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Functionality, membrane integrity and rheological properties are altered in red blood cells of smoking pregnant mother

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Abstract

Introduction: Decrease in the bioavailability of vasoactive nitric oxide (NO), derived from the endothelial nitric oxide synthase (NOS3), underlines vascular endothelial damage. Our expanding knowledge on adult red blood cells (RBCs) makes it supposable that RBCs might contribute to vascular function and integrity via their active NO synthetizing system (RBC-NOS3). This “rescue” mechanism of RBCs could be especially important during pregnancy with smoking habit, when smoking acts as an additional stressor and cause active change in the redox status.

Methods: In this study RBC populations of 143 non-smoking (RBC-NS) and 131 smoking (RBC-S) pregnant mothers were examined. Morphological variants were followed by confocal microscopy and quantified by a microscopy based intelligent analysis software. Fluorescence activated cell sorting was used to examine the translational and posttranslational regulation of RBC-NOS, Arginase-1 and the formation of the major product of lipid peroxidation, 4-hydroxy-2-nonenal. To survey the rheological parameters of RBCs like elasticity and plasticity atomic force microscopy-based measurement was applied.

Results: Significant morphological and functional differences of RBCs were found between the non-smoking and smoking group. The phenotypic variations in RBC-S population, even the characteristic biconcave disc-shaped cells, could be connected to impaired NOS3 activation and are compromised in their physiological properties. Membrane lipid studies reveals an elevated lipid oxidation state well paralleled with the changed elastic and plastic activities.

Conclusion: These features can form a basic tool in the prenatal health screening conditions, hence the compensatory mechanism of RBC-S population completely fails to sense and rescue the acute oxidative stress conditions.

Implications:

Under the influence of cigarette smoke RBCs become a source of reactive oxygen and nitrogen species and lose their characteristic structural and functional features. RBCs could be functionally impaired, far before their detectable morphological alterations. In case of endothelial dysfunction, consequently the functional loss in the RBC-NOS3 NO production is unavailable as a compensatory mechanism. Moreover, the changed protein expression profile might even augment and synergizes the development of vascular dysfunction/comorbidities

Introduction

Intensive tobacco smoking stands as a major etiological factor for the wide spectrum of cardiovascular morbidities.[1,2] Due to the socio-economic lifestyle burden, current meta-analysis data clearly depicts around 20-30% of the adult women population are addicted to tobacco smoking, even under full term pregnancy.[3,4] Pregnancy is a physiological state associated with an enhanced metabolism and demand for O_2, which may lead to the overproduction of reactive oxygen species (ROS). In general, chronic smoking habit causes redox homeostasis imbalance. Sustained smoking under the state of pregnancy acts as an additional stressor with increment in the cellular ROS levels. From the clinical purview, several obstetric complications like spontaneous miscarriage, preterm delivery, preeclampsia, ectopic pregnancy, intrauterine growth restriction, placental abruption, perinatal death etc. already has been reported as an impact of smoking during pregnancy.[5–7] The physiological levels of cellular ROS regulates various signalling pathways in the female reproductive system, but augmented intrinsic and extrinsic reactive entities leads to an imbalance between the pro-oxidant and antioxidant levels causing oxidative stress conditions.[8] Smoking sharply triggers the endogenous metabolic reactions with excess release of ROS, like superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO−). These factors majorly paralyze the vascular endothelial nitric oxide (NO) producing pathway.

Recent reviews emphasized on the low systemic bioavailable level of the key vasoactive regulator NO, along with other inclusive factors to the acute oxidative stress conditions.[9] NO in the endothelial cells is majorly synthesized by post translational modifications of endothelial nitric oxide synthase (NOS3).[10] The activation of NOS3 involves three key stages like dimerization, membrane dislocation and post translational modification of NOS3 in their regulatory pathway to release the bioavailable NO. The interplay between different signalling molecules like tetrahydrobiopterin, NADPH, FAD *etc.* as cofactors and common substrate L-arginine concentration for both Arginase-1 and NOS3 have major influence on NOS3 active dimer state.[11]

Red blood cells (RBCs) play an important role in tissue oxygenation and regulating blood pressure. Since long period of time RBCs were considered as a sink for endothelium-derived NO, synthetized by NOS3, to limit the bioavailable NO for vasodilation. Our understanding of RBC's biological function has expanded with ground-breaking data *i.e*. RBCs themselves are able to synthetize and release bioactive molecules including NO, NO metabolites and ATP that makes them a vital sensor entity under any vascular/endothelial

dysfunction.[12,13] The responsiveness of RBCs under any vascular pathological condition, via the RBC-NOS function, defines a notable alternative pathway to regulate the level of the vasodilatory bioavailable NO with its effect in the blood rheology, tissue protection and vascular homeostasis.[14]

