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Review Article

Recent Advancement in Nitric Oxide Research in India

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Discovery of nitric oxide (NO), as endothelial derived relaxing factor that has been awarded with Nobel Prize, highlights its importance as master signalling molecule in diverse systems. Recent research has unfolded several unknown facets of this intriguing biomolecule in cardiovascular system, immunomodulation neurotransmission and plant physiology. NO produced by nitric oxide synthases (NOSs), is a short lived radical that reacts with superoxide radicals to generate reactive nitrogen species and potent oxidant, peroxynitrite implying damage in various pathological conditions. This adds another level of complexity in the understanding of pathological conditions associated with NO paucity or due to increased reactive oxygen nitrogen species (RONS). Researchers from India have been instrumental in unfolding various important and novel functions of this molecule and associated signalling by using multipronged approaches in different systems. Association of NO signaling with increased burden of life style diseases in recent years provide sufficient rationale to investigate NO in diverse pathophysiological conditions in Indian perspective. Here we review recent and important contributions of Indian science during last five years in understanding of NO signaling fundamentals in human, animals and also plants, its association with diverse pathological conditions and therapeutic targeting with possible ameliorative strategies.

Keywords: Nitric Oxide; Nitric Oxide Intermediate; Oxidative Stress; Cardiovascular System; Central Nervous System; Immunomodulation

Introduction

Several systems and their functions are regulated at local levels though autacoid signaling, where auto means "self" and acos means "relief". An imbalance of such autocoids at the level of synthesis, release or signal tranductions may contribute to pathological conditions. Nitric oxide (NO), an important autacoid, is released from the cell and act as local environment with short activity duration to regulate physiological functioning based on its chemically diffusible property. NOis generated by reactive oxidation of nitrogen by many cell types in vertebrates and non-vertebrates following enzymatic catalysis (Fig. 1). Imbalance in NO signaling causes development of patho-physiological conditions such as allergy, hypersensitivity, inflammation, neurotransmission, cardiovascular disorders and ischemia-reperfusion injury. Anti-oxidants and anti-inflammatory agents are being suggested as the interventive agents for many of these associated pathologies. Recent research work on this intriguing molecule has identified more facets of its contribution in various disease physiology and pathophysiology. Together, recent upshot in life style diseases, cardiovascular diseases burden since last decade and association of NO signaling has been an active rationale to investigate nitric oxide biology and its implications in Indian perspective. Furthermore, role of NO has been implicated in plant physiology including plant-pathogen interaction, stress responses and food biotechnology field that is an national priority as agriculture based country and thus provide sufficient reasons to better understand nitric oxide research in diverse systems. In this current review, we overview major research work performed by Indian authors along-with their collaborators abroad in the field of nitric oxide biology. We have covered publications from year 2012-2017 (five years) for purpose of this review. Though there are significant amount of publications that have used NO and its

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byproducts as inflammatory markers in association with other inflammatory molecules and similarly high number of publications have reported modulations of NO pathways using natural products and other approaches, we sincerely apologize that we have not accomodated all of such publications to this review.

Nitric Oxide Synthase isoforms and Generation of NO

NO is produced enzymatically by the action of nitric oxide synthase (NOS) isoforms. NOS enzyme oxidizes the guanidine group of the enzyme substrate L-arginine. Arginine is converted into N^w-hydroxyarginine and then to citrulline and NO. The enzyme activity that required dimerization of NOS, is regulated by the number of co-factors such as tetra-hydrobiopterine (BH4), FAD, FMN and NADPH (Fig. 1). This process also requires acceleration by calcium calmodulin as an activator. The endothelial NOS isoform was discovered for the first time in vascular endothelium and now being reported to be present in many other cell types too. Eventually induced NOS (iNOS) and neuronal NOS (nNOS) were also identified. These nNOS, iNOS and eNOS are also defined as NOS1, NOS2 and NOS3 respectively. These three isoforms differ in their genetic locations. NOS1, NOS2 and NOS are located on chromosome 12, 17 and 7 respectively with single copy of the gene in the haploid human genome. All these three different isoforms have different catalytic properties, inhibitor sensitivity and have around >50% homology between human isoforms. Interestingly, two of these i.e., nNOS and eNOS are constitutive in nature and depend on calcium levels for activation, while iNOS is inducible as suggested by its name and trigerred by various cytokines and other factors in a calcium independent manner. Interestingly, various L-arginine analogues can inhibit NO production and enzyme activity, though these NOS isoforms differ in terms of their sensitivity to these analogues. Many of the single gene level variants are also found out and getting many more cannot be negated. NO is highly unstable molecule and gets transformed into nitrite and nitrate that are commonly used as indicator of nitric oxide signaling in various systems. NO also reacts with superoxide radicals to generate reactive nitrogen species (RNS) and potent oxidant, peroxynitrite implying damage in various pathological

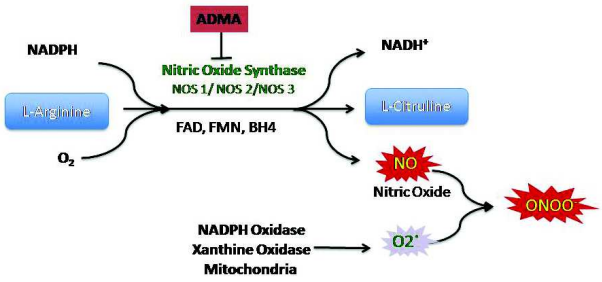

Fig. 1: NO generation by Nitric Oxide Synthase (NOS) and its association with oxidants

conditions (Fig. 1). This cause scavenging of NO and superoxide to cause reduction in their functions, while adds pathological insults through peroxynitrite and other reactive oxygen -nitrogen species (RONS) mediated damage.

