Ref: J. lipid. Res. Nov. 2010:51(11):3364-3369)

The enzymatic method used for cholesterol in plasma has limited value for assay in the cells.

MATERIALS AND METHODS

Study Design: Case Control Study

Study Setting: Tertiary Care Hospital.

Study Duration: October 2022 to Sepetember 2023.

Study Population: involves patients who are diagnosed with Coronary artery disease and are having Ischemic symptoms, ECG changes indicative of ischemia like ST Depression or elevation, Development of pathological Q waves in ECG, ECHO cardiographic evidence of new regional wall motion abnormality, Troponin positive.

Sample Size: A sample size of 100 patients who are diagnosed with CAD and are attending Cardiology OP and who are admitted in Cardiology Ward are considered.

Controls: 100 Healthy attendants matched for same age and gender with cases.

Inclusion Criteria: The cardiac disorder (CAD), Associated with risk factors will be grouped into 5 categories

a. Cases of CAD
b. CAD+hypertensive
c. CAD+Diabetic
d. CAD+ Alcoholic
e. CAD+Smokers.

Exclusion Criteria: Healthy People who are not willing to participate in the study were excluded.

Ethical considerations: Approval from the institutional ethics committee is obtained before commencement of the study. The study participants were explained the purpose of the study, and written consent is taken from the subjects in the local language after briefly explaining the purpose of the study. Approval from the head of the institute and head of the department is ta before starting the study. Confidentiality of the study participants is maintained strictly throughout the study. This study is purely descriptive in nature, and no drug intervention is included.

Methods of Collection

A. Peripheral Blood samples of both cases and controls will be collected after12 hrs overnightfasting in standard vacutainertubes
B. A prior consent from the patient Plasma and Red cells are separated

The hemolysate as well as the ghost (RBC) solution prepared as per standard procedure will be stored at -20°c of study population baseline clinical characteristics and laboratory findings will be compared

Methods and Technique

- RBC membrane protein assay
- Estimation of esterified and free cholesterol in RBC membrane preparations---KIT method

- Total phospholipid–ELISA detection-Detection range 20micro unit/ml500micro u/ml
- Sialic acid estimation ELISA, Detection range-16nmol/ml-980nmol/ml
- Interleukin -8 ELISA,50ngms/L-1000ng/L
- MDA –ELISA, Range 31-2000ngs/ml
- SOD –ELISA ,5u/ml-150u/ml
- Catalase activity –ELISA, Reference range -0.6ngs/ml-20ngs/ml
- Human glutathione peroxidase-ELISA - 30u/l-70u/l
- Ceruloplasmin activity –ELISA, Reference range 3 pg/ml -200pg/ml

Parameters the study

The EDTA samples will be utilized for clinical hematology studies, the RDW of the red cells will be studied taking the guidance of pathologist (for rheotological studies)

The sera samples will be estimated for antioxidant parameters and prooxidant species and tissues due to inadequate quantitative recovery of free cholesterol and cholesterol esters in enzyme compatible solvents Therefore a suitable solvent system yielding excellent cholesterol recovery by a simple Fluorometric enzymatic assay is used and the results are said to be comparable to that of GC- MS(gas chromatography-Mass spectrometry) Following the extraction of Red cell membrane contents, cellular total cholesterol is extracted in hexane isopropanol and then the total cholesterol is resolubilised in isopropanol- Nonidet P-40(NP-40)9:1(V/V)solvent.

- Free cholesterol quantification

Estimation of total phospholipid Content of Red cell membrane Sialic acid Estimation Interleukin-8(IL-8) estimation

Estimation of Serum Malondialdehyde (MDA):by the method of K.sabot (1978) clinica chemica acta1978:90:PP37-43

Assay of superoxide dismutase(SOD) (Method of beau champ and Fedowich,1976).

Assay of catalase activity (Titrimetric method of Radha krishnan and sarma,1963) Assay of peroxidase activity(glutathione peroxidase) method of Rotruck etal,1973)

Assay of Ceruloplasmin activity (enzymatic with P-PDA) Ref :J.Clin. Investi. 34:1485,1955- Markowitz et al and standard methods.clin.chem.4,39(1963)-Bandrowski base method

DATA COLLECTION AND STATISTICAL ANALYSIS

Data will be entered MS excel and analysed using SPSS Vertion 20. The biochemical parameters will be expressed as mean and standard deviation Students T test will be performed to find significance of res1,1lts Level of significance will be taken as $p< 0.05$.

