4.1 Chemicals

Chemicals used in the present study have been listed below:

Sigma (USA) - Acrylamide, Agarose, Aminobenzoic acid hydrazide (ABAH), Ammonium per sulphate (APS), Aproteinin, Apyrase, Ascorbate, Bis acrylamide, Bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene (DETA-NONOate), Bovine serum albumin (BSA), Citric acid, Dextran T-500, 4,5-Diaminofluorescein Diacetate (DAF-2DA), 2,7-Dichlorofluorescein diacetate (DCF-DA), Diethyl Pyro Carbonate (DEPC), Di-isopropyl fluorophosphates (DFP), Diphenyleneiodonium chloride (DPI), Di sodium hydrogen phosphate (Na_2HPO_4), Ethidium bromide, Ethylenediamine tetra-acetic acid (EDTA), Flavin adenine mono and dinucleotide (FMN, FAD), Formaldehyde, Giemsa, 3,3- 2′,7′-4-Imidazole, HEPES, Horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, L-Arginine, MOPS sodium salt [3-(N-Morpholino) propane sulfonic acid- sodium salt], N-acetyl-L-cysteine (NAC), Nicotinamide adenine di nucleotide phosphate (NADPH), N^{ω}-nitro-L-arginine methyl ester (L-NAME), N,N,N′,N′-Tetramethylethylenediamine (TEMED), N,N,N',N'-tetraacetic acid (EGTA), Paraformaldehyde, Penicillin G, Pepstatin A, Phorbol 12-myristate 13-acetate (PMA), Phenylmethanesulfonyl fluoride (PMSF), Pipes, Ponceau-S, Poly L-lysine, Potassium chloride (KCl), Potassium dihydrogen phosphate (KH_2PO_4), Propium iodide, RPMI 1640, S-nitroso-N-acetylpenicillamine (SNAP), Sodium Chloride (NaCl), Sodium dodecyl sulfate (SDS), Streptomycin, Tetrahydrobiopterin (BH_4), Tri Sodium citrate, Triton X 100, Trypan blue, Tween 20.

Invitrogen (Carlsbad, USA) - TRI reagent

Fermantas Life Sciences (Vilnius, USA) - Revert aid H^- first strand cDNA synthesis kit, 2X PCR master mix, SYBR green 2X master mix, DNA ladders

Alexis (New York, USA) - DMPO (5, 5-dimethyl-1-pyrroline-N-oxide)

Cayman (Michigan, USA) - Vinyl-L-NIO, 1400W, anti DMPO antibody

BD Biosciences (California, USA) - Anti CD61 antibody.

Amersham Biosciences Corp. (Uppasala, Sweden) - Polyvinylidene difluoride (PVDF), X-ray Hyperfilms, Percoll

Merck (Darmstadt, Germany) - Isoporopanol, Chloroform and Ethanol

Santa Cruz Biotechnology (California, USA) - Polyclonal nNOS, eNOS, p47 phox, Rac2, monoclonal iNOS, CD 3, CD 14, CD 15 antibody.

Eurofins Genomics India Pvt Ltd (Bangalore, India) - Primers (oligos).