Research on fundamental of NOS isoforms regulation is highly important and has provided different isoforms specific inhibitors. Recent research in this area has identified interesting phenomenon like NOS uncoupling that provides superoxide instead of NO. In India, laboratory of Dr. Koustubh Panda at Kolkata is focusing on basic regulation of NOS system. Pyrimidine imidazoles inhibits NOS dimerization, required for activity but precise mechanism of their action has remained unclear. A recent study using pyrimidine imidazole and its derivative (PID) identified mechanism of iNOS inhibition using rapid stopped-flow kinetic, gel filtration, and spectrophotometric analysis (Nagpal *et al*., 2013). Precisely, PID bound to iNOSheme generated an irreversible PID-iNOS monomer complex that could not be converted to active dimers by tetrahydrobiopterin and L-arginine. PID also caused irreversible monomerization of active iNOS dimers (Nagpal *et al*., 2013). This study established PID as a versatile iNOS inhibitor for complete physiological inhibition of iNOS in inflammatory, immunological, and neurodegenerative diseases. Interestingly, NO plays a regulatory role as signaling molecule at low concentration, while high NO and associated nitrogen species cause insult to cardiovascular, central nervous system and other tissues and implicated in various diseases (Fig. 2). Following sections will discuss NO signaling in these systems in details.

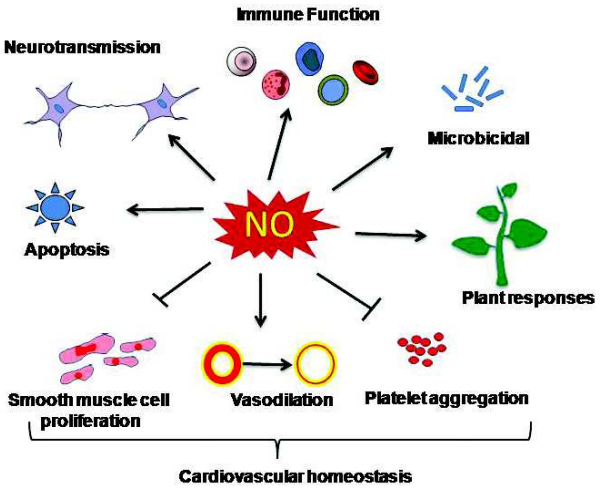

Fig. 2: Major functions of NO

Studies on Cardiovascular System and Related Pathological Conditions

In cardiovascular system, NO is mainly produced by eNOS constitutively expressed in the endothelial cells. In addition, diverse blood cells also contribute to NO availability in the vicinity to endothelium. Together, NO plays key role in maintenance of vascular function and vasodilation. The endothelial cells derived NO prominently from eNOS plays a central role in vascular tone regulation via acting as endogenous vasodilator. Usually, cytosolic eNOS is not catalytically active, while active enzyme is localized at the plasma membrane where NO generation takes place that subsequently released into extracellular environment and the abdominal side of the blood vessels. Continuous generation of NO is favourable towards maintenance of integrity of cardiovascular system. Further NO in association with other free radicals and their balance with anti-oxidant system is vital for normal cellular functioning and imbalance of which causes risk for cardiovascular health. Consistently, deficiency in production and/or bioavailability of NO have long been associated with endothelial dysfunction and cardiovascular diseases [summarized in a recent review (Charles *et al*., 2017)]. At equimolar ratio, NO and superoxide form speroxynitrite that decides cellular fate towards necrosis or apoptosis depending on its concentration in various cardiovascular disorders(Islam *et al*., 2015). NO and reactive oxygen intermediates (ROS) also mediatepost translational modifications such as tyrosine nitration, cysteine S-nitrosylation and S-glutathionylation that regulate functions of several proteinsimportant in cardiovascular and diabetes biology. Dr. Srinivas Gopala group's at Thiruvananthapuram has recently reviewed the functional importance of NO signaling in many mitochondrial and cytosolic proteins in diabetic heart using nitrated proteome elucidation studies (Jayakumari *et al*., 2014). Laboratory of Dr. Madhu Khullar at Chandigarh has also significantly contributed in understanding of NO and eNOS pathway in diabetic cardiomyopathy, hypertension nephropathy, preterm labour and rheumatoid arthritis. In addition, epigenetic regulation of myocardial NOS has also been suggestedin diabetic cardiomyopathy (Khanna *et al*., 2014) and eNOS gene polymorphism link was revealed in type 2 diabetic Asian Indians (Cheema *et al*., 2013). Dr. Madhulika Dixit at IIT Madras has been investigating role of NO Signaling in endothelial dysfunction, atherosclerosis and edema. Catestatin (CST), an endogenous antihypertensive/ antiadrenergic peptide regulates cardiovascular physiology. A recent study has revealed association of the naturally-occurring human CST-Gly364Ser variant with increased risk of systemic blood pressure and hypertension in human populations, possibly via diminished endothelial derived NO production due to altered interactions of CST-364Ser peptide with beta-adrenergic receptors (Kiranmayi *et al*., 2016).

Dr. Suvro Chatterjee's group at Anna University, Chennai has been investigating NO-cGMP signaling in endothelial permeability, nonalcoholic fatty liver disease (NAFLD), atherosclerosis and during mechanical stresses in RBCs(Balaguru *et al*., 2016, Nagarajan *et al*., 2016, Saran *et al*., 2017, Seth *et al*., 2017) and are summarizedhere. Rho GTPases downstream effecter, Rho-associated protein kinase (ROCK) isapotential target for cardiovascular diseases.Interestingly, ROCK inhibitorY-27631 was found to modulate NO production in endothelial cells in a biphasic manner suggesting caution for its use in cardiovascular diseases (Kolluru *et al*., 2014) and advocated a combination therapy of chemotherapeutic drugs and cGMP analogs, which would confer better protection against chemotherapy mediated vascular dysfunctions in cancer patients (Gajalakshmi *et al*., 2013). RBCs-eNOS contributes to intravascular NO pool and regulates physiological functions. Nagarajan *et al*., have shown that mechanical stimuli perturb RBC membrane that in turn triggered a signaling cascade to activate the eNOS via phosphorylation of