Clinical Implication of the study

- When RBCs are challenged with pro-oxidant species, they can provide a pro-oxidant signal to vascular cells.
- This could be counteracted by providing with antioxidant drugs that decrease the species generations. In this way, RBC can lessen the oxidative stress of other cells.
- Redox changes of RBCs could hypothesized to play a role in the pathogenesis of hypertension.
- In addition to the prompt response of RBCs to prooxidant species, any promising drug taking into consideration of reactive oxygen or Nitrogen species, the study of events in RBCs could represent a useful tool to investigate their vascular effects.

More in general, the improvement of clinical laboratory analysis aimed at evaluation of RBC integrity and function such as Red cell morphological parameters, expression of surface antigens and RBC redox state, could provide useful information in the clinical practice in the long run.

RESULTS

- In the present study, RBC and WBC counts, hemoglobin, hematocrit, mean corpuscular volume (MCV), and RDW levels will be measured and especially, RDW will be focused more than others
- RDW represents the variability in RBC volume distribution and can be considered as an index of heterogeneity in the size of circulating erythrocytes. Therefore, it is expected to obtain higher RDW levels in cases of coronary artery disease, especially with a higher mortality.
- As Coronary artery disease is associated with a sort of narrowing of artery with an overlying obstruction symptomatology interleukin-8(IL-8) is expected to raise.
- The deformable nature of red cells due to oxidative stress may probably lead to a neuraminidase like activity, releasing sialic acid into the matrix and it may lead to raised levels of sialic acid in serum and in membrane content expected.
- Erythrocyte membrane fluidity may be affected leading to agglumerational properties, in the patients of CAD, may be probably due to the release of free radicals and the counteracting mechanism to their limited extent.
- Therefore, it is expected that the corresponding in lipid peroxidation products (MDA)and counteractive antioxidant activity of many enzymes involved in it, as expected. But in reality, there might be observations which may not be hypothesized or logically guessed.

Futhermore, changes in Red cell glutathione, redox status in cardiac disease suggest the formation of a redox system which may posses a compensatory response allowing for the minimization of structural and functional destabilization of erythrocyte membrane.

It may suggest for an expectation of unstable shift of glutathione redox potential for S- glutathaonylation process.

DISCUSSION

As Coronary artery disease is associated with a sort of narrowing of artery with an overlying obstruction symptomatology interleukin-8(IL-8) is raised. Thedeformable nature of red cells due to oxidative stress may probably lead to a neuraminidase like activity, releasing sialic acid into the matrix and it may lead to raised levels of sialic acid in serum and in membrane content expected. Erythrocyte membrane fluidity may be affected leading to agglumerational properties, in the patients of CAD, may be probably due to the release of free radicals and the counteracting mechanism to their limited extent.

Therefore, the corresponding in lipid peroxidation products (MDA)and counteractive antioxidant activity of many enzymes involved in it, as expected. But in reality, there might be observations which may not be hypothesized or logically guessed. Furthermore, changes in red cell glutathione, redox status in cardiac disease suggest the formation of a redox system which may posses a compensatory response allowing for the minimization of structural and functional destabilization of erythrocyte membrane.

It may suggest for an expectation of unstable shift of glutathione redox potential for S- glutathaonylation process.

CONCLUSION

ROS represent important second messengers within the heart, since they are involved in multiple physiological processes including differentiation, proliferation, and excitation- contraction coupling. However, when the production of ROS exceeds the buffering capacity of the antioxidant defense systems in the heart, oxidative stress arises, resulting in cardiac dysfunction, ischemia reperfusion injury, hypertrophy, cell death, and heart failure. Endogenous ROS in the heart are generated by mitochondria, xanthine oxidoreductase, uncoupled nitric oxide synthase, NADPH oxidase, cytochrome P450, and monoamine oxidases. ROS are also involved in the onset of some complications related to specific clinical settings, including chemotherapy induced cardiotoxicity and POAF, as well as in the onset of diabetic cardiomyopathy, which represents a disorder of the heart in diabetic patients in the absence of other comorbidities related to diabetes. Multiple ant oxidative stress therapies have been tested through different approaches: inhibition of oxidative stress producers, improvement of endogenous antioxidant capacity, and improvement of antioxidant capacity by supplementation of exogenous antioxidants. However, the results from these clinical trials suggest that although targeting oxidative stress is theoretically logical, the majority of the current strategies fail to improve patient outcomes and prognosis. The results obtained using drugs with anti- inflammatory, antioxidative stress and antidiabetic properties appear to be more promising. The improvement of experimental settings and knowledge about the pharmacokinetic of antioxidants, as well as the identification of more specific markers and the use of larger study cohorts, will lead to the identification of novel, More effective therapeutic approaches for heart disease.

FUTURE PLANS BASED ON EXPECTED OUTCOMES IF ANY

This study may be used as a continuation study as regulation of Gene expression, post translational modifications of proteins, and signalling Transduction.

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