Millipore (Massachusetts, USA) - Chemiluminescence detection reagents

Table 5 - Composition of various reagents/ buffers

Reagents/ Buffer (pH)	**Composition**
HBSS (pH 7.4)	NaCl 138 mM; KCl 2.7 mM; Na_2HPO_4 8.1 mM; KH_2PO_4 1.5 mM; Glucose 10 mM.
PBS (pH 7.4)	NaCl 138mM; KCl 2.7mM; Na_2HPO_4 4.3mM; KH_2PO_4 1.5mM
Normal saline	0.9% NaCl
Trypan blue	2mg/ml Trypan blue in PBS
Tyrode's buffer	HEPES 10mM pH 7.4; NaCl 145mM; KCl 5mM; dextrose 5.5mM; $MgCl_2$ 1mM
Sodium citrate (pH 6.5)	3.8% Sodium Citrate
Stop buffer	Tris 10mM; NaCl 0.9%; Aproteinin 1µg/ml; PMSF 100µg/ml; Pepstatin A 20µg/ml; EDTA 1mM; pH-7.4 and DFP 5mM.
Laemmli sample buffer	Tris-HCl 50 mM pH 6.8; SDS 2%; glycerol 10%; β-mercaptoethanol 1%; EDTA 12.5mM; bromophenol blue 0.02%
SDS-running buffer	Tris 25mM; Glycine 192mM; SDS 0.1%
Transfer buffer	Tris 25mM; Glycine 192mM; methanol 20%; SDS 0.1%
TBST (pH 7.4)	Tris 10mM; NaCl 150mM; Tween 20 0.1%
Stripping buffer	Tris-Cl 62.5mM pH 6.7; mercaptoethanol 100mM; SDS 2%
Relaxation buffer	KCl 100mM; NaCl 3mM; $MgCl_2$ 3.5mM; EGTA 1.25mM; EDTA 1mM; NaF 10mM; PMSF 1mM; Pipes 10mM pH 7.3; Na_3VO_4 2 mM; pepstatin 20µg/ml; trypsin inhibitor 20µg/ml.
Urea lysis buffer	Urea 7M; thiourea 2M; CHAPS 4% w/v; Tris 20mM; DTT 10 mM ; EDTA 1mM ; PMSF 1mM.
Rehydration solution	Urea 7M; Thiourea 2M; CHAPS 2%; DTT 65mM; Triton X-100 1% v/v; bromophenol blue 0.01%; ampholytes 0.8% (pH 3-10)
SDS denaturing solution	Urea 6M; SDS 2%; glycerol 30%; Tris-HCl 50mM (pH 8.8);
Cathode buffer	Glycine 192 mM; SDS 0.1%; pH 8.3
equilibration buffer	Urea 6M; DTT 2%; Iodoacetamide 2.5%; glycerol 30%; Tris 45mM; SDS 1.6%; bromphenol blue 0.002%
RIPA buffer	PBS containing EDTA 1mM; sodium orthovanadate 1mM; sodium fluoride 1mM; aprotinin 1µg/ml; PMSF 100µg/ml; pepstatin 20µg/ml
Wash buffer (pH 8.0.)	Tris 50mM

4% PFA (pH 7.4)	Paraformaldehyde 4% in PBS
Culture medium (pH 7.4)	RPMI-1640 containing $NaHCO_3$ 0.2%; glutamine 2mM; FBS 10% (v/v); penicillin 100 units/ml; streptomycin 100µg/ml
5 x formaldehyde gel running buffer	MOPS 0.1 M (pH 7.0); Sodium acetate 40 mM; EDTA 5 mM (pH 8.0.
Ponceau S	Ponceau S 0.1% (w/v) in 1% (v/v) acetic acid

4.2 Human subjects

Blood was collected in citrate-phosphate-dextrose (CPD) (1:7) from healthy volunteers only after prior consent. A detailed medical history and physical examination was carried out before phlebotomy. The donors were free from heart, lung, kidney disease, cancer, epilepsy, diabetes, tuberculosis, abnormal bleeding tendency, allergic disease, sexually transmitted diseases, jaundice, malaria, typhoid, thyroid myeloid or any other endocrine disorder. Donors were also free from any prior medication for last 72 h. Bone marrow cells were obtained from patients after informed consent by aspiration from the posterior superior iliac spine, who were undergoing routine bone marrow examination. Patient details have been summarized in **table 6**. The patients included in the study had normal hematopoiesis and also exhibited normal myeloid origin cells, as was clear from their myeloid to erythroid ratio (M:E ratio), which was usually within or less than 10:3 for normal hematopoeisis. The study was performed in accordance with the ethical committee of the institute.

Table 6 – List of patients included in the study

Patient ID	Age (Years)	Sex	Disease	M:E Ratio	Hematopoesis
P1	3.5	F	Retinoblastoma	7:1	Normal
P2	26	M	Massive splenomegaly	4:1	Normal
P3	44	M	Retinoblastoma	3:1	Normal
P4	80	M	Anaemia	1:2	Normal
P5	9	M	Dengue fever	2:1	Normal
P6	0.6	M	Pneumonia	3:1	Normal
P7	40	F	Anaemia	2.5:1	Normal
P8	55	F	Lymphoma	3:1	Normal
P9	40	F	Pyrexia of unknown origin	2:1	Normal
P10	30	F	Retinoblastoma	3:1	Normal

4.3 Isolation of various blood cells from human blood.

4.3.1 Neutrophil

Human neutrophil were isolated according to **Fuchs *et al,* (2007)** by centrifuging at 200 g (TJ6, Beckman, USA) for 10 min to remove platelets. This was followed by further centrifugation at 700 g for 10 min (TJ6, Beckman, USA). Platelet poor plasma was discarded and buffy coat layer containing WBCs was collected in separate tube. 6% Dextran was added to the buffy coat, total volume was made upto 10 ml, kept at room temperature for 30 min. Supernatant (8ml) was loaded on 4 ml Percoll (1065/1080, equal volume), centrifuged at 700 g for 15 min at room temperature (3K30 Sigma Centrifuge, Germany). Supernatant was removed and the WBCs band containing neutrophil were collected and washed once with HBSS before resuspending in it. Purity of the population was ascertained by Giemsa staining and CD 15 surface labeling.

