NITRIC OXIDE IMPLICATION IN DIFFERENT AGED RED BLOOD CELLS

A PROJECT REPORT

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BONA FIDE CERTIFICATE

Certified that this project work titled **"NITRIC OXIDE IMPLICATION IN DIFFERENT AGED RED BLOOD CELLS"** is the bona fide work of Ms. SHATHYA YOGARAJAN (Reg. No. 1120203014), who carried out the research under my supervision. Certified further, that to the best of my knowledge the work reported herein does not form part of any other thesis or dissertation on the basis of which a degree or award was conferred on an earlier occasion on this or any other students.

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ABSTRACT

Nitric oxide (NO), one of the most important vascular signaling molecule, is primarily produced by endothelial NO synthase. Recently, a functional NO synthase (NOS) was described within red blood cells (RBC NOS) and it was shown to significantly contribute to the intravascular NO pool and to regulate physiologically relevant mechanisms. However the regulatory mechanisms and clinical implications of RBC NOS are unknown. The present study aimed at investigating RBC NOS activity in erythrocyte age distribution. In addition, comparative study on NO production in non-diabetic and diabetic RBC age fraction was done on the light of the fact that NO production is impaired during diabetes. Results of the study confirm the NOS expression in RBC. Diabetic subjects showed a different erythrocyte age distribution, with an almost lower NO production compared with controls. In young erythrocytes, NO level was higher and gradually reduced with middle age, older age erythrocyte separation. These observations may have important physiological implications within the microvasculature and for pathophysiological disruption of NO homeostasis in diabetes.

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CHAPTER 1

1.1 INTRODUCTION

Nitric oxide (NO) is a key vertebrate biological messenger, playing a role in a variety of biological processes. NO is the first gas known to act as a signaling molecule. NO is able to diffuse directly across the plasma membrane of its target cells and has paracrine and autocrine effects. It is short lived, usually degraded or reacted within a few seconds.

NO is synthesized by nitric oxide synthase (NOS) in a reaction that converts arginine and oxygen into citrulline and nitric oxide. There are three isoforms of the NOS enzyme: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). eNOS and nNOS are calcium-dependent and produce low levels of gas as a cell signaling molecule. iNOS is calcium independent and produces large amounts of gas which can be cytotoxic. Co-factors for NOS include oxygen, NADPH, tetrahydrobiopterin and flavin adenine nucleotides.

NO is an important regulator and mediator of numerous processes in the nervous, immune and cardiovascular systems, including smooth muscle relaxation thus resulting in vasodilation of the artery and increasing blood flow, neurotransmission in the nervous system and has been associated with neuronal

activity and various functions like avoidance learning, macrophage mediated cytotoxicity for microbes and tumor cells.

Endothelial nitric oxide synthase (eNOS)-derived nitric oxide is one of the most important mediators in vascular homeostasis. NO contributes to vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium. NO very rapidly reacts with red blood cell-derived free hemoglobin, limiting its bioactivity. Therefore, RBCs have been considered as a major trap for NO. However, within the last few years, this paradigm has been rejected.

Apart from the vascular endothelium, RBCs have now been identified as a vascular sources of NOS-dependent NO formation. It has been shown that RBCs from humans express an active and functional endothelial-type NOS (eNOS). This enzyme is active under basal, resting conditions and releases bioactive NO supplying the basal NO level within the circulation.

Patients

Impaired NO production which is a hallmark of vascular diseases associated with diabetes, hypercholesterolemia, atherosclerosis and hypertension is characterized by endothelial dysfunction and reduced endothelium-mediated vasodilation. Of several vascular disease states, patients with diabetes mellitus have particularly marked deficits in NO-mediated endothelial function and an increased risk of thrombosis and accelerated atherogenesis.

Vascular complications are the leading cause of increased mortality in patients with diabetes mellitus. Hyperglycemia which is a primary cause of

diabetic vascular complications is shown to reduce the nitric oxide production in endothelial cells. It can also result in inactivation of NO by oxygen-derived free radicals, and/or increased production of endothelium-derived contracting factors, which oppose the protective activity of NO.

Erythrocytes are the most abundant cells in the human body (5.4 million cells/mm) blood in a healthy male and 4.8 million cells/mm blood in a healthy female). Erythrocytes are biconcave discs with an average diameter of 7.8m. The flexible, biconcave shape enables erythrocytes to squeeze through narrow capillaries, which may be only 3m wide. Mature erythrocytes are quite simple in structure. They lack a nucleus and other organelles.

Human erythrocytes (red blood cells) have an *in vivo* life span of approximately 120 days and are selectively removed from circulation via phagocytosis. During its life span, the erythrocyte undergoes progressive physical and chemical changes, such as the decrease on cell volume with cell aging. This is presumably due to the loss of potassium and to the loss of membrane patches by microvesiculation, resulting in an increase on cell density. Aged cells exhibit decreased deformability, electric mobility and lower surface negative charge. The membrane zeta-potential (which assesses the cell surface charge), together with the morphological and mechanical properties, are important structural and functional parameters of erythrocytes.

Red blood cell-derived NOS has common, but also distinct regulatory mechanisms when compared with eNOS. The NOS-dependent conversion of L-arginine in RBCs is comparable to that of cultured human endothelial cells. RBCs seem to be devoid of various eNOS regulatory mechanisms in endothelial cell,

because of the lack normal cell organelles. RBC NOS significantly contribute to the intravascular NO pool and to regulate physiologically relevant mechanisms. However, the regulatory mechanisms and clinical implications of RBC NOS are unknown.

Red blood cells play an important role in the onset of complications associated with diabetes. The RBCs of diabetics are known to have a weakened defence mechanism against oxidizing molecules. In addition, RBCs in diabetics are known to be less deformable than those in healthy individuals. Glycosylation impairs the vasodilator function of RBCs within a physiological range of tissue oxygenation. These findings may represent an important contribution to reduce NO bioavailability in the microvasculature in diabetes.

1.2 OBJECTIVES

- To study NOS activity and NO production in RBC of different age distribution.
- To compare the NO production in RBC age fraction from non-diabetic and diabetic subjects.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Nitric Oxide

Nitric oxide (NO) was discovered to be a potent vasodilator, inhibitor of platelet aggregation, and active species of nitroglycerin before the discovery of endothelium-derived relaxing factor (EDRF) in 1980 (Palmer *et al.,*1987). The discovery that NO is the endothelium-derived relaxing factor changed our understanding of NO from that of a noxious gas to a molecule with a broad spectrum of biological activities. (Ignarro *et al*., 1986; Moncada *et al*., 2006). NO is considered as an important chemical in the body that the journal *Science* named it the "Molecule of the Year" in 1992, and in 1998 three scientists Louis.J.Ignarro, Robert Furchgott and Ferid Murad won the Nobel Prize in Medicine for researching its role in the body.