RBCs' high plasticity or deformability property allows them to dynamically adapt to the continuous changing flow conditions along the vascular system. Any alteration in the redox state of RBCs can directly modulate their hemorheological properties, so increased formation of oxidants and NO are important indicators of various pathological processes effecting the vascular hemodynamic profile.[15]

The aim of this study was to recognize smoking induced molecular and functional alterations in the RBC population during pregnancy and assuming these parameters as the blueprints for vascular conditions.

Materials and Methods

Human samples

In compliance with the rules and regulations as per the Declaration of Helsinki, informed consents were taken from healthy pregnant heavy smoker volunteers (n_S=75) (with at least 10 cigarettes per day) and non-smoker healthy pregnant women as the control (n_{NS}=82), to withdraw blood from their peripheral veins. Blood samples were collected in the Department of Obstetrics and Gynaecology at the University of Szeged, Hungary. The Ethics Committee of the Department of Obstetrics and Gynaecology approved our study protocol (16/2014). The study protocol excluded pregnant women with complicated pregnancy terms, clinical history of infection or inflammatory conditions, comorbidities like cardiovascular diseases, diabetes mellitus, malformations or evidence of genetic disorders and aged below 18 years. The nutritional status of the mothers during pregnancy was satisfactory; no cases of malnutrition was reported. The blood samples were subjected to centrifuge at 200g for 10 min at 4 °C, where only the lower two-third portion of the RBC phase was collected. The RBCs were washed twice with 2 volumes of isotonic saline solution at pH 7.0. The RBC population was immunostained with RBC specific mouse anti-Glycophorin A antibody to test the sample homogeneity. Purity of the samples was > 95% for the isolated RBC preparation. Fresh samples were immediately processed for morphological analyses, immunocytochemistry and Atomic Force Microscopic studies. For classical biochemical studies aliquots were stored at -80 °C until processing.

Immunohistochemistry with Fluorescence activated cell sorting (FACS) and Microscopic Analysis

RBCs were fixed and stained as per the indicated standard protocol. Generally, cells were fixed in 4% (w/v) paraformaldehyde (PFA) in 0.05 M phosphate-buffer (PB) with pH 7.0 for FACS analysis, at 4°C for 60 min. After washing with PB cells were permeabilized at room temperature for 30 minutes using 0.1% Triton-X100. To block nonspecific binding, 4% bovine serum albumin and 5% normal goat serum in PB were used. RBCs were immunolabelled with primary antibody in single or in combination staining and kept overnight at 4 °C. On the following day samples were washed and further incubated with conjugated secondary antibodies for 2 h at room temperature. After washing, RBCs were either processed for quantitative analysis (FACS, BD FACSCalibur™, BD Biosciences) or were mounted in Immunohistomount (Sigma Aldrich, Saint Louis, Missouri, USA) and

examined under confocal laser-scanning microscope (Zeiss LSM 880, Axiocam 503 mono, 40x oil immersion objective, numeric aperture: 1.4 (Carl Zeiss Microscopy GmbH, Germany). Eight bit pictures were taken by ZEN 2.1 (black) software (Carl Zeiss Microscopy GmbH 1997-2015) and analysed by Image J 1.51n and ZEN 2.1.

Determination of Annexin V binding

Cell apoptosis rate was assessed by Annexin V-FITC/PI apoptosis detection kit (Abcam, ab14085) as referred. Briefly, 1×10^5 RBCs were collected and re-suspended in 500 µl 1 × Annexin V Binding Buffer. Then cells were double-stained with 1/100 volume of Annexin V-FITC and Propidium Iodide for 10 min at room temperature in the dark, followed by flow cytometry quantification (BD Biosciences, San Jose, CA, USA), using FITC signal detector.

Determination of $ONOO^-$ level

To determine the $ONOO^-$ levels, spectrophotometric measurements were performed at 302 nm, using GENESYS 10S UV-Vis spectrophotometer (Thermo Fischer Scientific, Madison, WI, USA). The hemolysate of each samples were diluted into 1 M NaOH solution in a ratio of 1:250. The increase of absorbance was measured until it reached a stable equilibrium, then samples were added into 100 mM PB (pH-7.4) in the same ratio as a reference. On this neutral pH, the $ONOO^-$ decomposed, and the decrease in absorbance was observed till the equilibrium point.[16] The final results were normalized with protein concentration (µmol/mg protein).