4.3.2 Peripheral blood mononuclear cells (PBMCs), monocytes and lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by loading diluted blood (1:1 with saline) on Histopaque 1077 and centrifuged at 400 g for 30 minutes at 20°C (3K30 Sigma Centrifuge, Germany). PBMCs collected from the interface were washed twice with Hank's Balanced Salt Solution (HBSS) by centrifugation at 100 g for 10 minutes at 20^0C and adhered in RPMI-1640 containing 10% FBS for 1h **(Repnik *et al,* 2003)**. Adhered monocytes and floating lymphocytes were collected for subsequent analysis. Viability of the isolated blood cells was more than 90% as assessed by Trypan blue exclusion test.

4.3.3 Platelets

Human platelets were isolated from whole blood as described by **Prakash *et al,* (2011)**. The platelet-rich plasma (PRP) was separated by centrifugation of the citrated blood at 200 g for 20 min. Apyrase (1 unit) and acid citrate dextrose (ACD) were added to the PRP, and the platelets were pelleted by further centrifugation at 800 g for 10 minutes. The platelets ($2x10^8$ cells/ml) were washed once with modified Tyrode's buffer containing apyrase (1 unit) and then resuspended in modified Tyrode's buffer.

4.3.4 Red blood cells (RBCs)

Blood was centrifuged at room temperature at 800 g and the plasma was removed. Blood cells were diluted and washed with phosphate-buffered saline solution and centrifuged at 800 g at room temperature. Supernatant and buffy coat were removed by aspiration, a process that was repeated three times. Packed RBCs ($5x10^8$ cells/ml) were sonicated with ultra sonicator (Heat System, USA) (three 10-second bursts, setting 5) at 4°C. The preparation was checked for intact RBCs if any under the microscope **(Kang *et al*, 2000)**.

4.4 Isolation of neutrophil precursor cells from human bone marrow

Neutrophil precursors from bone marrow were isolated according to method described by **Cowland and Borregaard (1999)** with minor modification using Percoll density gradient (1.065, 1.080, and 1.086 g/ml). Briefly, bone marrow was collected from patients (having unaffected myeloid origin) in 3.8% sodium citrate diluted in HBSS (1:9) and put for dextran sedimentation for 30 min at room temperature. Following sedimentation, supernatant containing leukocytes was removed and the cells were recovered by centrifugation at 200 g for 10 min at room temperature. The pellet was resuspended in HBSS and added slowly onto the Percoll density gradients (1.065, 1.080, and 1.086 g/ml) and centrifuged at 1,000 g for 20 min. After centrifugation, bone marrow cells were separated into three bands having MBs/PMs, MCs/MMs, and BCs/segmented neutrophil (SCs) in Bands 3, 2, and 1, respectively.

4.5 Immunolabeling of surface markers

Lineage specific CD markers present on the cell surface offers a wide range of choices to identify the cells of interest from a mixed population. Neutrophils, monocytes, lymphocytes, platelets express CD15, CD14, CD3 and CD61 respectively. 10^6 cells were resuspended in 100µl HBSS and incubated with 5 µl of anti-human CD15-PE (for neutrophil), CD14-PE (for monocytes), CD3-PE (for lymphocytes), CD61-PerCP (for platelets), CD15-PE and CD11b-Pac blue (for band 3), CD15-PE, CD11b-Pac blue and CD16-FITC (for band 2) and CD11b-PE and CD16 (for band 1) antibodies for 30 minutes at 4^0C. Matched isotype controls were also put up simultaneously. Following

incubation cells were washed twice with HBSS and acquired by flow cytometer (FACS Calibur, Becton Dickinson, USA).