Subsequent studies done after discovery of NO as an endogenous vasodilator have shown that NO is synthesized by mammalian cells from L-arginine through a complex oxidation reaction catalyzed by enzyme NO syntheses (NOS). (Moncada *et al*., 1990). NOS catalyzes the NADPH and oxygen dependent oxygenation of L-arginine to NO plus L-citrulline .The mechanism of nitric oxide synthesis involves the transfer of electrons between various cofactors, including flavin adenine dinucleotide, flavin mononucleotide, nicotinamide

adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin, and heme. Finally, one atom of oxygen from oxygen binds with the terminal guanidine nitrogen from arginine to form nitric oxide.

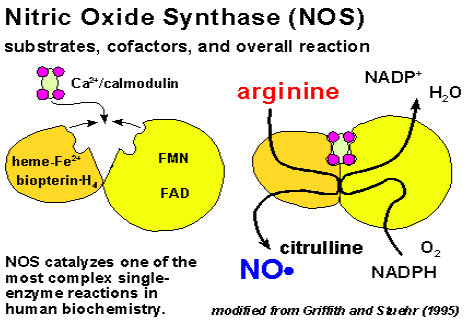


Fig: 2.1 NO biosynthesis by Nitric oxide synthase.

There are three distinct enzyme isoforms that synthesize NO: one is the endothelial NO synthase (eNOS), which was the first to be identified in the vascular endothelium; later on an enzyme in the neuronal tissue, mainly in the brain, was identified and is known as neuronal NO synthase (nNOS); and a third isoform, an inducible NO synthase (iNOS) that was identified first in macrophages. (Ivan *et al*., 2005). The three isoforms of NO synthase are encoded by three different genes present in different chromosomes in humans. The nNOS is encoded by a gene present in chromosome12, the eNOS is encoded by a gene

present in chromosome 7 and the iNOS is encoded by a gene in chromosome 17. Cloning of NOS enzymes indicates that, both brain constitutive (NOS1) and endothelial constitutive (NOS3) are constitutive NOS (cNOS) and the third isoform is inducible (NOS2).

NOS enzymes are highly homologous and have a common dimer structure; each monomer contains a reductase and an oxygenase domain, implicating the importance of molecular oxygen. The reductase domain encloses binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and nicotinamide adenine dinucleotide phosphate oxidase (NADPH). The oxygenase domain contains a heme center and binding sites for L-arginine and tetrahydrobiopterin (BH4). A calmodulin (CaM) binding site that regulates the transfer of electrons from the reductase to the oxygenase domain is located between the two regions. (Crane *et al*., 1997) The main difference between the three isoforms is related to their Ca^{2+} dependence: nNOS and eNOS are Ca^{2+}-dependent and require elevated levels of intracellular Ca^{2+} to become activated, whereas iNOS does not. (Nathan *et al*., 1991).

NO acts through the stimulation of the soluble guanylate cyclase, which is a heterodimeric enzyme with subsequent formation of cyclic GMP. Cyclic GMP activates protein kinase G, which causes phosphorylation of myosin light chain phosphatase, and therefore inactivation of myosin light-chain kinase, and leads ultimately to the dephosphorylation of the myosin light chain, causing smooth muscle relaxation. (Surks *et al*., 2007).

Nitric oxide plays a wide variety of roles in numerous processes in the nervous, immune and cardiovascular systems. It is induced by several factors, and

once synthesized by eNOS, it results in phosphorylation of several proteins that cause smooth muscle relaxation. NO inhibits platelet aggregation and vascular smooth muscle cell proliferation. (Ozlem *et al*, 2008) NO is a requisite component of endothelial cell migration and wound healing (Sumathy *et al.,* 2008).

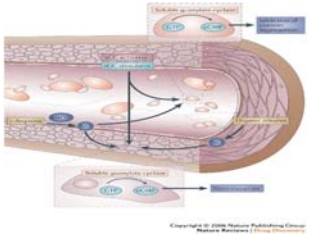


Fig: 2.2 Mechanism of action of NO. NO activates sGC to release cGMP which cause vasorelaxation and platelet aggregation.

The vasodilatory actions of nitric oxide play a key role in renal control of extracellular fluid homeostasis and are essential for the regulation of blood flow and blood pressure. (Joseph *et al*., 2008).Nitric oxide also serves as a neurotransmitter between nerve cells, part of its general role in redox signaling. NO-cGMP cascade is involved in learning and memory through the maintenance of long term potentiation. (Hopper *et al*., 2006). Nitric oxide is an important non-adrenergic, non-cholinergic neurotransmitter in various parts of the gastrointestinal tract. It causes relaxation of the gastrointestinal smooth muscle. Macrophages produce nitric oxide in order to kill invading bacteria. (Nathan *et al*., 1991)

Nitric oxide also acts on cardiac muscle to decrease contractility and heart rate. NO contributes to the regulation of cardiac contractility. (Furchgott *et al*., 1980; Hayward *et al.,* 1999). NO also inhibits the contractility of the smooth muscle wall of the uterus. NO affects secretion from several endocrine glands, stimulating release of gonadotropin releasing hormone from hypothalamus, amylase from pancreas, adrenaline from adrenal medulla etc.,

Nitric oxide has been shown to have therapeutic potential as a drug. (Nicholas *et al*., 2004; Kinsella *et al*., 2000). Nitric oxide is considered an antianginal drug: it causes vasodilation, which can help with ischemic pain known as angina by decreasing the cardiac workload. By dilating the veins, there is less blood returned to the heart per cycle. (Abrams, 1996).Nitroglycerin is used to prevent or treat acute chest pain. Nitric oxide/oxygen blends are used in critical care to promote capillary and pulmonary dilation to treat primary pulmonary hypertension in neonatal patients. Nitric oxide might serve as an inflammometer in conditions like asthma (Ashutosh *et al*., 2000). Nitric oxide therapy has the potential to significantly increase the quality of life and, in some cases, save the lives of infants at risk for pulmonary vascular disease.

2.2. Endothelial NO production

Nitric oxide (NO) is produced by many cells in the body; however, its production by vascular endothelium is particularly important in the regulation of blood flow. Endothelium (Tunica Interna) forms the innermost lining layer of blood vessels, which enables metabolic exchange between blood and tissue by virtue of its permeability and proximity to all cells. Tunica externa made of collagen fibres and Tunica media made up of smooth muscle cells and elastic fibres form the outer layers of endothelium. NO released from endothelium

diffuses to the nearby smooth muscle cells and exerts its effects. (Ignarro *et al*., 1989)

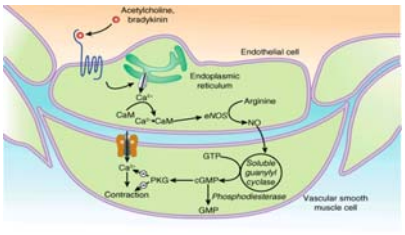


Fig2.3 Regulation of vasorelaxation by endothelial-derived nitric oxide

Endogenous vasodilators activate NO synthesis leading to NO production. NO diffuses into smooth muscle cells, where it activates soluble guanylyl cyclase and cGMP synthesis activates protein kinase G (PKG), resulting in an overall reduction in calcium influx, and inhibition of calcium-dependent muscle contraction. PKG can also block other pathways that lead to muscle contraction.