the serine-1177 moiety of RBC-eNOS and promoted important endothelial functions such as migration and vascular sprouting (Nagarajan *et al*., 2016). This study implicated mild mechanical/physical perturbations (like excersize) to sensitize RBC-eNOS for NO production *in vivo* and during storage to improve viability of RBCs in blood banks (Nagarajan *et al*., 2016). Tip cell formation from single leader endothelial cell is an essential process in angiogenesis, studies have performed on the role of eNOS-NO-cGMP signalling during this process that confirmed loss of eNOS suppressed tip cell formation (Priya *et al*., 2015). Further, dissection of NO downstream signaling using pharmacological inhibitors and inducers indicated that NO via sGC/cGMP pathway in the tip cells led angiogenesis (Priya *et al*., 2015). A comparative study of NONOate based NO donors and linking NO release dynamics with physiological functions suggested spermine NONOate applicability for angiogenesis (Majumder *et al*., 2014). Thalidomide treatment to pregnant women, causes limb deformities. Interestingly, NO was found to prevent limb deformities in thalidomide affected chick and zebrafish embryos by promoting angiogenesis, reducing oxidative stress and inactivating caspase-3 dependent apoptosis (Siamwala *et al*., 2012). Similarly donating NO can be a preventive measure for cadmium mediated teratogenicity (Nagarajan *et al*., 2013, Veeriah *et al*., 2015). Secreted Frizzled-Related Protein 4 (sFRP4), a secreted glycoprotein caused endothelial dysfunction followed by suppression of angiogenesis. Saran *et al* dissected the mechanism of sFRP4 mediated inhibition of angiogenesis that envisaged NO-cGMP signaling and elevated corresponding ROS levels and apoptosis for the induction of endothelial dysfunctions (Saran *et al*., 2017).

During physiological conditions, NO levels can be regulated at multiple levels. Constent to this, endogenous asymmetric dimethyl arginine (ADMA) in serum competitively inhibits NO synthase. Interestingly, serum ADMA/NO ratio has been shown to be better predictive marker for the severity of the coronary artery disease in patients at the risk of angina pectoris or myocardial infarction(Shivkar and Abhang, 2014). NO augmentation by sports and supplements are currently used for physical fitness of the sport persons. These products exhibited increase in circulating levels of nitrite and nitrate and in saliva (Jacob *et al*., 2017). Mercury exposure led to loss of endothelium-dependent vaso-relaxation due to reduce NO bioavailability via enhanced oxidative stressand can function as an early trigger to consequent cardiovascular complications (Omanwar and Fahim, 2015). Organochlorine, endosulfan pesticides that are associated with cardiovascular disease (CVD) and atherosclerosis cause endothelial dysfunction by decreasing eNOS activity(Ghosh *et al*., 2017). Decreased NO production or bioavailability was also found in chronic kidney disease (CKD) patients (Reddy *et al*., 2015). Further adiponectin specially IL-6 was negatively correlating with circulating NO levels in CKD patients (Ambarkar *et al*., 2016). Many of amino acid such as arginine, lysine, glycine and methionine intake has significant impact on the NO levels and cardiovascular parameters. Glycine supplementation in hypercholesterolemic rats significantly increased total NO concentration (Venkatesh *et al*., 2017). L-Arginine supplementation a well tolerated safe amino acid that improved endothelial dysfunction, ameliorates arterial stiffness and oxidative stress in chronic kidney disease mediated corroborating NO levels (Reddy *et al*., 2015). Organic "nitro" compounds like nitroglycerine are useful in acute coronary syndrome, but the mechanism remains speculative. Using different anti-anginal agents, Bank *et al*., found that organic nitro compounds, acetyl salicylic acid, insulin and glucose activate NOS in the arterial endothelial cells to generate continuous NO that seems to control the chest pain in acute coronary syndrome(Bank *et al*., 2017). In yet another study, NO donors sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP) and S-nitrosoglutathione (GSNO) were found to inhibit ion channel pannexin 1 (Panx1) mediated currents in HEK-293 cells (Poornima *et al*., 2015). Role of NO in ischemic preconditioning (IPC)-induced cardioprotection is suggested. Ovariectomyalso reduces atrial natriuretic peptide (ANP) with subsequent reduction in the level of NO. IPC-mediated cardioprotection was thus significantly attenuated in ovariectomized rat (Vishwakarma *et al*., 2017). Further perfusion with ANP protected that was attenuated by perfusion with N(ω)-nitro-l-arginine methyl ester hydrochloride (L-NAME) suggesting ANP mediated availability of NO as central in cardioprotection (Vishwakarma *et al*., 2017). Administration of recombinant erythropoietin (rEPO)

is often associated with systemic and pulmonary arterial hypertension in animals and human. A recent study showed that even a short-term exposure of erythropoietin impaired endothelial function through inhibition of NO production (Sultan *et al*., 2017). During pregnancy, hypertension is the most common medical problem, that is associated with maternal endothelium. Recently Gaikwad *et al*., observed a decrease in NOx and Thiol levels in pregnancy induced hypertension (PIH) (Gaikwad *et al*., 2017). Interstingly S-nitrothiols were increased in PIH, suggesting nitrosative stress a potential factor for this clinical manifestations. While in another study fractional exhaled nitric oxide (FENO) was failed to differentiate controlled and uncontrolled asthma with FENO value of 16(11-23) ppb and 13 (11-25) ppb, respectively (P=0.26) in children (Meena *et al*., 2016). A study aimed to investigate effect of stored RBC transfusions that increase cell-free Hb on NO availability in postoperative surgical patients, results obtained suggested decrease in NO metabolites irrespective of stored RBC transfusions, but most likely due to hemodilution (Nagababu *et al*., 2016).