Atomic force microscopy

Instrumental specifications:

All elasticity experiments were carried out with Asylum Research MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA; driving software IgorPro 6.32A, Wavemetrics). Cells were mounted on a Zeiss Axiovert 200 optical microscope for initial positioning. The experiments were performed with a V-shaped tip that was mounted on a gold coated silicon nitride rectangular cantilever (BL RC150VB-A), the nominal spring constant was kept at 30 pN/nm and resonant frequency at 37 kHz during the procedure. Freshly prepared RBC solution of 20 µl was dropped onto 1mg/ml PEI coated Petri Lid surface. Measurements were carried out in 0.9% NaCl solution. In each case, 15x15 μm^2 area was selected, divided into 40 lines by 40 points with a force distance curve recorded at each point.

Each force-distance curve was recorded at 8μm/s speed, having maximal load of 0.5nN and 2kHz sampling frequency.

Image processing and data analysis

Eosin stained smears' image processing was done in the scientific calculation software MatLab. All the images went through quality control as a pre-processing step in the initial phase of the analysis, which consisted of illumination correction using the MatLab tool CIDRE[17] and exclusion of poor quality ones.

An intelligent analysis software, Advanced Cell Classifier[18] (ACC, available on http://cellclassifier.org) was utilized to (automatically) identify and populate distinct phenotypes present in the data. Based on such machine learning method, all cells were predicted with a given phenotype. In data analysis, the reports created in ACC provided insights to the distribution of each phenotype of interest compared to the control, along with their indicated frequency.

Statistical Analysis

All statistical analysis was calculated with one-way analysis of variance (ANOVA) (GraphPad Statistical Software version 4.0) using Newman-Keuls multiple comparison test. Significant differences were accepted at *$p<0.05$, **$p<0.001$, *** $p<0.001$and ****$p<0.0001$. In exception, atomic force microscopy data was analysed using Kruskal-Wallis rank test at *$p\leq0.05$.

Results

Mapping the NOS3 activation pattern in the phenotypic RBC variants

Variations in shape and size of RBCs serve as a preliminary platform for many clinical diagnoses. Altogether more than 20000 RBCs, collected from adult smokers (RBC-S; n= 13058 cells/15 independent samples) and non-smoker (RBC-NS; n=9173 cells/12 independent samples), were screened, using the Advanced Cell Classifier, an intelligent bio-image analytical tool. Three major phenotypic variations were followed; 1. regular biconcave shaped "healthy looking" cells, 2. echinocytes with spiny projections and 3. dacrocytes with a teardrop shape. A significantly increased number of echinocytes was detected in the RBC-S population (~2 fold), while the frequency of teardrop-like cells exhibited an elevated tendency, but this increase was not significant (Supplementary Figure 1A, B).

Recently, a new "erythrocrine function" of adult human RBCs was proposed, playing role in the liberation of bioavailable NO. Anti-NOS3/p-NOS3 double-labelled RBCs were analysed under confocal microscope, to interrelate the impaired NOS3 functionality with the varying phenotypes. Using the Image J software tool, we analysed middle three slices across the Z-stack images of the biconcave shaped cells from RBC-NS, RBC-S and those of the RBC-S echinocytes. The images were quantified for NOS3 (Figure 1A) and p-NOS3 (Figure 1B) intensity levels. No changes in the NOS3 intensity were found between RBC-NS and RBC-S populations, regardless of their phenotypes. However, compared to the RBC-NS population, significant reduction in the phosphorylated state of NOS3 were detected within RBC-S sub-populations; 20% in the regular biconcave shaped cell and 44 % in the echinocytes. As a consequence, the ratio of NOS3/p-NOS3 intensity increased significantly by 1.5 fold in the biconcave shaped cells and 2.0 fold in the echinocytes with S origin (Figure 1C).

In parallel with microscopic examination, the anti-NOS3/p-NOS3 double-labelled cells were also quantified by FACS analysis. During the evaluation of the histograms, an intensity value of 10^2 was set as a borderline between the basal and high NOS3 expressing populations (Supplementary Figure 2A-C). The basal and high NOS3 intensity and frequency remained unaltered in the RBCs with S origin. However, a significantly lower levels of phosphorylation were detected in both the basal and high NOS3 expressing populations, a 4- and 3-fold reductions, respectively (Supplementary Figure 2D-G).