In the endothelium, NO is synthesized by the constitutive, calcium (Ca^{2+})/calmodulin (CaM)-dependent endothelial NOS (eNOS). eNOS initially resides in the Golgi complex, where it is anchored to the membrane via one myristoyl and two palmitoyl groups (Luc *et al*., 1999), eNOS is transported to the plasma membrane via vesicular transport, where it is associated to caveolae. These invaginations of the plasma membrane are rich in cholesterol and sphingolipids.

In caveolae, eNOS binds to caveolin-1 (cav-1) via its scaffolding domain. This interaction inhibits eNOS catalytic activity because cav-1 interferes with the

binding of CaM at low concentrations of cytosolic Ca^{2}. Upon Ca^{2+} influx, CaM binds to eNOS, thereby disrupting the eNOS–cav-1 interaction, leading to activation of the enzyme. Meanwhile, eNOS is depalmitoylated and the enzyme translocates to the cytosol.

The protein kinase B (PKB; also known as Akt) phosphorylates eNOS at the serine amino acid group at position 1177, leading to further activation of the enzyme. Association of eNOS with heat shock protein 90 (Hsp90) facilitates the binding of CaM and mediates the interaction between eNOS and Akt. A decrease in Ca^{2+} concentration and dephosphorylation of eNOS leads to the rebinding of eNOS with cav-1 and the relocation of the enzyme to the Golgi complex, where it is repalmitoylated, enabling eNOS to be transported to the caveolae. By contrast, the nitric oxide synthase interacting protein (NOSIP) and the nitric oxide synthase traffic inducer (NOSTRIN) can negatively regulate eNOS localization in the plasma membrane.

2.3. NO production in RBC

To date, human blood and, in particular, hemoglobin-carrying red blood cells (RBCs) have been considered as a major sink of NO. (Ulker *et al*., 1987). Most importantly, a NOS-dependent formation and release of NO-related species from RBCs has not been shown so far. In fact, the diffusion-limited chemical inactivation of NO by intra-erythrocytic hemoglobin would suggest that even if RBCs contain NOS, NO production from such would represent a futile vestigial function derived from an earlier stem-cell precursor (prior to RBC hemoglobinization). Current information on the NOS isoform, its localization,

and functional activity within RBCs is still inconsistent and subject to considerable debate.

Indeed, the characterization and proof of a functional NO in RBCs has been hampered by the high content of hemoglobin. First, the complex and oxygen-sensitive biochemistry of NO with intracellular and extracellular proteins (Pawloski *et al.*, 2001) demands a composite analysis of the various constituents of the circulating NO pool to assess RBC-based NOS activity in blood. Second, the extraordinary high protein content of RBCs precludes standard procedures for characterization of NOS protein.

More recently, it has been shown that RBCs expresses a functional endothelial nitric oxide synthase (Kleinbongard *et al.,* 2006). Using immunofluorescence confocal microscopy, standard thin section and immunogold cryosection, freeze-fracture electron microscopy, Western blotting, and reverse transcriptase–polymerase chain reaction (RT-PCR) a unequivocal evidence that human RBCs express an active NOS was given.

Red blood cell (RBC)-derived NOS has common, but also distinct regulatory mechanisms when compared with eNOS. Its NOS activity depends on the intracellular Ca^{2+} level and phosphorylation at serine 1177 regulated by phosphatidylinositol-3 kinase (PI3K) (Kleinbongard, P. *et al*, 2008). RBCs seem to be devoid of various eNOS regulatory mechanisms because they lack normal cell organelles, such as a nucleus, endoplasmic reticulum and Golgi complex. (Bratosin,. *et al*, 2001). Experiments demonstrated unique properties of RBC NOS in the sense of primary regulation via its substrate L-arginine. (Kleinbongard, P. *et al*, 2008).

2.3.1 NOS- relevant proteins in red blood cells

In RBCs, several NOS relevant proteins have been reported. They include membrane-related as well as cytosolic proteins and cofactors serving as possible interaction partners of RBC NOS. Calcium is considered to be mostly, and perhaps exclusively, located in the erythrocyte membrane (Harrison *et al.,* 1997). In addition to its role in CaM binding, the rise of cytosolic Ca^{2+} in RBCs triggers a sequence of biochemical and morphologic changes that finally result in the release of hemoglobin-containing exovesicles, which is a protection strategy against destruction by the complement. Elevated cytosolic Ca^{2+} levels cause alterations in the membrane protein pattern (Anderson, D.R. *et al.*, 1977), activating potassium efflux that causes the loss of cell water and concomitant shrinkage of the RBCs. The Ca^{2+} pump of RBCs extrudes the incoming Ca^{2+}, maintaining an intracellular calcium level of 0.634 µg/ml.

Application of acetylcholine (Ach) to RBCs increased the concentration of NO metabolites, namely nitrite and nitrate, via an interaction with erythrocyte membrane muscarinic receptor. On ECs, the ACh receptor (AChR) is a very prominent eNOS activator. Two main classes of AChR have been identified on the basis of their responsiveness: the muscarinic receptors and the nicotinic receptors. RBCs respond to choline being the agonist of the muscarinic receptor. Treatment of blood with (Ach) resulted in increased deformability and decreased aggregation of RBCs.

Human CAT1 (hCAT1) shows a selective expression pattern in erythroid-lineage cells, being detected at levels approximately eight times higher in

glycophorin-A-positive nuclear cells from cord blood. Furthermore, CAT1 seems to be highly expressed in relatively mature erythroid cells and is possibly involved in late (or terminal) erythroid maturation via a modulation in NO formation.

2.3.2 Potential RBC NOS regulation

RBC NOS is detected in the cytoplasm and along the plasma membrane, where it is exclusively expressed on the plasmatic face of the bilayer plasma membrane. Its expression is 20% to 30% greater in the membrane than in the cytoplasm (Kleinbongard, *et al*, 2006). The major difference to EC is a consequence of the fact that mature RBCs lack cell organelles, which play a pivotal role in NOS regulation in EC. The RBC NOS regulation mechanism might also be a circular process comparable to eNOS. But to date the exact mechanism remains theoretical.