4.6 Free radical generation from neutrophil

4.6.1 Flow cytometry (DCF-2DA)

Human neutrophil ($2x10^6$ cells) were incubated with vehicle or various interventions [DPI (10μM), ABAH (100μM), N-acetyl-L-cysteine (NAC, 5mM)] for 5 min at 37°C, loaded with DCF-DA, (10μM) for 5 min, and finally diethylenetriamine NONOate (DETA-NO, 1μM)-1mM] was added to induce the free radical generation, which was assessed by FACS Calibur (Becton Dickinson, USA) **(Sharma *et al*, 2004)**.

4.6.2 Western blotting (DMPO nitrone adduct)

Neutrophil ($2x10^6$cells/ml) were incubated with vehicle or various interventions [DPI (10μM), ABAH (100μM), N-acetyl-L-cysteine (NAC, 5mM)] for 5 min at 37°C and finally SNAP (100μM), DETA-NO (100μM) and PMA (50nM) was added to the suspension and incubated for 30 minutes, 1hr., 2hrs. and 3hrs at 37^0C on rocking. 50mM DMPO (5, 5-dimethyl-1-pyrroline-N-oxide) was added to each tube and again incubated at 37^0C for 5 min. Reaction was stopped by pelleting (1000g, 5 minutes at RT) followed by adding ice cold stoping buffer for 5min at 4^0C. Cells were lysed by ultrasonicator for 1 min with 30 duty cycle and 5 sec pulse at 4^0C followed by centrifugation at 13000g for 10min at 4^0C. The supernatant was used as whole cell protein lysate. Protein concentration was quantified using protein assay reagent (Pierce). Equal amount of protein samples (30μg) were heated with Laemmli sample buffer at 95^0C for 10min and quickly chilled on ice. Samples were centrifuged at 15,000rpm for 5min and then resolved onto 10% SDS-PAGE. The components of polyacrylamide gel have been mentioned in **Table 7.**

Table 7 - Composition of polyacrylamide gel

Components	8% resolving gel (for 5 ml)	10% resolving gel (for 5 ml)	12% resolving gel (for 5 ml)	5% stacking gel (for 2 ml)
Deionized water	2.3ml	1.9ml	1.6ml	1.4ml
30% acrylamide mix	1.3ml	1.7ml	2.0ml	0.33ml
1.5M Tris-Cl (pH 8.8)	1.3ml	1.3ml	1.3ml	
1.0M Tris-Cl (pH 6.8)				0.25ml
10% SDS	50µl	50µl	50µl	50µl
10% APS	50µl	50µl	50µl	50µl
TEMED	3µl	2µl	2µl	2µl

Samples were run on the chilled SDS-running buffer using Mini electrophoresis cell (Amersham). SDS-PAGE was run at 70 Volts till the dye entered in the stacking gel and then at 100 Volts to separate proteins in resolving gel. PVDF membranes were activated in methanol for 30 seconds. Membrane was placed over the gel and assembled in gel transfer cassettes (Amersham) to transfer the separated proteins from gel on to the membrane. Cassettes were fitted into the electrophoresis module and transfer was performed at constant current of 150mA for 4 hrs. in chilled transfer buffer. To detect the transferred proteins on the membrane, membranes were washed once in deionized water and dipped in 0.2% Ponceau-S stain. Membranes were thoroughly washed with TBST, pH 7.5 and blocked overnight with 5% skimmed milk (in TBST) at 4^0C. Subsequently, membranes were washed 3 times with TBST. Membranes were incubated with Anti-DMPO antibody (1:3000 in 1.5% skimmed milk TBST) for 2 hours at RT. After 10 times washing with TBST (5 min each) membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:20000 in 1.5% skimmed milk TBST for 2 hours). After further washes, bound antibody was revealed by enhanced chemiluminescence detection reagents (Millipore) and blots were exposed to the Hyperfilms (Amersham Pharmacia Biotech) **(Detweiler *et al,* 2002).** Blots were stripped by incubating the membrane at 65^0C for 20 min in stripping buffer with shaking. Membrane was washed thoroughly with TBST and reprobed with β-actin antibody.