Increase in the Arginase-1 level

To evaluate the alterations that may underlie the difference in NOS3 activation between groups RBC-NS and RBC-S, Arginase-1 and NOS3 double stained RBCs were analysed for frequency and intensity distribution by FACS analysis (Figure 2). In general, Arginase-1 expression was low in the RBC-NS populations, there was a significant 2.5-3-fold difference in the intensity levels between the two groups (Figure 2A). The distribution of the basal and high level Arginase-1 expressing cell was 90 and 10% in the RBC-NS population, respectively (Figure 2B). On the other hand, in the RBC-S group, 64% of the cells showed high intensity of Arginase-1 (Figure 2B), and around 90 % of this high Arginase-1 expressing cells expressed low level of NOS3 (Figure 2C and Supplementary Figure 3A-C).

Elevated pro-oxidant-, lipid peroxidation- and Annexin V-positivity- level in the smoking population

Sustained smoking tends to enhance the intrinsic pro-oxidant content. We measured the level of peroxynitrite ($ONOO^-$), one of the strong oxidant formed *in vivo*. A significant, 1.5-fold increase was detected in the RBC-S population (Figure 3A).

In agreement with the elevated ROS production, we detected a significant increase in the lipid oxidation. 4-hydroxynonenal (4-HNE), a known product of lipid peroxidation, readily forms an adduct with protein residues, resulting in a signal specific response. The isolated RBCs were double stained with anti-NOS3 and anti-4HNE antibodies and were subjected to FACs analysis to follow the extent of lipid peroxidation. Within the total NOS3 expressing cells in the RBC-S group there was a 5-fold increase in the 4-HNE intensity, compared to the RBC-NS (Figure 3B). The 4-HNE level in the basal and high NOS3 expressing cells was 3- and 2-fold higher than in the matching RBC-NS population respectively (Supplementary Figure 4A-C).

The increased frequency of echinocytes and lipid peroxidation indirectly signifies a loss of membrane integrity and function in the RBC-S population. By Annexin-V staining we could detect and quantify the level of phosphatidylserine in the outer leaflet of RBC plasma membranes. The frequency of Annexin-V positive cells in the RBC-S population was significantly higher (~ 24%), than the RBC-NS group (~17%) (Figure 3C and Supplementary Figure 5A,B).

Determination of rheological parameters of RBCs by Atomic Force Microscopy

There are existing limitations and challenges to measure the parameter of deformability in the RBCs. The very direct and robust technique available to determine the nanomechanical properties is Atomic Force Spectroscopy (AFM). More than 500 cells from RBC-NS and the RBC-S populations were screened to distinguish the nanomechanical characteristics. The indenting (Elastic Work) and remanent energies (Plastic Work) were calculated, using a custom-made MatLab® routine on AFM data. The ratio of Plastic Work / Elastic work defines the Elastic Index (EI). The extent of the Elastic Work remained almost equal for RBCs derived from either origin but the extent of Plastic work significantly lowered in the cells with S origin; it was about half of the value measured in RBC-NS. Cumulative ratios of both the Elastic and Plastic Work, the EI, represents significant ~1.5-fold decrease in the RBC-S with respect to the RBC-NS population (Figure 4A,B and Supplementary Figure 6A-D).

Discussion

Smoking induced endothelial dysfunction highlights the vitality of RBCs to sense and respond under varying physiological and pathological conditions. It is especially important during pregnancy where there is a wide increase (20-30 %) in the RBC mass to meet the physiological demand. We believed that RBCs would reflect to the effects of harmful substances that originated from an improper maternal lifestyle and can serve as a real-time sensor for the overall vascular micro-environmental status. Data in this study illustrate the underlying molecular mechanisms of the morphological, functional and physiological alterations in response to *in vivo* oxidative stress conditions. We provided evidence that 1. RBCs could be functionally impaired, far before their detectable morphological alterations; 2. the NOS3 phosphorylation and Arginase-1 expression level inversely proportional in RBCs of smoking mothers; 3. RBCs from smoker origin become a source of reactive oxygen species in parallel with increased rate of lipid peroxidation and apoptosis, and lastly 4. we demonstrated an altered plastic work activity in correlation with the lessened rheological properties.