Two possible interaction partners inhibiting RBC NOS activity seem to be evident within RBCs: cav-1 and flotillin. Although admittedly speculative, a possible circular process might proceed as follows: RBC NOS and cav-1/flotillin are transported from the cytoplasm to the membrane, where the NOS protein is associated to the lipid raft proteins (Fig:2. 4 b).The interaction of cav-1/flotillin inhibits RBC NOS activity, and this inhibition is augmented when RBCs, through their flow along the vascular walls, are continuously exposed to shear stress signals, which lead to changes in the membrane potential and the opening of cation channels.

These changes lead to a Ca^{2+} influx into the cytoplasm. Ca^{2+} binds to CaM, which enhances the linkage of CaM to RBC NOS. Upon increasing Ca^{2+}

concentrations, the interaction between RBC NOS and cav-1/flotillin is weakened and displaced by the binding of RBC NOS with CaM. In this active state, RBC NOS becomes enzymatically active and translocates to the cytoplasm, where Hsp90 facilitates NO production by stabilizing and protecting RBC NOS. A Ca^{2+} drop weakens the linkage of RBC NOS to CaM and strengthens that of cav-1/flotillin to NOS again, thereby inactivating the enzyme. Alternative RBC-NOS-activating mechanisms are likely to be modulated by coupling of ACh to the AChR at the outer site of the RBC membrane and the phosphorylation at serine 1177 regulated by the PI3K. Furthermore, NO is possibly oxidized to nitrite and nitrate or it is bound to SNO-Hb. NO is also exported out of RBCs through the anion exchanger AE1.

Possible regulatory mechanism of RBC NOS is shown in Fig.2.4. This hypothetic cycle shows that (i) RBC NOS is bound to cav-1 in the cytoplasm and might are (ii) transported to the membrane via vesicle transport. The RBC NOS is (iii) located near the lipid rafts and is inactive. (iv) Shear stress signals or agent-dependent receptor stimulation initiate serine phosphorylation and a Ca2+ influx. This increase (v) CaM binding to RBC NOS, thereby activating the enzyme and inducing the translocation to the cytoplasm where the NOS (vi) bound to Hsp90 synthesizes NO. (vii) The decrease in Ca2+ concentration facilitates the binding of RBC NOS to cav-1/flotillin again. (viii) Possible routes of NO might include: NO has bioactive potential and regulates functions of the RBC itself; it is oxidized to nitrite and nitrate; or it is bound to SNO-hemoglobin. NO is then either exported out of RBCs through the anion exchanger AE1 or oxidized to nitrite and nitrate subsequently.

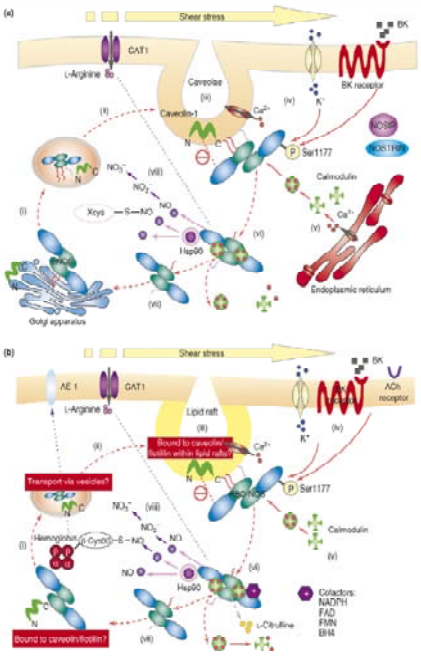


Fig: 2. 4 eNOS regulation and potential RBC NOS regulation.

2.3.3. Functions of RBC NO

RBC-NOS–dependent NO formation is hypothesized to alter functional characteristics of RBC membrane. In human RBCs, NOS-derived NO regulates deformability of RBC membrane and inhibits platelet function. (Kleinbongard *et al.,* 2006). Stimulating RBC-NOS activity suppresses platelet activation and aggregation, whereas NOS inhibition increased platelet aggregation. The RBC-NOS–dependent modulation of platelet function may be mediated by NO itself or by NO-related intermediates such as plasma nitroso compounds, which have been shown to suppress platelet activation. Studies have shown that RBCs release NO in addition to ATP in response to a fall in hemoglobin O_2 saturation.

2.4. NO and vascular diseases.

A number of disorders are associated with reduced synthesis and/or increased degradation of vascular NO. These include hypercholesterolaemia, (Casino *et al*., 1993), diabetes mellitus (Creager *et al*.,1996: Hadi *et al*.,2007) and hypertension (Panza *et al.,*1995) .The endothelial dysfunction caused by these disorders contributes to the alterations in vascular reactivity observed in these conditions. The reduction in the activity of vascular NO is also likely to play a significant role in the development of atherosclerosis.

Atherosclerosis is associated with impaired endothelium-mediated vasodilatation in vitro and in vivo, in laboratory animals and in man. (Förstermann *et al*.,2000). Endothelial NO can play a dual role in atherosclerosis. High levels of NO produced from iNOS in endothelial cells can induce injury to

the endothelium. Peroxynitrite (ONOO-), the product of NO interaction with superoxide, is produced in significant amounts in atherosclerotic lesions.

Hypercholesterolemia is associated with an impaired endothelial NO production, and as a consequence, alterations in eNOS abundance and activity were proposed to constitute early events in the development of atherosclerosis (Flavahan *et al.,* 1992; Casino *et al.*, 1993). The reduced ability of the endothelium to produce biologically active NO could also predispose the vascular wall to platelet adhesion and the constrictor effects of substances released as a result of platelet disruption. Interestingly, even coronary arteries showing significant intimal thickening and plaque formation usually maintain a morphologically intact endothelial cell layer.

Nitric oxide is crucial for maintenance of normal blood pressure and therefore the role of NO in essential hypertension has been an area of intense investigation. Impaired NO-mediated vasodilations have been shown in animal models of hypertension and hypertensive patients.

2.5. Endothelial nitric oxide and diabetes

Diabetes mellitus, which essentially represents a heterogeneous group of disorders that have hyperglycemia as a common feature, is characterized by endothelial dysfunction (Hadi et al., 2007). Human IDDM (insulin dependent diabetes mellitus)and NIDDM (non-insulin dependent diabetes mellitus), and animal models of IDDM are all associated with reduced bioavailability of nitric oxide and impaired endothelium-dependent relaxation (Williams *et al.,* 1996).

Under hyperglycemic conditions, human aortic endothelial cells have been shown to have either reduced or increased eNOS expression. Human glomerular endothelial cells on the other hand exhibit increased eNOS expression but reduced NO formation under hyperglycemic condition. Interestingly, eNOS knockout mice exhibited accelerated diabetic nephropathy supporting a role for deficient eNOS-derived NO production in the pathogenesis of diabetic nephropathy. Under hyperglycemic conditions, *in vivo* in rats, mesenteric arteries produced reduced NO and exhibit reduced hsp90-eNOS complex formation. Metformin, a bioguanide derivative (dimethylbiguanide) and one of the most commonly used drugs for the treatment of type 2diabetes, dramatically attenuated high glucose-induced reduction in the association of hsp90 with eNOS in bovine aortic endothelium, resulting in increased NO bioactivity and a reduction in overexpression of adhesion molecules and endothelial apoptosis caused by high glucose exposure. Thus, in the setting of hyperglycemia, enhancing eNOS activity and NO bioavailability has beneficial effects.