4.7 Neutrophil activation and sub-cellular fractionation

Human neutrophil (10^7cells/ml) were treated with PMA (50nM) and SNAP (100μM) for 30min., 1hr, 2hrs., 3hrs. at 37^0 C in dry incubator with continuous shaking. Reactions were stopped by adding ice-cold reaction stopping buffer. Neutrophils were then centrifuged (1000g; 5 min; 4°C), and resuspended in relaxation buffer. 5mM DFP (di-isopropyl fluorophosphates) was added to relaxtion buffer and incubated at 4^0C for 10min. The cells were then disrupted by five 15-s cycles of sonication at 4°C using a ultra-sonicator (Ultrasonics, W-385). Unbroken cells and nuclei were pelleted by centrifugation at 500 g for 10min at 4°C. The obtained supernatants were further centrifuged twice at 10,000 *g* for 5 min at 4°C and thereafter, subjected to an ultracentrifugation at 100,000 *g* for 1hr. at 4°C. Supernatant obtained was referred as cytosolic fraction, it was mixed with Laemmli sample buffer. The membrane fraction (pellet) was resuspended in relaxation buffer and centrifuged again at 100,000 *g* for 30 min at 4^0C. The pellet thus obtained represented membrane fraction, it was resuspended in ice-cold relaxation buffer having 1% Triton X-100 on ice for 1hour before Laemmli sample buffer was added **(Forsberg *et al*, 2003).**

4.8 p47 phox subunit migration

20 μg of both cytosolic and membrane proteins were separated on 10% SDS PAGE and transferred on PVDF membrane. Overnight blocking of membrane was done with 3% skimmed milk in TBST (pH 7.4) solution at 4^0C followed by 5 times washing and further incubation with anti p47 (1:500 in 1.5% skimmed milk TBST) for 2 hours at RT. After 10 times washing with TBST (5 min each) membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG diluted 1:20000 in 1.5% skimmed milk TBST for 2 hours. After further washes, bound antibody was revealed by enhanced chemiluminescence detection reagents. Membrane was stripped and reprobed with β-actin antibody.

4.9 2D SDS-PAGE for nNOS and iNOS protein in human neutrophil

Immediately after immunoprecipitation (by using nNOS or iNOS antibodies) purified proteins were resuspended in a urea lysis buffer and stored at 4^0C for 5min.

Samples were then centrifuged at 21,000g for 15 min at 4^0C. Supernatant was taken and mixed with rehydration solution containing IPG buffer and centrifuged at 21,000g for 15 min at room temperature. Supernatant was loaded onto a 11 cm IPG strip, pH 3–10 and left for overnight at room temperature. Isoelectric focusing (IEF) was performed with a low initial voltage and then by applying a voltage gradient up to 8000 V with a limiting current of 50 μA/strip. The strips were then equilibrated with equilibration buffer and loaded onto the second dimension using 0.5% agarose solubilized in cathode buffer for the interfacing and run on 8% SDS-PAGE followed by Silver staining.

4.10 Interaction between iNOS and Rac2

4.10.1 Co-immunoprecipitation

To study the physical interaction of iNOS and Rac2, neutrophil lysate (750μg) was incubated with 1μg of Rac2 antibody for overnight at 4°C followed by incubation with 40 μl Protein A sepharose for 1 h at 4°C. The immunoprecipitate was collected by centrifugation at 13000g for 5 min at 4°C, and washed three times with 1 ml RIPA buffer and once with wash buffer. Protein precipitated by Rac2 antibody was separated by 8% SDS-PAGE, and transferred on to PVDF membrane. Overnight blocking of membrane was done with 3% skimmed milk in TBST (pH 7.4) solution at 4^0C followed by 5 times washing and further incubation with iNOS Ab (1:500 in 1.5% skimmed milk TBST) for 2 hours at RT. After 10 washings with TBST (5 min each), membrane was incubated with horseradish peroxidase-conjugated anti-mouse (for anti-iNOS) diluted 1:20000 in 1.5% skimmed milk TBST for 2 hours. After further washes, bound antibody was revealed by enhanced chemiluminescence detection reagents (Millipore) and blots were exposed to the Hyperfilms (Amersham Pharmacia Biotech).

4.10.2 Co-immunolabeling

To explore the interaction between iNOS and Rac2 in neutrophil, cells were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS (pH 7.4) at 25^0C for 30 min and washed twice for 5 min each with PBS containing 0.5% (w/v) glycine. The washed cells were allowed to adhere on 0.01% (w/v) poly-L-lysine coated cover slips, permeabilized with 0.2% (v/v) Triton X-100 (5 min) and blocked with 10% (v/v) goat serum in PBS for 30

min. Cells were incubated overnight at 4^0C with iNOS and Rac2 antibodies at a dilution of 1:200 and subsequently stained with alexa flour 488 secondary antibody (1:500), at 4^0C for 4 h in dark. Nuclei were stained with Hoechst 33258 dye (3 μg/ml) at 25^0C for 15 min. Cover slips were mounted in the mounting medium (Oncogene, CA, USA) and images were acquired under NIKON Eclipse TE 300 Confocal Microscope using 63 X 1.4 NA Plan Apochromate lens. Adobe Photoshop software was used for further analysis and presentation of images. Control samples were processed similarly as mentioned above, omitting the primary antibody **(Saini *et al*, 2006)**.