Studies on Central Nervous system and Related Pathological Conditions

Nitric oxide is prominently produced by nNOS in brain and playsa keyrole in the central nervous system pathophysiology including regulation of stress(Gulati *et al*., 2015). It is important to mention significant contribution made by Dr. Kavita Gulati and Dr. Arunabha Ray, New Delhi and others in NO regulation in CNS physiology and dysfunction. NO and its stable metabolites (nitrite, peroxynitriteetc) cumulatively cause nitrosative stress in brain especially in nigrostriatal systemthat is implicated in Parkinson's disease. Consistently, NO Sinhibitors have moderately rescued Parkinson's disease (Gupta *et al*., 2014), which was also suggested by the earlier findings from Dr Madhu Dikshit's lab. Datta *et al*. revealed that localization and number of astrocytes decided dopaminergic neuron survival and function under 6-hydroxydopamine (6-OHDA) stress, as astrocytes from midbrain provided better dopaminergic neuronal (TH) cell survival in comparison to forebrain and hindbrain through BDNF secretion (Datta *et al*., 2017). Further BDNF released from astrocytes is mediated through autocrine and paracrine signaling of NO, as NOS inhibitor mitigated this BDNF release, while NO donor (DETA-NO) increased BDNF release (Datta *et al*., 2017). In other *in-vitro* study, 6-OHDA or lipopolysaccharides (LPS) has significantly decreased the viability of astrocytes, by inducing iNOS, nitrite level, ROS and decrease in mitochondrial membrane potential (Gupta *et al*., 2015). Role of iNOS was further confirmed by using iNOS inhibitor aminoguanidine that significantly attenuated the 6-OHDA/LPS induced cell death including mitochondrial activity, ROS levels (Gupta *et al*., 2015). Another view of NO role in Parkinson disease has also been suggested that NO from nNOS causes neurodegeneration, while NO produce from iNOS in proliferating microglia mediates the disease progression. In addition antagonistic pleiotropic effects of NO has been suggested in the pathophysiology of Parkinson's disease (Tripathy *et al*., 2015). Intra-cerebroventriculars trepto zotocin (STZ) model of cognitive impairment in rats exhibited increased NO, oxidative stress, inflammatory cytokines, increased expression of Rho kinase in cortex and hippocampus. Taurine, the essential amino acid exerted neuro-protective and beneficial effects for cognitive impairment of Alzheimer's type by suppressing above-mentioned parameters (Reeta *et al*., 2017). In mouse model of STZ induced chronic hyperglycemia, NO (using SNP) caused oxidative stress in addition to molecular alteration in the neurons and glial cells through neuroinflammation via NF-κB signaling (Richa *et al*., 2017). Consistently in a rat model of ischemia, NOS Inhibitor, L-NAME exhibited neuro-protective effects by mitigating glutamate excitotoxicity, inflammation and oxidative stress mediated by decreased nitrate/nitrite content (Pramila *et al*., 2015). Hyper-ammonemia found in many neurological disorders is associated with urea cycle dysfunction and altered brain energy metabolism. Glutamate-NO-cGMP pathway on modulation of glutamate receptors and transporters altered important cerebral processes causing cerbraledema and cell death (Natesan *et al*., 2016). In another study, Zinc-induced nigrostriatal dopaminergic neurodegeneration was found to be dependent on reduction in nitrite content and total/nNOS activity/expression. NO donors discernibly alleviated Zn-induced neurobehavioral impairments, neurodegeneration, and other associated changes (Singh *et al*., 2017). Curcumin have significantly attenuated vincristine induced neuropathic pain in a mice model owing to its

anti-nociceptive, calcium inhibitory and anti-oxidant effects (Babu *et al*., 2015). The hyper-angelic pain induced by CNS stimulant Modfinil was reversed by NOS inhibitors indicating role of NO pathways (Gupta *et al*., 2014). In addition, NO precursors have exacerbated and NOS inhibitor attenuated low frequency magnetic field induced OCD like behaviours by modulating levels of NO (Salunke *et al*., 2014). Chronic alcohol administration altered the functioning of CNS with increased ROS and NO levels and decrease in mitochondrial complex I, III and IV activities (Reddy *et al*., 2013). Opioid agonist, morphine protected from restraint stress induced anxiogenesis and neurobehavioral suppression in rats that was associated with reductions in oxidation products (NOx) of NO in the brain (Anand *et al*., 2012). Importantly, NO levels were rescued with morphine. Further, L-arginine synergized with sub-effective doses of morphine to protect stress-induced anxiety, whereas L-NAME blocked morphine mediated protection (Anand *et al*., 2012). In another study, morphine and L-arginine pre-treatment ameliorated stress mediated effects via decrease inHSP-70 levels and demonstrated involvement of NO in brain (Joshi *et al*., 2015).Chronic predictable and unpredictable stresses also modulated immunological responses by decreasing IgG type antibody and delayed type hypersensitivity. Stable metabolite of NO including peroxinitrite formation through 3-nitrotyrosine (3-NT) formation impacted immuno-modulation. Pre-treatment with iNOS inhibitor amino guanidine attenuated effects of stress in decreasing NOx and 3-NT levels indicating involvement of iNOS during modulation of adaptive immunity to stress (Thakur *et al*., 2017). Acute and chronic restraint stress causes anxiety, while both acute and chronic restraint stress correlated with increased HSP-70 levels, only acute restraint stress led to decrease in NOx level. Acute restrained stress induced anxiogenesis is more in male rats than female rats, that can be associated with increased level of ADMA and reduced level of NOx in brain homogenates (Chakraborti *et al*., 2014). Markedly higher level of gastric ulceration was observed in male rats than female rats upon cold restraint stress (Gulati *et al*., 2015). These effects were associated with the reduced brain and plasma NOx and GSH levels while MDA levels were elevated. L-Arginine pre-treatment prior to cold restraint stress prevented ulceration while NO synthase inhibitor L-NAME pre-treatment increased it significantly (Gulati *et al*., 2015). Together suggests that estrogen and its interactions with oxidative stress including NO are central to gender based differences in cold restraint stress induced gastric ulceration (Gulati *et al*., 2015). In a study, hypobaric hypoxia using high altitude simulation chamber (294.4 mmHg) for 24 h resulted in elevation of arterial blood pressure, renal sympathetic nerve activity, right ventricular systolic pressure, lung wet to dry weight ratio and Evans blue dye leakage (Sharma *et al*., 2015). These responses were significantly attenuated after lesioning posterior hypothalamus or after chronic infusion of GABA receptor agonist muscimol into posterior hypothalamus. Interestingly, chronic infusion of the NO donor SNAP into the posterior hypothalamus mitigated such attenuation (Sharma *et al*., 2015). Together during hypobaric hypoxia over-activity of posterior hypothalamic neurons via local decrease in GABA-ergic inhibition increased the sympathetic drive and thus pulmonary hypertension and edema.