It is crucial to include the importance of iNOS in diabetic pathophysiology since recent reports have revealed decreased expression of endothelial nitric oxide synthase concomitant with increased expression of iNOS and nitrotyrosine during the progression of diabetes in rats (Nagareddy *et al.,* 2005). This finding suggests that induction of iNOS in cardiovascular tissues is dependent on the duration of diabetes and contributes significantly to the depressed pressor responses to vasoactive agents and potentially to endothelial dysfunction.

Hyperglycemic conditions induce endothelial production of superoxide and synthesis of antioxidants enzymes. In animal models of diabetes, impaired endothelium-dependent relaxation was abrogated after acute inbubation with

superoxide dismutase, a superoxide scavenger. These results are suggestive of a major role of destruction of NO by O_2^- in diabetes associated vascular dysfunction.

2.6 Separation of RBC into age distribution

The behavior of the 2 sialidase form present in the erythrocyte membrane was investigated in subjects with type 2 diabetes mellitus and given the important role of sialic acid in erythrocyte aging, to investigate if mean erythrocyte age is modified by the diabetic condition and, secondarily, to verify in both healthy and diabetic subjects, the content of the 2 sialidases in erythrocytes of different ages. (Bruno Venerando, 2003)

Study was to evaluate if fibrinogen-erythrocyte binding is dependent on *in vivo* cell aging, to assess the life span of the specific receptor and to get a further insight on its nature. Our data indicate that increasing erythrocytes aging, there is a significant decrease on the fibrinogen binding, by decreasing the frequency of its occurrence but not its binding strength. For the binding between fibrinogen and erythrocytes to occur, a lower fibrinogen concentration is needed on young erythrocytes than for the older one

The biological age of a mouse influences erythrocyte metabolism and erythrocyte aging *in vivo*, blood samples were collected from Male C57/BL6J mice. The age of erythrocytes in mice of all ages was directly related to density. Also, in older erythrocytes compared with younger erythrocytes, decreased concentrations of total free thiol and reduced glutathione, and decreased glutathione reductase activity were observed. (Edathara, 1995)

2.7 Red blood cell role in diabetes complications

Red blood cells play an important role in the onset of complications associated with diabetes. Diabetes mellitus is shown to decreases RBC life-span; (Laura *et al*., 1992) therefore, it may change the plasma membrane by acting through its effect on the aging process. During aging, several variations appear in red blood cell (RBC) plasma membrane such as peroxidative damage of membrane lipids; reduced insulin receptor number, variations in total cholesterol content, phospholipids content, cholesterol/phospholipids ratio (Luly *et al*.,1988) and in RBC cytosol such as reduced activity of glycolytic enzymes and decreased intracellular K^+ concentration. Abnormalities in membrane proteins may play a role in reduced erythrocyte deformability associated with diabetes mellitus.

Disregulation of certain proteins has been reported in red blood cell membrane of diabetic patients. Increased expression of arginase-1 in diabetic patients might down regulate NO production through competition with NOS. Flotillin-1, a recently discovered membrane protein of RBC lipid rafts also appears to be affected in the disease.

The RBCs of diabetics are known to have a weakened defence mechanism against oxidizing molecules. In addition, RBCs can be deformed as they flow through blood vessels and RBCs in diabetics are known to be less deformable than those in healthy individuals. Investigations were done how oxidants affect the release of adenosine triphosphate (ATP) from RBCs. RBCs release ATP when they are deformed. ATP triggers nitric oxide production which causes blood

vessel widening and inhibits platelet aggregation. When RBCs stiffen, nitric oxide production is disrupted and this can cause vascular problems.

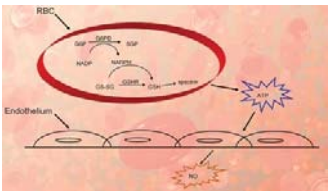


Fig: 2.5 A mechanism by which RBC disrupts NO production by endothelial cells.

CHAPTER 3

MATERIALS AND METHODS

3.1. Blood samples and RBC preparation

Whole blood was collected in 4 ml vacutainer tubes (K2 EDTA spray dried) from IDRF Hospital and LIONS blood, Egmore, Chennai. Venous blood samples from non-diabetic and diabetic human male and female volunteers, aged between 20 and 80 years were collected.

For separation of RBC from plasma, initially centrifugation was carried out at 1200 rpm for 5 minutes at 4°C. Following that the RBCs were washed three times with physiological saline (0.9% NaCl). Each saline washing entailed the addition of five times the volume of pellet, vortexing and centrifugation at 1000 g for 5 min at 4°C.The final saline wash was discarded, and the cells were resuspended in phosphate-buffered saline (PBS; pH 7.4) to an approximate hematocrit of 50%. Of the total cells in the sample, the washed RBC fraction contained on average 98 % RBCs. All the experiments were done using freshly prepared RBC suspension and in case RBCs need to be used further, storage was done at 4^0C.

3.2 RBC separation using Percoll density gradient

3.2.1 Preparation of discontinuous Percoll gradients

Human erythrocytes of different biological age, from both controls and diabetic patients, were separated as described by Bruno Venerando *et al*., 2013 .The following solutions were prepared at 26°C.
HEPES-buffered stock solution (HBS stock): 2.66 M NaCl, 0.09 M KCl, 0.2 M HEPES, pH 7.4;

Solution A (BSA-HEPES-buffered solution, pH 7.4): 19 volumes of 5.25% (w/v) BSA in water solution added to 1 volume of HBS-stock solution;

Solution B (BSA-Percoll-HEPES-buffered solution, pH 7.4): 19 volumes of 5.25% (w/v) BSA in Percoll added to 1 volume of HBS-stock solution.

Solutions A and B were mixed to form 5 solutions at final "Percoll" concentrations of 60%, 66%, 70%, 74%, and 80% (vol/vol; density 1.087-1.098 g/mL, pH 7.4). Discontinuous 5-step gradients were prepared by overlayering 1.0 mL of 80%, 8.0 mL of 74%, 8.0 mL of 70%, 8.0 mL of 66%, and 4.0 mL of 60% of Percoll concentration using a peristalting pump in 36 mL tubes. The same discontinuous Percoll gradient was successfully applied for diabetic patient erythrocyte separation.