4.11 Translocation of iNOS and Rac2

To assess the translocation of Rac2 and iNOS from the cytosol to neutrophil membranes, cytosol and membrane fractions were prepared from cells ($1X10^7$cells) pre-treated with PMA (50 nM) or vehicle at 37^0C for 30 min **(Forsberg *et al*, 2003)**. Cytosol and membrane fractions were immunoprecipitated with iNOS antibody. The immune complex was resolved on 8% SDS-PAGE and subsequently transferred to PVDF membrane. For Rac2 migration, 20 μg of cytosol and membrane fractions were run on 10% SDS–PAGE. The membrane was blocked overnight, incubated with iNOS and Rac2 antibody (1:500 in 1.5% skimmed milk TBST) for 2 h at room temperature. Both proteins were detected after incubation with HRP-conjugated anti mouse (for iNOS) and anti rabbit (for Rac2) IgG (1:20,000) for 2 h by enhanced chemiluminescence detection reagents (Millipore, USA).

4.12 NOS and Rac2 labeling in NETs

To study the presence of nNOS, iNOS and Rac2 in NETs, cells were fixed in 4% (w/v) PFA in PBS (pH 7.4) at 25^0C for 30 min and washed twice for 5 min each with PBS containing 0.5% (w/v) glycine. The washed cells were allowed to adhere on 0.01% (w/v) poly-L-lysine coated cover slips, permeabilized with 0.2% (v/v) Triton X-100 (5 min) and blocked with 10% (v/v) goat serum in PBS for 30 min. Cells were incubated overnight at 4^0C with nNOS, iNOS and Rac2 antibodies at a dilution of 1:200 and subsequently stained with alexa flour 488 secondary antibody (1:500), at 4^0C for 4 h in dark. Nuclei were stained with Hoechst 33258 dye (3 μg/ml) at 25^0C for 15 min. Cover

slips were mounted in the mounting medium (Oncogene, CA, USA) and images were acquired under NIKON Eclipse TE 300 Confocal Microscope using 63 X 1.4 NA Plan Apochromate lens. Adobe Photoshop software was used for further analysis and presentation of images. Control samples were processed similarly as mentioned above, omitting the primary antibody **(Saini *et al*, 2006)**.

4.13 Stimulation of extracellular traps formation

Neutrophil were seeded on 0.001% poly-L-lysine coated plate and allowed to settle down for 30 min. Then treated with DETA-NONOate (100μM) and PMA (50nM) or left unstimulated for 3hrs. Effect of NADPH oxidase/NOS (DPI; 10 μM) and MPO inhibitor (ABAH; 100μM) was monitored on NETs release by incubating with neutrophil for 15 min at 37°C and then treated with DETA-NONOate for 3 hr. Neutrophils were fixed with 4% PFA, washed with PBS and stained with Propidium iodide and extracellular trap formation was counted. The percentage of NETs formed was calculated by quantifying the number of neutrophil forming NETs out of the total number of neutrophil observed under 40 high-power magnification fields. The NET was defined as a discrete area of red fluorescence larger in size than neutrophil **(Fuchs *et al*, 2007)**

4.14 RNA isolation

RNA was isolated from human blood cells, bands 3, 2, and 1 by using Tri reagent by a modified method of **Chomczynski *et al*, (1987)**. Briefly, cells ($5x10^6$) were lysed in 0.8 ml of tri reagent by repeated pipetting and kept at room temperature for 5 min. Chloroform (0.2 ml) was added and shaken vigorously for 15 seconds followed by an incubation of 3 min at room temperature to precipitate the proteins. The samples were centrifuged at 12000g for 15 minutes at 4°C (Biofuge Stratos, Heraeus, Germany). The aqueous phase containing RNA was aspirated into a different tube where 0.5 ml of isopropanol was added to precipitate the RNA. The samples were incubated for 10 min at room temperature and subsequently centrifuged at 12000g for 15 min at 4^0C (Biofuge Stratos, Heraeus, Germany). RNA pellet was washed with 75% alcohol by centrifuging at 7500g for 5min at 4^0C. The RNA pellet was finally resuspended in 20 μl DEPC water. RNA was quantified and subjected to RT-PCR.