Nitric Oxide in Dyslipidaemia, Insulin Resistance, Sepsis and Diseases

Here we would like to mention that studies from our group have been instrumental to demonstrate significant alterations in the NO signaling in experimental models of thrombosis, hypoxia-reoxygenation, sepsis, and in CNS disorder patients. NO has been suggested to play an important role in the initiation of dyslipidaemia induced insulin resistance (IR) with contrary reports. Recently by using iNOS KO mice, our group has reveal an altered glucose and lipid homeostasis in liver and adipose tissue that pre-dispose to insulin resistance (Kanuri *et al*., 2017). The respiratory exchange ratio (RER), volume of carbon dioxide (VCO_2), and heat production were lower as compared to WT mice. Significant reduction in eNOS and nNOS gene expression, hepatic and adipose tissue nitrite content, circulatory nitrite suggest a link between the NO status with systemic and tissue specific IR. Furthermore, a potential link between NO, leptin and adipocyte insulin responsiveness has been suggested (Gupta *et al*., 2017). Recently chronic hyper-leptinemia was found to induce insulin signaling disruption in adipocytes through increased expression of iNOS. Further, leptin effects on insulin signaling were mitigated by pharmacological depletion of iNOS and were absent in iNOS knockout animals (Gupta *et*

al., 2017). Reduced NO generation in the kidney is associated with hypertension in insulin resistance. Interestingly insulin was found to increase NO production in mouse renal inner medullary collecting duct cells via increased p-eNOS (Ser1177) levels (Pandey *et al*., 2015). Other experiments suggested contribution of reduced insulin receptor signaling in renal inner medullary collecting duct cells towards hypertension in the insulin-resistant state (Pandey *et al*., 2015). Active nitrogen molecules have been suggested to play an important role in vascular instability of septic shock. Plasma levels of nitrite and nitrate in systemic inflammatory response syndrome (SIRS), sepsis and septic shock has revealed the association of active nitrogen molecules in the progression of septic shock. Plasma nitrite and nitrate were high in patients with sepsis and septic shock, which increases with severity of sepsis (Kothari *et al*., 2012). Endogenous ADMA inhibits NOS and thus regulates vascular tone. A recent study revealed the association of ADMA and diabetes induced kidney injury. Significant elevation in plasma ADMA levels was observed in T2DM micro and macroalbuminuric patients, suggesting a causative role of ADMA in the development of kidney injury in terms of renal fibrosis (Jayachandran *et al*., 2017). This study also suggested 0.66muM/l of plasma ADMA level as a predictive risk threshold for diabetic nephropathy in south Asian Indian population (Jayachandran *et al*., 2017). Tobacco smoke induced oxidative damage to lung proteins, activated pro-inflammatory Rtp801 that triggers nuclear factor kappa B and consequent iNOS mediated overproduction of NO to induce oxido-nitrosative stress and lung protein nitration (Gupta *et al*., 2016). Interestingly, lung protein nitration was inhibited with lung-specific inhibition of iNOS using N6-(1-iminoethyl)-L-lysine, dihydrochloride (L-NIL) but fails to inhibit/reverse the oxidative lung injury (Gupta *et al*., 2016). Ascorbate or vitamin C, a dietary antioxidant substantially prevented tobacco smoke-induced lung protein oxidation as well as Rtp801 activation and iNOS/NO-induced nitration and thus provided holistic prevention to pulmonary emphysema.A recent review article advocated role of oxidatively nitrated histones in the initiation/ progression of autoimmune inflammatory diseases. Interestingly, systemic lupus erythematosus and rheumatoid arthritis sera shows oxidatively and nitrated modified histones involve in the initiation and progression of autoimmune diseases (Khan *et al*., 2017).

Nitric Oxide and Host-pathogen Interaction

Role of nitric oxide in host and pathogen interplay has also been a point of focus. Recently, high NO levels were found in the samples with high mononuclear cell counts and chronic tuberculous meningitis thus suggesting important role of NO (Kumar *et al*., 2017). Autophagy is important innate immune defense mechanism though lysosomal degradation. Sustained activation of Raw264.7 macrophages by IFN-γ and LPS has limited autophagy in NO dependent activation of AKT-mTOR signalling. Using Si-RNA approaches authors have demonstrated AKT was responsible for glycolytic shift, decreased mitochondrial potential and autophagy inhibition in activated macrophages (Matta and Kumar, 2015). Interestingly, *Plasmodium falciparum* parasite drive cerebral malaria exhibited persistent debilitating neurological deficit due to blood brain barrier disruption, endothelial cell activation, NO bioavailability, apoptosis and neuro-inflammation (Hora *et al*., 2016). In Northeast India, the Jaintia tribes utilize aqueous extract of the medicinal plant Carexbaccans to control helminthiasis. A recent study identified that phytochemicals resveratrol- and alpha-viniferin decrease acetylcholinesterase and NOS in helminth parasite *Raillietina echinobothrida* (Giri and Roy, 2015). This study highlights NO signaling in helminth intracellular communications through neuromuscular system and potential for anthelmintic potential purpose (Giri and Roy, 2015). In another study, rabies virus induced pathologies in mouse model were reduced with U0126 (inhibitor of MEK1/2) treatment (Manjunatha *et al*., 2017). The better survival was positively correlated with reduced viral load and reduced viral spread in the brain. RV-infected mice were present with higher levels of serum NO, iNOS, and TNF-α. $CD4^+$, $CD8^+$ T lymphocytes and NK cells were increased in blood and spleen of U0126-treated group (Manjunatha *et al*., 2017). Furthermore, intra-macrophage survival of *L. donovani* depended on the availability of extracellular L-arginine (Mandal *et al*., 2017). Leishmania, resulted in upregulation of L-arginine transport while Leishmania survival was greatly impaired when the L-arginine transporters CAT-2 were blocked either using inhibitor or siRNA-mediated downregulation (Mandal *et al*., 2017). NO also plays an indispensable role in killing of invading