3.2.2 Fractionation of erythrocytes according to biological age

Tubes containing 2.0 mL each of packed blood (hematocrit 1ml mixed with 1ml of 10mM HEPES buffer, pH 7.4, containing 133 mM NaCl, 4.5 mM KCl, were layered on the discontinuous gradient. Centrifugation was carried out in a swing-out rotor at 3500 rpm for 15 minutes at 20°C. The centrifuge was slowly decelerated over 3 minutes to prevent gradient disturbance. The plasma remaining above the gradient was removed, and all cell fractions were collected manually over the liquid interface by aspiration from the top of the gradient .The following age-dependent red cell parameters were characterized in each fraction.

3.3 Characterization of aged fraction

3.3.1 Preparation of membrane protein (Vincent *et al.*,1967)

1. Packed red cells washed twice in 0.9% NaCl,; lysed in 10 vol of 5 m Tris-HCI, pH 7.0, 1 mM EDTA, (a) mixed 15-20 min; centrifuged 25,000 *g*, 30 min. Plasma and buffy coat aspirated off
2. Red cells resuspended in (a) medium, mixed, centrifuged 25,000 g, 10 min and supernatant discarded
3. (b) Red cells suspended in discarded 0.05 M Tris-HCl, pH 7.0, 0.50 M NaCl, 1 mM EDTA;2 centrifuged 25,000 g, 10 min. supernatant discarded.
4. Red cells (now faint pink in discarded color) suspended in 5 mM Tris-HCI, pH 7.0, 1 mM EDTA. Centrifuged 25,000 g, 10 min. supernatant discarded.
5. Ghosts suspended in same medium, centrifuged 25,000 g, 10 min, supernatant discarded.
6. Ghosts (now milky white) suspended in small volume (0.5 ml/l ml pellet) of 0.05 M Tris-HCl, pH 7.0.

(a) This and all other operations after the first hemolysis were carried out in the cold. It was necessary to centrifuge the initial hemolysate at 25,000 g for 30 min to insure the complete sedimentation of all ghosts from the dense and viscous hemoglobin-containing medium. This high speed was used in subsequent steps to shorten the centrifugation times.

(b) Treatment of ghosts with this high salt to pH 7.0 with 2 M Tris, was an important step in rendering the ghosts free of most of the residual hemoglobin. The protein content of the final ghost preparations was determined by the Lowry procedure with bovine serum albumin as the standard.

The ratio of protein 4.1a to 4.1b was analyzed on ghost membranes purified from each subset, since this is a useful index of red cell age. Ghosts were prepared from RBCs as described, and total membrane proteins were analyzed by electrophoresis in 7.5% SDS–polyacrylamide gel. Gels were silver stained and ratio between 4.1a and 4.1b proteins was determined

3.3.2 Protocol for silver staining

Silver staining is much more sensitive than Coomassie, typically you can see 10-50 ng of a protein. It does vary, however, with the glycosylation and physical properties of the protein. (Protocol from Jeff Brodsky to Tamara.)

Materials (enough for one mini gel)

A. 100 ml of a solution containing: 50 ml Ethanol, 12 ml Acetic Acid, 38 ml dH2O and
50 µl of 37% formaldehyde

B. ~200 ml of 50% Ethanol (in water)

C. 100 ml of a solution containing: 10 mg of sodium thiosulfate (Na2S2O3) in 100 ml dH2O

D. 100 ml of a solution containing: 100 mg of silver nitrate (AgNO3) in 100 ml dH2O

E. 100 ml of a solution containing: 3 g sodium carbonate (Na2CO3), 50 µl 37% formaldehyde, 2 ml of Solution C (sodiumthiosulfate solution) and 98 ml of dH2O

F. 100 ml of 25 mM EDTA

1. Fix the gel by washing (with rocking) with Solution A for 1 hour. Discard solution.
2. Wash the gel 3 times with ~70ml Solution B, for 20 minutes each. Discard solutions.
3. Treat the gel for 1 minute in Solution C. (Do not over do this, it can prevent good staining). Discard solution. Wash the gel 3 times with dH2O 3 times for 20 seconds each.
4. Stain the gel for 30 minutes in Solution D. Discard unwanted solution and wash the gel 3 times with dH2O 3 times for 20 seconds each.
5. Develop the gel for 10 min to 2 hours in Solution E. You need to watch the gel closely, as it is easy to over develop. However, it is often necessary to overdevelop somewhat in order to see low abundance proteins.
8. Stop developing by washing with Solution F for 20 minutes. Stop a little lighter than you want the gel at the end as it continues to develop a bit after stopping.
9. Wash in dH2O for 10 minutes.
10. Properly cut and thoroughly wet two clear plastic drying sheets with water. Place the gel in between the two sheets in a drying frame with plenty of water and clamp the frame pieces together. Make sure to remove any air bubbles, otherwise

the gel might crack upon drying. Allow the frame to sit in a vertical position overnight and the gel should be dry in the morning.

3.4 NO estimation

3.4.1. Griess Assay

It is an indirect method to investigate NO formation by measuring nitrite. It uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. Nitrite reacts with Griess reagents to give an azo compound which is estimated spectrophotometrically at 540nm.The limit of detection is 2.5µM (125pmol) nitrite.

To study the effect NO production in RBC age fraction, Griess assay was performed. Isolated RBCs (young, middle,old age) were centrifuged at 7500 rpm at 37^0C for 10 minutes. The supernatant was collected and incubated with Griess reagent for 30 minutes at 37˚C in dark. The absorbance was read at 540nm by using Elisa reader.

To compare the NO production in non-diabetic and diabetic RBCs, Griess assay was performed using isolated RBC age fractions from normal and diabetic blood samples.

3.4.2 DAF-FM

For the measurement of NO in RBC age distribution, isolated RBCs were loaded with 3µM DAF- FM (Molecular Probes, Eugene, Oregon) in PBS buffer

and incubated at 37^0C for 20 minutes. Following incubation, cells were taken in 96 well plate and reading were taking at wavelength 495/515 fluorimeter

3.4.3 Immunofluorescence microscopy for eNOS and Ser1177P-eNOS

Immunofluorescence was performed to visualize the sub-cellular distribution of eNOS and phosphoserine 1177 site in RBC age fractioned eNOS. Antibodies directed towards eNOS was used for immunofluorescence to show localization of eNOS in RBC and phosphoserine 1177 antibodies were used to show that Serine 1177 residue is getting phosphorylated during eNOS activation.

Isolated RBCs were placed on poly-L-lysine coated glass slides and were fixed in 0.25% Glutaraldehyde in phosphate-buffered saline (PBS) at 4˚C temperature for 30 minutes. After extensive washing with 0.5% Tween in PBS, they were incubated for 1 hour in PBS containing 1% bovine serum albumin (BSA) to block nonspecific binding and 0.05% Tween 20 for permeabilization. RBCs were immunolabeled with primary monoclonal rabbit antibodies against eNOS (ABCAM) at a 1:1000 dilution and against Serine 1177P-eNOS (ABCAM),which is a phosphorylated form of eNOS, at a 1:1000 dilution for 1 hour and kept overnight at 4˚C. Cell smears were then washed with 0.1 M PBS and Tween and incubated with anti-rabbit FITC-conjugated secondary antibodies for 1 hour. The slides were examined with a fluorescent microscope (Olympus IX71); pictures were taken with a CCD camera and processed by an Olympus DP controller program version 3.2.1.276.