Quantification of RNA

The quantity of RNA was determined by measuring absorbance (OD) at 260 nm. Where, 1OD was equivalent to 40 µg/ml (for single stranded RNA).

Gel preparation

0.5 g agarose was dissolved in 5 ml of 10x MOPS (pH 7.0) and 36.5 ml of water was added, solution was boiled and further kept for cooling at 55^0C. Then 8.5 ml of formalin and ethidium bromide (5µg/ml) was added to the gel mix.

RNA sample preparation

RNA samples were prepared for electrophoresis as below:

RNA	4.0 µl (2-3 µg)
DEPC water	1.0 µl
6X loading dye	1.0 µl

Samples were loaded and gel was run at 3 V/cm in 1x MOPS buffer, till the dye reaches 2/3 of the gel. Gel was visualized under UV and the image was captured using gel doc system (Bio-Rad)

4.15 cDNA synthesis

cDNA was synthesized using Revert aid H^- first strand cDNA synthesis kit (Fermantas) as per manufacturer's protocol. In brief the reaction mixture for cDNA was prepared as follows:

Template RNA	5 µg
Oligo dT primer	1 µl
DEPC treated water	up to 12 µl

- The reagents were mixed gently and spun down for 3-5 second in a micro centrifuge. The mixture was incubated at 70^0 C for 5 min, chilled on ice and drops were collected by light centrifugation
- The tube was placed on ice and the following component were added in the indicated manner

5X RT buffer	4 µl
dNTP mix (10 mM)	2 µl
RNAse inhibitor	1 µl
RT enzyme	1 µl

- The reagents were mixed gently and drops were collected by light centrifugation
- The mixture was incubated at 42^0 C for one hour.
- The reaction was stopped by heating at 70^0 C for 10 min and then chilled on ice. The prepared cDNA was used for carrying out PCR.

4.16 Primers

To explore different isoforms of NOS in human RBCs, Platelets, PBMCs and neutrophil, gene specific primers were used. Following primers were used for the present study **(Table 8)**.

Table 8 - Primers and PCR conditions for amplification of different genes.

Gene	Primers sequence (5'-3')	PCR conditions	Reference
nNOS	F - TCTAACAGGCTGGCAATGAAG R - TCTCTAAGGAAGTGATGGTTGAC	95°C for 5min 95°C for 15s 57°C for 45s 72°C for 30s 72°C for 10min	**Zhong *et al*, 2008**
iNOS	F - GTTCTCAAGGCACAGGTCTC R - GCAGGTCACTTATGTCACTTATC	95°C for 5min 95°C for 15s 55°C for 45s 72°C for 30s 72°C for 10min	**Zhong *et al*, 2008**
eNOS	F - ATTATATCCTACACAAGACTCCAG R - TCTTCAAGTTGCCCATGTTAC	95°C for 5min 95°C for 15s 56°C for 45s 72°C for 30s 72°C for 10min	**Zhong *et al*, 2008**

β-actin	F - AACTGGAACGGTGAAGGTG R – CTGTGTGGACTTGGGAGAGG	95°C for 5min 95°C for 15s 56°C for 45s 72°C for 30s 72°C for 10min	**Quattrone *et al*, 2004**
Testicular Exon 1-4	F – TAGGTGGGGGTTGAGAAATG' R - CAATGTGCTCTTAAGGTGG	95°C for 5min 95°C for 15s 45°C for 45s 72°C for 30s 72°C for 10min	**Wang *et al*, 1997**
Neuronal Exon 1d-2	F – ATGCCAGGGTGAG R – CGTCTGATGGCTGGTCTAGAAGT	95°C for 5min 95°C for 15s 52°C for 30s 72°C for 30s 72°C for 10min	**Bros *et al*, 2006**
Neuronal Exon 1f-2	F – CCGAGCGGACGGGCTC	95°C for 5min 95°C for 15s 52°C for 30s 72°C for 30s 72°C for 10min	**Bros *et al*, 2006**
Neuronal Exon 1g-2	F – TGCCCGGCTCGCCGT	95°C for 5min 95°C for 15s 52°C for 30s 72°C for 30s 72°C for 10min	**Bros *et al*, 2006**
Neuronal Exon 1i-2	F – GTTGCCAGTGCAGCATCT R – GGAAGTGATGGTTCACCAGG	95°C for 5min 95°C for 15s 52°C for 30s 72°C for 30s 72°C for 10min	**Wang *et al*, 1999**
Neuronal Exon 2-3 (PDZ domain)	F - ACCAGAGTCAGCCTCCAA R – GCACATTGCCCTTCCCCC	95°C for 5min 95°C for 15s 45°C for 45s 72°C for 30s 72°C for 10min	**Wang *et al*, 1999**