pathogens by enhancing RONS in immune cells. A study using novel and alternative approach for intracellular delivery of NO using inhalable microparticles (MP) containing NO donors has induced phagosome maturation and kill Mycobacterium tuberculosis Mtb H37Rv (Verma *et al*., 2013). Importantly, inhalable MP were able to target NO donors to the macrophage and NO release in cytosol to reduce Mtb (Verma *et al*., 2013). It would be interesting to further investigation these MP as an adjunct to standard anti-tuberculosis chemotherapy.

Nitric Oxide in Immune Cells, Hematopoiesisand Leukemia

Nitric oxide has been shown to have contributory role in hematopoietic cell growth and differentiation. To validate this presumption, our group recently has assessed the alterations in nitrite level in control and leukemic cell growth by using myeloid leukemias including AML and CML patients. The significant decrease in nitrite levels in the blood plasma, marrow fluid and cellular fractions in BM and blood of myeloid leukemia suggests towards decrease in NOS activity (Jain *et al*., 2017). Further current work is focused to unfold molecular targets for therapeutic role of NO modulators. Another study from Dr. Vaijayanti Kale's group has investigated an direct effect on hematopoietic potential, NO donor (SNP) treatment has led to high expression of CD34+ cells in murine bone marrow Lin-cells or sorted LSK-CD34-cells, suggesting upregulation of CD34, that has contrasting age-dependent effects on the functionality of murine hematopoietic stem cells (Jalnapurkar *et al*., 2016). Another interesting study has revealed the role of NO in migration and/or invasion of colon cancer cells by up-regulating cGMP-PKG-ERK1/2-AP1 pathway leading to increase expression of MMP-2/9 (Babykutty *et al*., 2012). DEPTOR endogenously inhibit mTOR complexes and are often deregulated in carcinogenesis. DEPTOR overexpression and silencing studies concluded that it promotes survival of cervical squamous cell carcinoma cells by reducing apoptosis mediated by differential effects of iNOS/eNOS expression, PI3K-AKT and P38-MAPK pathway (Srinivas *et al*., 2016).

Recent focus of our lab research has been investigation of NO signaling and its effect on neutrophil function and death (Fig. 3). Recent study has revealed a crucial role of NO/iNOS in neutrophil apoptosis via enhanced ROS generation and caspase-8 mediated activation of mitochondrial death pathway (Dubey *et al*., 2016). Prolonged treatment of human

