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Physiological properties of human erythrocytes in inflammation

C. Saldanha* and A.S. Silva-Herdade
Instituto de Medicina Molecular, Instituto de Bioquímica, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal

Abstract. The aim of this mini review was to show data that will could open new biotechnical applications. Physiological properties of the erythrocytes obtained in inflammatory conditions simulated *in vitro* or *in vivo* in experimental animal models of inflammation or *ex vivo* by analysis performed in blood samples from patients with vascular diseases are herein presented. Those data obtained *in vivo* have been utilized in mathematical models of vascular blood circulation on search biomechanical properties of blood components.

Behind the enlargement of scientific knowledge all data could open new questions giving support to create new therapeutic drugs and to ameliorate apparatus for non-invasive detection of vascular circulatory diseases and further surgical interventions.

Kewords: Erythrocyte deformability, inflammation, nitric oxide

1. Physiological erythrocytes properties- *in vitro* studies mimicking inflammatory acute conditions

Inflammation or inflammatory response is a natural process of the organism to fight an aggressive internal or external chemical, physical or mechanical stimuli or infection resulting from virus or bacterium presences. Redness, edema, pain and heat are the four cardinal signals of inflammation well known since the antiquity. In case of tissue injury, release of chemical signals like histamine, prostaglandins and nitric oxide (NO) by endothelial cells occurs. NO liberate to vessel lumen is scavenged by erythrocytes through band 3 protein [1]. The NO diffuse also to adjacent smooth muscle cells inducing relaxation [2]. Vasodilation occurs, increases blood flow, augment vessel wall permeability and leukocytes recruitment with transmigration to cell injury site for phagocytes pathogens and cells debris. Vascular endothelial cells changes its phenotypes to participate in the acute inflammation which involve a fast and a slower responses in close relation with the blood cells [3]. Acute phase proteins like fibrinogen, oxLDL as well as the non-cholinergic molecule acetylcholine (ACh) levels increase in plasma [4–6]. It was showed that the erythrocyte when in presence of ACh, the natural of substrate of the membrane acetylcholinesterase (AChE) release NO which efflux is quantified by amperometric measurement with amiNO-sensor [7, 8]. It was verified that the AChE-ACh enzyme active complex activate the protein kinase C (PKC) which in turn phosphorylate the protein tyrosine kinase (PTK) turning it from inactive to active state. PTK phosphorylate band 3 protein became able to receive NO in its thiol group from S-nitrosohemoglobin for delivery to extracellular medium [9–12]. PKC inhibited by phosphorylation protein tyrosine phosphatase (PTP) and also the phosphodiesterase 3 (PDE3) which consequently do not hydrolyzes the cyclic adenosine triphosphate (cAMP) [9, 10]. The AChE-ACh

*Corresponding author: C. Saldanha, Instituto de Medicina Molecular, Instituto de Bioquímica, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal. E-mail: carlotasaldanha@fm.ul.pt.

enzyme active complex joined to Gαi protein which inhibit adenylyl cyclase (AC) became it unable to cAMP formation form ATP [11]. Active AChE enzyme conformations are associate with the increase of NO efflux from erythrocyte, which is further potentiate by ACh [12].

The NO efflux measurements from erythrocytes is based in the ACh transduction pathway where AChE-, ACh, Gαi protein, band3 protein, PKC, PTK, PTP, AC and PDE3 are participants. The cAMP levels did not interfere. This is the scientific rational base of the efficient methodology for estimate erythrocyte bioavailability in NO [7–12].

The NO biosensor amiNO-sensor can be utilized in suspensions of endothelial cells from umbilical cord to quantify the NO liberated [13, 14].

Our studies showed that the ability of erythrocyte to scavenger NO is potentiated in presence of soluble fibrinogen with consequently decrease of NO efflux at physiological concentrations [15]. It is necessary to highlight that the erythrocyte membrane CD47 is the target of soluble fibrinogen [16]. CD47 which is a component of Rh group associate with Gαi protein that inhibiting AC decrease the level of cAMP [17]. When hyperfibrinogenemia is mimicked *in vitro* erythrocyte NO efflux increased in dependence of band3 protein phosphorylation returning to normal levels when in presence of either ACh or timolol (AChE inhibitor) showing dependency of the AChE enzyme conformational states [12, 18–20]. However if the AC completely inhibited by the specific inhibitor MDL, in presence of high fibrinogen levels and either timolol or 4N1K (a binding peptide of CD47) an increase of erythrocyte NO efflux is verified [21]. The levels of NO efflux return to normal with increased cAMP concentration achieved either by PDE3 enzyme dephosphorylated in inactive state (resulting from inhibition of PI3-K by worthamin) or by the AC activation by forskolin [21, 22]. Those results allows us to conclude that in acute or chronic inflammation where soluble fibrinogen concentration are above the normal range, the erythrocyte ability to scavenger maintaining NO or deliver it is dependent of band 3 protein phosphorylation degree, low cAMP levels and AChE enzyme molecule conformations [12].