. Immunofluorescence assay for RBC eNOS and Serine1177P-eNOS was carried out to show the presence of eNOS in RBC and to give evidence that on activation, eNOS gets phosphorylated at Serine 1177 site.

For the comparative study of presence of eNOS and Serine1177 phosphorylation in RBC eNOS, immunofluorescence assay was carried out using non-diabetic and diabetic RBC age fractions.

CHAPTER 4

RESULTS

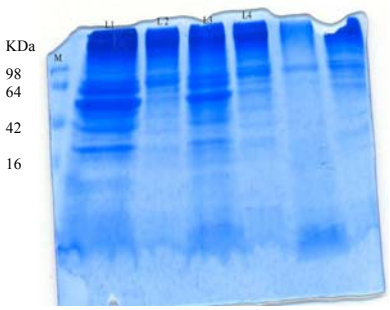
4.1. Separation of RBC using density gradient

In order to investigate the changes occurring in NO during aging, red blood cells were sub fractionated into three age dependent groups; young, mid age and old. This sub fractionation was done with Percoll density fraction. Four cell layers were identified after the fractionation of erythrocytes obtained from the studied subjects. According to reported criteria (Bruno Venerando *et al*., 2013), the different fractions were identified as follows: (a) fraction 1, at 66% Percoll, largely composed of “young erythrocytes” (b) fractions 2 and 3 at 70% and 74% Percoll, composed of “average age erythrocytes” (c) fraction 4 at 80% Percoll, prevalently made of “senescent erythrocytes”. Also in diabetics the erythrocyte fractionation by age showed 4 cell layers corresponding to those obtained in the controls.

Fig: 4.1 RBC separation using Percoll density gradient

4.2 Protein separation using SDS GEL

Ghost membrane protein from both Non -diabetic and Diabetic subjects were isolated and separated using 10% SDS gel. Later, it was stained with Coomassie Blue solution. Various bands of RBC membrane protein was analyzed



L1, L3- Non diabetic
L2- Diabetic
M- Marker

Fig: 4.2 SDS gel of RBC membrane protein

4.3. Characterization of RBC age fraction for band 4.1a and 4.1b

Characterization of different aged RBC using band 4.1a: 4.1b ratio confirmed as the isolated RBC was young, middle and old. Cells separated

according to this method are confirmed for its different age using increase in band 4.1a: 4.1b ratio (Vincent *et al*.,1967). Increase in band density is observed in old aged RBC. This fact showed that the bottom layer of centrifuged cells (high-density cells) contained preponderantly old red cells, while young red cells were concentrated in the top layer of centrifuged cells (lowdensity cells). These RBC are stored at 4˚C for further experiments.

Young Middle old Young Middle

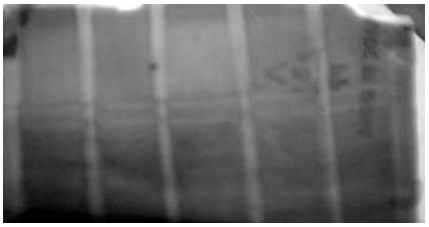


Fig: 4.3 Characterization of aging RBC by silver staining

4.4. Griess assay:

Griess assay is an indirect method for measurement of nitric oxide level. NO level in young RBC fraction was 34% increased than middle age RBC. Gradually NO level decreases in Older age RBC.

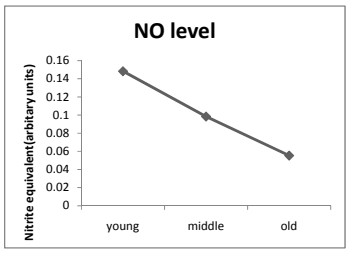


Chart :4.1 Nitric oxide estimation of different aged RBC using Griess assay

We was interested in testing NO level in Diabetic pateins. Whole Diabetic blood is checked for NO level. Diabetic blood had 25% less nitric oxid than Non-Diabetic blood.

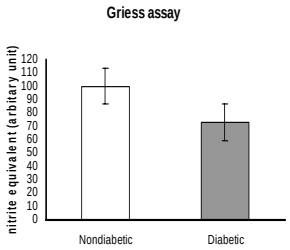


Chart 4.2: Griess assay showed around 25% less NO production in diabetic RBC.

Further comparative analysis of NO level for Diabetic RBC sub population showed same declined pattern as control RBC sub polpulations.

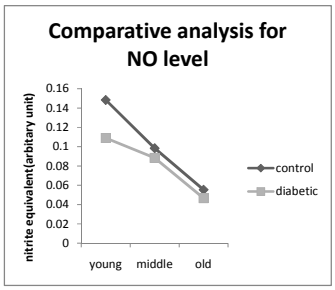


Chart 4.3: Comparative analysis of NO-nitrite level of different age fraction is less in diabetics comapared to control.

Diabetic young aged RBC showed significant difference in NO level compared to Control. But mid age RBC and old age RBC do not have much difference . Thus changes in NO level observed in Diabetic is mainly due to alteration in young aged Diabetic fraction.

4.5 DAF-FM

Amino acid arginine is proved to increase the activity of NO in cells. Arginine acts as an inducer molecule. Thus in all age RBC (young, middle, old), cells treated with arginine showed higher level NO compared to base level treatment.

DAF-FM is direct method for nitric oxide measurement. DAF-FM data showed high NO in mid age RBC population than young and older age RBC. It clearly states that Diabetic NO level is lower than Non-diabetic in all three age RBC.

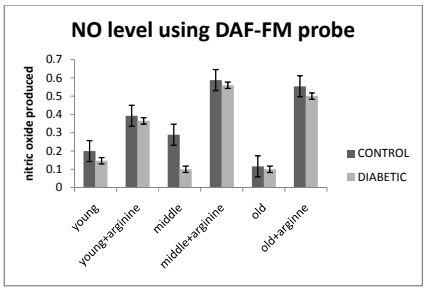


Chart 4:4 Direct measurement of NO with arginine activator

4.5 Comparison of presence of eNOS protein and Serine 1177P-eNOS in Non-diabetic and Diabetic RBC.

In order to compare the amount of eNOS and phosphorylated eNOS in non-diabetic and diabetic RBC, immunofluorescence experiment using eNOS antibody and phosphoSerine1177 antibody was carried out.

Among three age fraction (young, middle, old), young cells have higher eNOS expression. Immunofluorescence results supports previous data of Non-diabetic RBC age fractions have higher NO than Diabetic age RBC. This is confirmed by increased expression of eNOS and positive fluorescence spots in Non-diabetic age fraction. Since eNOS catalysed the NO production, increase in eNOS level simultaneously increases NO level.