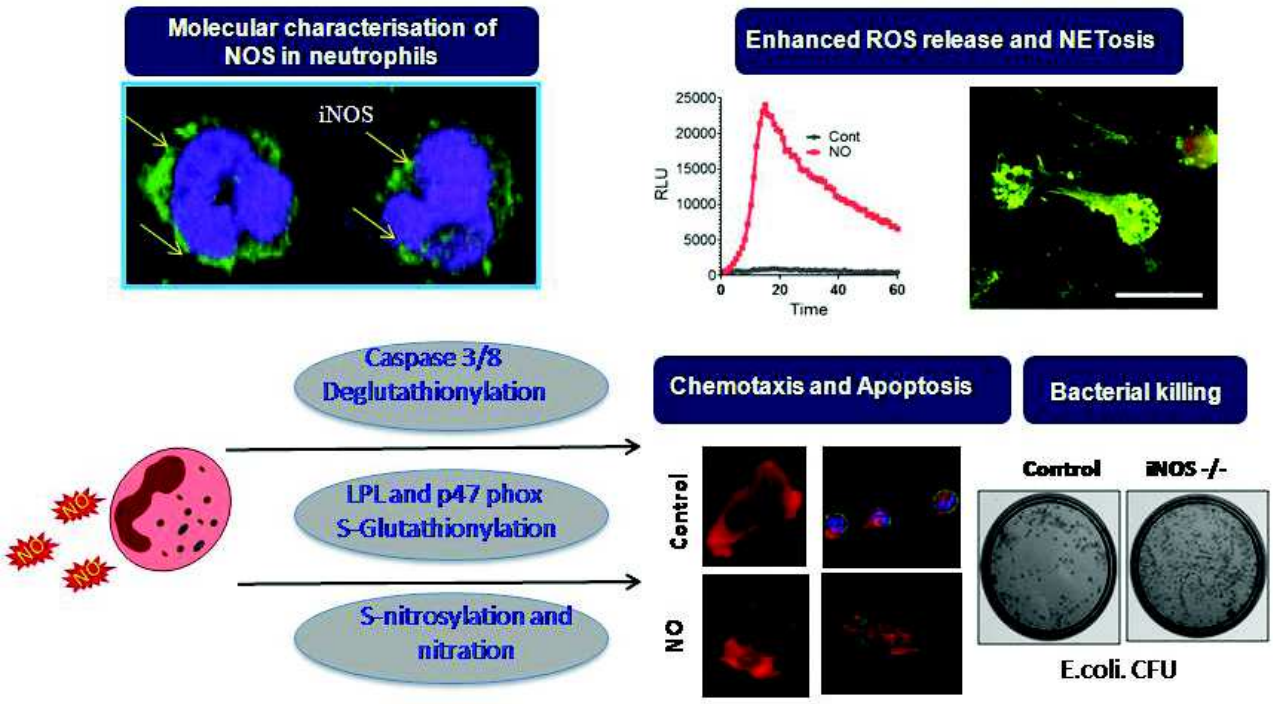

Fig. 3: NO signaling and its effect on neutrophils functions

PMNs or mice neutrophils with NO led to enhanced ROS generation, caspase-8/caspase-3 cleavage, and reduced mitochondrial membrane potential and finally cellular apoptosis (Dubey *et al*., 2016). Involvement of NOX2 in NO-induced apoptosis of PMNs was further suggested by inhibition of caspase-8 and BID cleavage in BMDN from neutrophil cytosolic factor-1 (NCF-1) knockout mice. Furthermore, ROS, NO generation and iNOS expression were enhanced in a time-dependent manner in PMNs undergoing spontaneous apoptosis. Pharmacological and genetic ablation of iNOS in human PMNs and mice BMDN significantly reduced the levels of apoptosis (Dubey *et al*., 2016). Furthermore, nitric oxide induced oxidative stress-related posttranslational modifications (PTMs) of cytoskeleton proteins were investigated in human PMNs by using *in vitro* and genetic approaches. Importantly S-glutathionylation of L-plastin (LPL) and β-actin promotes reduced chemotaxis, polarization, bactericidal activity, and phagocytosis. S-Thiolation diminished binding as well as the bundling activity of LPL (Dubey *et al*., 2015). Enhanced nitroxidative stress with LPL S-glutathionylation identified disease relevance in diabetic patients and db/db mice with impaired PMN functions (Dubey *et al*., 2015). In diabetes-associated vascular complications, lower levels of glutathione and increased oxidative stress have been reported. Thus provide a mechanistic basis for their impaired functions in diabetes mellitus(Sanchez-Gomez *et al*., 2013). Another study has identified interaction of iNOS with rac2 that has regulated ROS and RNS generation in the human neutrophil phagosomes to mediate microbial killing. During phagocytosis neutrophils showed significant elevation in NO and RONS, these responses were inhibited in iNOS, Nox2 and Rac2 silenced human or iNOS-knockout mice neutrophils. Interestingly iNOS-Rac2 complex formed translocate to phagosomes after phagocytosis accompanied by superoxide, NO, ROS/RNS. Rac inhibitor, NSC23766 that significantly abrogated NO release and microbial killing in vivo suggests its importance in inflammatory conditions (Jyoti *et al*., 2014). In a study focused to explore estrogen mediated regulation of immune responses, Estrogen through ER-α was found to differentially modulates β2-AR-induced immune response pathways, and NO that seems to be responsible for estrogen-induced immunosenescence and development of female-specific diseases (Priyanka *et al*., 2014).

Studies targeting NO in Health and Diseases

Several natural products have been evaluated for NO mediated cardiovascular diseases, neurodegeneration and inflammatory syndromes. Various flavonoids, carotenoids, phytoestrogen, phytosterols contribute to improve endothelium dependent vaso-relaxation by modulating availability of NO and has been reviewed recently (Upadhyay and Dixit, 2015). To discuss all of such studies in this review was not feasible. We recommend another review that has described the herbal plants and phytochemicals with hepatoprotective and Immunomodulatory via targting chemokines, and cytokines, and release of the inflammatory mediator (Ilyas *et al*., 2016).

Cardiovascular Associated Diseases

Traditional essential oil rich in curzerene, phenone, germacrone and other sesquiterpenes caused significant vaso-relaxant effects in ex vivo model system through NO dependent pathway (Shiva Kumar *et al*., 2017). Amaranth extract increases NO levels in circulation suggesting improvement of overall performance of sport persons (Subramanian and Gupta, 2016). A novel class of '1-(nitro-oxy) ethyl ester' group-containing NSAIDS as efficient NO releasing 'true' prodrugs of aspirin and naproxenwas reported recently with parallel bioactivity and exhibited protective effects in rats from gastric damage (Gund *et al*., 2014). Atorvastatin has ameliorated arsenic induced hypertension by improving lipid profile and aortic NO signalling that restored vascular redox homeostasis (Kesavan *et al*., 2014, Sarath *et al*., 2014). In another study, Gentianalutea (GL) and its component isovitexinexerted anti-atherosclerotic effects (Kesavan *et al*., 2016). GL aqueous root extract and isovitexin prevented endothelial inflammation and smooth muscle cell migration to block the onset and progression of atherosclerosis in STZ induced diabetic rats. Interestingly, GL treatment led to reduction of iNOS expression in aortic segments of diabetic rats (Kesavan *et al*., 2016). Sinapinic acid increased level of plasma NO metabolites in L-NAME induced hypertension model and protect from high blood pressure, cardiac fibrosis, cardiac dysfunction, kidney fibrosis and lipid metabolic (Silambarasan *et al*., 2014, Silambarasan *et al*., 2016). Widely used chemotherapeutic breast cancer drugs has been shown to dampen vascular functions by interfering with NO signaling in endothelium and these effects

could be recovered using pharmaceutical agonists of NO signaling pathway (Gajalakshmi *et al*., 2013). L-theanine, a non-protein amino-acid found in tea (*Camellia sinensis*), promotes NO production in endothelial cells (Siamwala *et al*., 2013) to improve vascular function and is linked to lowering the risk of cardiovascular disease.