The activation pathway of NOS3 can be influenced at many points. One of the critical steps is the phosphorylation at Ser1177 residue via phosphatidylinositol-3 (PI3) kinase and the downstream serine/threonine protein kinase Akt pathway.[11,19,20] In the majority of the cells from smoking origin showed no impact on NOS3 expression level, while the phosphorylation at Ser1177 position was significantly reduced. Importantly, the alterations were regardless of the phenotypic appearance; not only the echinocytes, but the regular, biconcave disc-shaped cells were also affected. The recognition of the functional loss of RBCs, against the conventional way of analysing the phenotypic variations, can serve as an early alert for changes in vascular environment.

Endothelial cells effectively respond to cigarette smoke induced pathological elevation of inflammatory agents, hypoxia and ROS in the vascular environment, by triggering the Arginase-1 activation.[21–23] Arginase-1 competes with NOS3 for their common L-arginine substrate, that simultaneously retards the bioactive NO production with an accelerated generation of superoxide ($O_2^{\cdot -}$). Under the influence of cigarette smoke L-arginine proposed to be preferentially metabolized by the Arginase-1 pathway, producing increased level of urea and L-ornithine, and L-ornithine derived polyamines contribute to pathological vessel wall remodeling.[24–26] We demonstrated that not only the endothelial cells but RBCs themselves respond to nicotine/ free radicals with altered L-arginine metabolism pathway; the impaired activity of NOS3 is well paralleled with an increased Arginase-1 level. The importance of this

result is underlined by recent findings that RBCs intensively contribute to vascular functioning and integrity. NOS3-derived NO export from RBCs mediate a rescue mechanism in case of a vascular dysfunction, but the pathway gets stringently regulated by Arginase-1. Recent publication pointed out that RBCs, isolated from patients with diabetes mellitus 2, detrimentally induced impairment in healthy rat aorta, by upregulation of Arginase-1. Elevated level of Arginase-1 inhibits NO export thus reducing NO bioavailability or increasing oxidative stress conditions by the ROS generation, causing serious endothelial dysfunction. This induced dysfunctionality can be prevented by inhibition of Arginase activity.[27,28] As it was mentioned above, an increase in Arginase-1 level results in an accelerated formation of $O_2^{\bullet -}$. One of the reasons behind this phenotype is Arginase-1 induced NOS3 uncoupling, where NOS3 produces $O_2^{\bullet -}$ instead of NO, resulting in an imbalance of the redox homeostasis.[10] The $O_2^{\bullet -}$ can readily react with available NO in a spontaneous reaction, to form strong pro-oxidant molecule $ONOO^-$. We demonstrated a significant high level of $ONOO^-$ in the RBC-S origin, which initiates free radical mediated processes that mark an array of macromolecular damages. The RBC membrane is particularly susceptible to oxidative damage due to a high content of iron, polyunsaturated fatty acids and its role as an oxygen transporter.[29,30]

Oxidative stress conditions prone to enhance the rate of membrane structure remodelling and lipid peroxidation, causing considerable alteration in the lipid bilayer ultrastructure. Membrane lipid asymmetry is a key factor for the signal transduction process where under normal circumstances the phosphatidylserine (PS) lies in the inner leaflet which binds to signal proteins like protein kinase C, the altered appearance of PS on the outer leaflet impair the membrane integrity and widely detected as a marker for cell death.[31,32] We demonstrated a loss in the lipid asymmetry, PS became exposed on the outer leaflet of the plasma membrane. Further, we detected an increased level of lipid peroxidation end product (HNE), formed from the oxidation of n-6 polyunsaturated fatty acids.[33] An intrinsic property of deformability in the RBCs makes them traverse freely through variable shear stress conditions along the macro and microvasculature. However, an enhancement of lipid peroxidation in the RBCs causes structural membrane damage with subsequent decrease in their cell deformability and fluidity, thus making it more rigid and non-deformable.[34,35]

Deformability of RBCs is a critical parameter of their need to continuously adapt to the changing flow conditions along the vascular tree. Nanomechanical properties of RBCs show considerable alterations in several pathology condition.[36] Inflammation related free iron