4.17 Real-Time PCR

The cDNA was amplified in PCRs. The primers used for nNOS, iNOS, eNOS **(Zhong *et al*, 2008)** and β-actin **(Quattrone *et al*, 2004)** amplified a 165-bp, 201bp, 160bp and 208bp products respectively. Real-time RT-PCR was performed with a Maxima SYBR Green RT-PCR Kit (Fermantas) on Roche light cycler. Reactions were 25-μL volumes including 12.5 μL of 2× Maxima SYBR Green RT-PCR Master Mix, 1 μL of cDNA template, and 0.2 μmol/L primers that were designed to amplify a part of each gene. The three-step PCR protocol applied for nNOS reactions consisted of 45 cycles of 95°C for 15 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, for iNOS consisted of 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds and for eNOS consisted of 45 cycles of 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. After PCR, a melting curve analysis consisting of 1 cycle: 95° C for 0 s, 70° C for 10 s, 95° C for 0 s, Cooling 1 cycle: 40°C for 3 min was performed to demonstrate the specificity of the PCR product as a single peak. A control, which contained all the reaction components except for the template, was included in experiment. The amplification reactions for β-actin 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.

4.18 Immunoprecipitation (IP) and Western blotting

Blood cells (750 μg protein) and Band1, Band2, Band3 cells were suspended in ice-cold RIPA buffer with 5mM DFP (di-isopropyl fluorophosphates) and incubated at 4^0C for 10 min. Cells were lysed with 1% Triton X 100 and 0.1% SDS at 4°C for 30 minutes. Immunoprecipitation was carried out with using 1μg of nNOS, iNOS and eNOS antibodies **(Saini *et al*, 2006)**. Western blotting was performed by using primary (1:500 in 1.5% skimmed milk TBST) and HRP-conjugated anti-rabbit IgG (for nNOS and eNOS) and anti-mouse (for iNOS) secondary antibodies (1:20000 in 1.5% skimmed milk TBST) followed by chemiluminescence detection (Millipore). β-actin was used to normalize the NOS expression by 1:25 dilution of total protein taken for NOS immunoprecipitation to avoid the dispersion of the band which otherwise would hinder in densitometry analysis.

4.19 Assessment of NO generation by flow cytometry

Flow cytometry is an effective tool to assess the real time generation of NO in live cells. NO production in different blood cells and Band1, Band2, Band3 was explored using a NO probe, DAF-2DA (**Sharma *et al*, 2003**) by flow cytometry. Band cells were incubated for 30 min with DAF-2DA (10 μM). Blood cells were incubated with NOS inhibitors (L-NAME 10 μM, Vinyl-L-NIO 100 μM or 1400W 100μM) in the presence and absence of NOS cofactors (BH_4 10μM, NADPH 1mM, FAD 5μM, FMN 25μM, L-Arg 0.15 μM, Ascorbate 1mM) and then with DAF-2DA (10μM). DAF-2DA reacts rapidly and irreversibly with NO to produce a highly reactive fluorescent product triazolo fluorescein (DAF-2T). Different cells were gated according to their distinct size and internal complexity using forward (FSC) and side (SSC) scatter characteristics. Response of at least 10,000 cells from each sample was acquired and analyzed by Cell Quest program (Becton Dickinson, USA). Results have been represented as shifts in the mean fluorescence corresponding to the increase in NO generation. Cells without DAF-2DA treatment were used as negative control.

4.20 Statistical analysis

Data have been represented as Mean ± SEM, from at least 5-10 independent experiments in each group. Comparisons between two groups were performed by unpaired "t" test and statistical analysis between multiple groups was performed by one-way ANOVA followed by Newman-Keul's post analysis test. Data were considered significant at $p<0.05$.