CNS Associated Diseases

Sesame which is reported lipid lowering agent of *Sesame indium* Linn (Pedaliaceae) corrected the aluminium chloride (AlCl3) induced cognitive dysfunction and memory impairment in rodents and also reversed NO and inflammatory cytokines in hippocampus and frontal cortex of these rodents (John *et al*., 2015). In another study, fisetin, a naturally occurring flavonoid, exhibited therapeutic benefits by modulating urea cycle enzymes in ammonium chloride induced hyper-ammonaemic rats. This effect was derived from decrease in iNOS and NF-κB in hyper-ammonaemic rats (Subramanian *et al*., 2014). Naringenin another flavonoid has reduced focal cerebral ischemia reperfusion injury by supressing neuro-inflammation and NF-kB mediated inflammatory pathway, thus improved functional outcome (Raza *et al*., 2013). Naringenin has also decreased oxidative stress by reducing increased lipid peroxide and NO in type-2 diabetes induced memory dysfunction in rats and improved cholinergic function (Rahigude *et al*., 2012). *Bacopa moneri* extract has been shown to reverse cognitive dysfunction in many neurodegenerative disorders/diseases. Brahmi has also increased the reduced age related NO production in lymphocytes in rats (Priyanka *et al*., 2013). Fish oil enrichment with quercetin has provided higher degree of neuro-protection in 3-nitropropionic acid rat model of neuro-degeneration by attenuating oxidative stress in brain regions (striatum/cerebellum) as observed reduction in reactive species, hydropreoxides and NO levels (Denny Joseph and Muralidhara, 2013, K and Muralidhara, 2014). Pre-treatment of NO mimetic, L-arginine as well as melatonin reduced aminophylline induced anxiogeneic response including anxiety and seizures while NO synthase inhibitors L-NAME and 7-NI aggravated it (Gulati and Ray, 2014).

Inflammatory Syndromes

Recently, a novel chemically modified, non-carbonyl compound enriched *Curcuma longa* L. extract (CMCE) was found to exert potent anti-inflammatory activity and cytotoxic effect. Interestingly, CMCE induced a significantly decrease in LPS-induced nitrite, aortic iNOS expression, and thus rescue vascular dysfunction and thus suggests a therapeutic potential for its use in sepsis and leukemia (Rana *et al*., 2016). Interestingly, Curcuma oil was also found to ameliorate insulin resistance and associated thrombotic complications in hamster and rat models (Singh *et al*., 2015). Curcuma oil also reduces endothelial cell-mediated inflammation in post myocardial ischemia/reperfusion in rats (Manhas *et al*., 2014).

NO and Plant Physiology

NO also plays role in plant stress responses including infection resistance and tolerence. Dr. Sanjay Ghosh and others are understanding the plant-pathogen interaction and nitrosative stress participation in plant responses. In a recent study, salicylic acid (SA) and NO using SNP were found to mitigate injury symptoms of saline stress in *Pisumsativum* L. through substantial decline in reactive oxygen species accumulation and inducing effects on activities of superoxide dismutase, catalase, guaiacol peroxidase and ascorbate peroxidase (Yadu *et al*., 2017). Thus may be efficiently utilized to overcome the adverse signatures of salinity stress (Yadu *et al*., 2017). Furthermore, NO is an important signaling molecule in plants under physiological and stress conditions. A recent review has described the influence of NO on chloroplasts possibly by influencing photophosphorylation, electron transport activity and oxido-reduction state. In addition, NO can change the gene expression to influence the photosynthetic apparatus and its functions (Misra *et al*., 2014).A recent finding demonstrates that sunflower seedling roots exhibit high sensitivity to salt stress in terms of nitrite accumulation. Salt stress cause reduction in S-nitrosoglutathione reductase (GSNOR) activity that was restored with dithioerythritol (Jain *et al*., 2017). Opposite patterns of S-nitrosylation in seedling cotyledons and roots was observed using LC-MS/MS analysis, suggesting S-nitrosylation as a key mechanism of salt stress sensing in sunflower seedling (Jain *et al*., 2017). Another study has revealed ROS/NO regulation of phenolic metabolism under water stress and abscisic acid (ABA) by using tolerant and sensitive wheat cultivar (Kaur and

Zhawar, 2017). Sufficient endogenous ROS/NO signalling was present in tolerant cultivar under water stress which susceptible cultivar lacked but showed growth improvement on exogenous ROS/NO applications (Kaur and Zhawar, 2017). Under hypoxia, plants produce high levels of NO but its role in plant-adaptive response to hypoxia remained unknown. A recent study identified that under hypoxic conditions, wheat roots produced NO apparently via nitrate reductase (Wany *et al*., 2017). While scavenging of NO led to a marked reduction in aerenchyma formation. Hypoxically induced NO was also found important for induction of the ethylene biosynthetic genes encoding ACC synthase and ACC oxidase (Wany *et al*., 2017). Another study has shown that improvement of photosynthetic performance using exogenously nitrate application in tomato (*Solanum lycopersicum* L.) under arsenic toxicity (Agnihotri and Seth, 2016). Furthermore, nitrate treatment revamped nitrogen metabolism and also enhanced the total nitrogen and NO content while membrane electrolytic leakage and malondialdehyde content were remarkably decreased (Agnihotri and Seth, 2016). Authors also suggested it as a cost effective approach in amending arsenic toxicity. Cadmium (Cd) exposure to mustard plant (*Brassica juncea* L.) has induced oxidative stress (H2O2 and lipid peroxidation) to inhibit growth and photosynthesis (Per *et al*., 2017). Interestingly, exposure of NO (using SNP) reversed the effects of Cd through superoxide dismutase, ascorbate peroxidase, glutathione reductase stimulation and reduced glutathione and thus scavenged ROS and increased plant growth, photosynthesis (Per *et al*., 2017).

Conclusions

Together, in recent years Indian researchers have contributed significantlyinNO biology and related research that has significant role in health and diseases. Particularly, there are excellent groups that have contributed to better understand the basic NO biology in cardiovascular, central nervous and immune system. While many other studies have investigated disease pathologies and their correlation with NO, NOS and other metabolites. It is encouraging to observe scientific validation with high translational and applied research publications using traditional natural products, that we could not cover fully in this review. With recent development in gasotransmitter regime, further studies are required to focus on chemical/biochemical network of NO signaling with other gasotransmitters (H_2S, CO, CH_4etc), an emerging area in nitric oxide research. Furthermore, research progress in population based research directions and solid basic understanding in this area of research using next-generation sequencing (NGS), RNA-seq analysis for transcriptome, high resolution proteome and metabolomeareanticipated in near future to better understand NO dysfunctions in diverse pathological conditions with strong translational output and healthy lifestyle.

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