level in blood might induce nanomechanical alterations in the membranes and underlying network of RBCs.[29] It was found that RBC stiffness increases with age and shows considerably higher values in cigarette smokers.[37] Furthermore, smoking-induced rheological and roughness alterations was reported for RBCs of heavy smoking persons suggesting that these alterations might result in hemolysis.[38]Although the sample in these studies were collected predominantly from male persons, their results point towards the devastating effect of smoke-carried toxins induced alterations in RBCs rheology. Deformability of RBCs is highly determined by the elastic properties of the membrane-cytoskeleton complex. Our goal was to characterize the differences in nanomechanical properties of RBCs from different origin. We have mapped freshly isolated RBCs, effectuating an indentation curve at less than half a micrometre resolution. The reconstructed maps showed slightly increased stiffness of RBC-S population, in good agreement with the findings of Lekka and co-workers in adult patients with coronary disease, hypertension, and diabetes mellitus.[37] Furthermore, our results revealed a remarkable decrease in the recovering ability of the cells after indentation. The observed loss in the ability of RBC-S samples to regain their initial shape compared to RBC-NS points towards serious alterations in deformability and rheology. Differences found by Pretorius and co-workers and Masilamani and co-workers in membrane ultrastructure might be strongly related to the observed plastic behavior.[38,39] However, due to the complexity and variety of smoke-related toxins, the identification of exact mechanisms leading to the observed elevated plasticity is beyond the limit of the present study.

The involvement of RBC NOS3-produced NO in the regulation of RBC deformability was proposed/presented by many groups,[40–42] however, these findings were not confirmed by other laboratories.[43,44] In recent publication it was suggest that NO itself does not affect RBC deformability under physiological circumstances, but might preserves it in conditions of oxidative stress.[45]

Conclusion:

We draw the conclusion, that under the influence of cigarette smoke, an increase in the redox homeostasis imbalance in RBCs enhance the rate of macromolecular damage with membrane stiffness and loss in their intrinsic functional and elastic activities. As a consequence of it, in case of endothelial dysfunction with low bioavailable level of NO the RBC NOS3-NO production is unavailable as a compensatory mechanism. Moreover, because of the wide increase in the RBC mass during pregnancy the elevated Arginase-1 level might even augment and synergizes the development of vascular dysfunction/comorbidities. And

lastly, we believe that the alterations in NOS3 activation pathway and Arginas-1 expression could serve as a real-time sensor and could be used as an early prognostic marker for not only RBC-linked anomalies but also for endothelial dysfunction and several vascular comorbidities.

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Declaration of interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1: Relationship between RBC-NOS3 activation in correlation to their different phenotypic variations from RBC-NS and RBC-S origin

RBCs were randomly selected from both RBC-NS and RBC-S populations, representing altogether 100-100 regular biconcave-shaped cells from 5 independent samples. In RBC-S we have in total 50 echinocyte types from 5 independent samples. Graphical plots represent the average intensity levels of NOS3 (A), p-NOS3 *i.e.* the activated state of NOS3 (B). (C) indicates the ratio between NOS3 and p-NOS3 intensity level. The results were accepted statistically by one-way ANOVA using the Newman-Keuls multiple comparison test at $p<0.05$*, $p<0.01$**, $p<0.001$*** and $p<0.0001$****.

Figure 2: The reciprocal relationship in the expression level of Arginase-1 and NOS3 from the RBC-NS and RBC-S population.

The total sample volume of RBC-NS (n=44) and RBC-S (n=35) were considered. Panel (A) represents the Arginase-1 intensity in the total cell population, (B) shows the distribution of the cell populations expressing Arginase-1 in the both basal and high intensity level and in (C) there lies the percentage distribution of NOS3 in particular within the high Arginase-1 expressing cells. Statistical significances were accepted by one-way ANOVA using the Newman-Keuls multiple comparison test at $p<0.01$** and $p<0.0001$****.

Figure 3: Estimation on the array of macromolecular damage by the level of pro-oxidants, extent of lipid peroxidation and cell death

Graphical summary on RBC-NS (n=44) and RBC-S (n=35) samples. Panel (A) represents the spectrophotometric measurements of ONOO- level. Panel (B) indicates the level of 4-HNE within the total NOS3 positive cell population and panel (C) showing the percentage distribution of the apoptotic cells. *Marks the significant difference accepted by one-way ANOVA using the Newman-Keuls multiple comparison test at $p<0.05$* and $p<0.01$**.

Figure 4: Measurement of the deformability capacity in RBC populations by atomic force microscope.

The ratio of Plastic Work / Elastic Work (EI) represented by box and whisker plots (A) calculated from RBC-NS and RBC-S (n=50 cells/from each of the 10 independent samples). *Marks the significant difference based on Kruskal-Wallis rank test at $p<0.05$. Outliers are marked with red plus signs. Values of Plastic Work versus Elastic Work are shown in (B) as mean +/- SD represented by error bars.