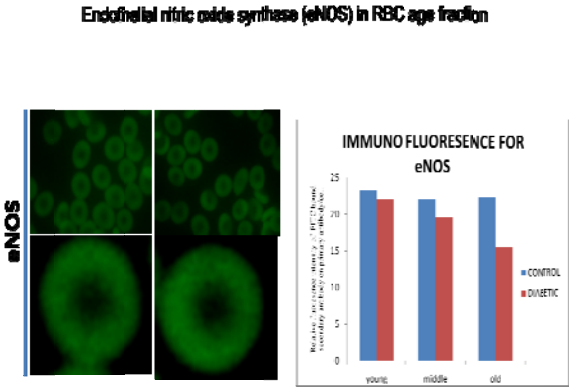


Fig: 4.4 IF imaging of different aged RBC targeting eNOS enzyme

Phosphoserine 1177 immunofluoresence showed that more fluorescent spots were present in Non-diabetic RBC indicating that more phosphorylated serine1177 sites were present in Non-diabetic RBC age fraction. p-eNOS expression decreases gradually in Non-diabetic age fraction. Young RBC cells showed high expression of phosphoserine 1177.

In comparison for phosphoserine site between Non-diabetic and Diabetic, Significant variation was observed in young RBC.

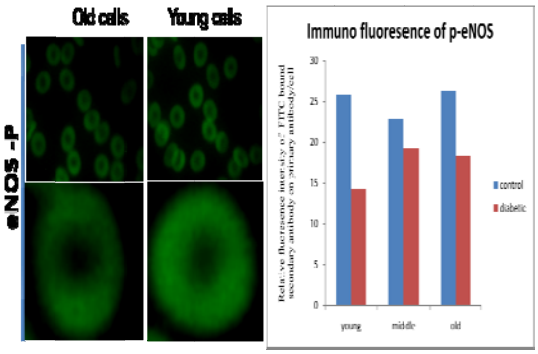


Fig: 4.5 IF imaging of different aged RBC targeting p-eNOS enzyme

CHAPTER 5

DISCUSSION

To date, human blood and, in particular, hemoglobin-carrying red blood cells have been considered as a major sink of NO. Although early reports postulated a NOS resident in RBCs (Mehta *et a*l, 1998) it was only recently discovered that RBC expresses a functional NOs **(**Kleinbongard *et al*., 2006). Our studies demonstrated the presence of active eNOS and NO production in RBC.

Earlier studies documented the NO presence in RBC by indirect methods like Griess assay, citrulline assay and oxyhemoglobin assay (Kleinbongard *et al*., 2006; Baskurt *et al*;2009). Our studies used DAF FM which is direct methods for NO estimation which showed real time NO production in RBC, which has not been shown before.

Our studies using DAF FM, IF and Griess assay showed that NO production in RBC can be increased 20-25% by supplementation of L-arginine which is a substrate of eNOS. In endothelial cells, it is known that on activation, eNOS migrates to cytoplasm from plasma membrane and associates with Akt leading to eNOS phosphorylation and further activation of the enzyme. The exact mechanism is hypothesized to be applicable to RBC eNOS (Kleinbongard *et al*., 2008).

Analysis of our immunofluoresence results showed the presence of eNOS and phosphorylated serine 1177 site in RBC eNOS. Using immunofluoresence

confocal microscopy, the presence of active eNOS in RBC has already been shown (Kleinbongard *et al*., 2006). Our studis shows that NO production in RBC occurs by eNOS activation by phosphorylation through Serine 1177 site. Since no other phosphorylation sites have been yet reported in RBC eNOS, we can conclude that eNOS activation in RBC depends predominantly on a phosphorylation at serine 1177 and young age RBC are phosphorylated in high level than older age.

. RBC NOS is detected in the cytoplasm and plasma membrane with its presence 20%-30% greater in plasma membrane than in cytoplasm. (Kleinbongard *et al*., 2006). Our eNOS immunofluoresence images are also showing the localization of eNOS more in plasma membrane.

The functions of RBC NO reported to date include the prevention of platelet aggregation and leukocyte adhesion to blood vessels, improving RBC deformability thus maintaining vascular homeostasis. (Kleinbongard *et al*., 2006).

Another focus of our study was comparative study of NO production in non-diabetic and diabetic RBC age fraction. Our studies documents that diabetic RBC has 25% less NO production. In search of possible reason for decreased NO production in diabetic RBC, we have checked the presence of eNOS in diabetic RBC age fraction. Strikingly, the presence of eNOS and serine1177-P eNOS are observed to be less in all three age fractions of diabetic RBC. Younger Diabetic age fraction show more decrease in eNOS expression. Earlier studies have shown the altered expression of endothelial NOS in diabetes (Catravas *et al*., 2008). Our study gives an indication that expression of RBC NOS is also altered during diabetes.

Various alterations of RBC plasma membrane appear both in diabetes mellitus and during the physiological aging process. Diabetes mellitus decreases RBC life-span; therefore, it may change the plasma membrane by acting through its effect on the aging process. (Bertoli *et al*., 1992). In order to check the correlation between the RBC aging occurring during diabetes and eNOS activity, we have checked the NO production in various age groups of RBC. RBC aging provoked a progressive reduction of eNOS activity in both non-diabetic and diabetic RBC. Our studies showed difference in NO production in various age groups of diabetic and nondiabetic RBC. But our studies suggest a possibility that eNOS can be used as a marker for RBC aging. Another focus of our study was comparative study of NO production in non-diabetic and diabetic RBC age fraction. Our studies documents that diabetic RBC age fraction has less NO production. To make this results stronger Western blotting of different age fraction targeting eNOS protein has to be included.

At this point, the question of the clinical implications for RBC NOS is evolving. RBC NOS will serve as a common denominator for fields such as atherosclerosis, diabetes, and RBC aging and microcirculatory diseases. Development of new therapeutic strategies for enhancing the expression and activity of RBC NOS is highly recommended.

CONCLUSION

In summary, RBC expresses a functionally active eNOS and produces NO which has potential effects on maintenance of vascular tone. NO and eNOS activity was found to be continuously decline from younger to older age fraction. RBC eNOS is tightly regulated by its substrate L-arginine and diverse interacting proteins. eNOS is localized more in plasma membrane and its activation occurs via phosphorylation at serine residue 1177. Among three RBC age fraction(young, middle, old), young cells play crucial role in NO production. RBC NO is implicated in pathological conditions like diabetes. Impaired NO production in RBC age fraction during diabetic condition is attributed to the low expression of RBC eNOS. In future, novel and effective therapeutic interventions enhancing RBC NOS activity might gain importance in the therapy of diverse diseases including diabetes.

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