It was demonstrated that S-nitrosoglutathione (GSNO) molecules that preserve NO inside erythrocytes are released from them in presence of ACh or timolol [23]. Under unstimulated erythrocytes did not occur any GSNO efflux and it is regarded as a potential therapeutical agent, acting as a store or donor of NO [23].

It is very interesting that the activation of AC enzyme by forskolin, which normalize the levels of NO efflux from erythrocytes in hyperfibrinogenemia, is nowadays used for lower body weight in obese human and to alleviate patients with glaucoma [24–26]. Obesity and glaucoma are inflammatory diseases where erythrocytes form patients with these diseases showed increase NO efflux [27, 28]. So, one explanation for the forskolin success in both inflammatory diseases could be the ability of the erythrocytes capture the NO avoiding the oxygen and nitrogen reactive species formation. This is a clue to be explored in patients under forskolin medication.

Pay attention to the patients's clinical profile it is always needed. For example, the oxidized LDL, at high concentration, decreases the NO efflux from erythrocytes [5]. So, if the same occurring in patients with atherosclerosis, erythrocyte promote a vascular protection by decreasing the nitrogen reactive species. One possible signaling mechanism, need of validation, could results from the binding of oxLDL to its receptor CD36 disturbing the interaction with band 3 protein knowing as the route for entrance and sort of NO [5]. Previous study showed that autologous plasma LDL sub-fractions induce increase in erythrocyte aggregation what may also be a positive factor to enhance the erythrocyte scavenger ability of NO [29].

It is not yet completely understood the participation of erythrocyte in acute inflammation mechanism. Some shadows and questions existed pointing clues need it to be clarified. These are particularly important when thinking in potentiate the drug's effects therapy and exploration of new non-invasive instrumental devices for its amelioration for blood flow microcirculatory visualization in healthy persons and in patients with vascular diseases.

2. Physiological erythrocytes properties- *in vivo* experimental animal models of acute inflammation

Behind several applications, animal models of inflammation are useful for hemodynamic, hemorheological, biomechanical and molecular characterization of the early phase of leukocytes approach to endothelium cells [30]. The intravital microscopy coupled with fluorescence detectors and confocal microscopy allow us to visualize video- images in time of acute inflammation development. Sophisticated software treated them to obtain hemodynamic data, leukocyte rolling velocity, number of leukocytes rolling and adherent in to endothelial wall. These data are used by mathematicians' able to construct mathematical models useful to give the biomechanical characterization of the shear stress appearance in each or in several leukocytes surface forcing them to interact with each other's, interfering with their approach to the endothelial cell membrane.

A study conducted *in vivo* on an animal model of lipopolysaccharide (LPS) induced inflammation aimed to evaluate the effects of ACh on the leukocyte-endothelial cells evidenced that ACh has an anti-inflammatory effect showed by a decrease in TNF alpha plasma levels and by the decrease of the number of adherent leukocytes [30]. In a previous study the same authors using equal animal model protocol showed that the rolling leukocytes velocity was reduced without changes in plasma levels of IL-1beta [31].

In animal model of acute inflammation with labeled neutrophils and using intravital microscopy it was verified increased number of neutrophil rolling and adhesion. The erythrocyte deformability decreased favoring increase whole blood viscosity and consequently pouching the neutrophil to post-capillary venules endothelium [32]. The efflux of NO from erythrocyte decrease during the acute phase of inflammation [32].

Systemic hypertension in Sprague-Dawley rats induced by L-nitro-L-arginine methyl ester (L-NAME) is recovered by Valsartan, an angiotensin II AT1- receptor antagonist [33]. While systemic blood pressure increase the NO efflux from erythrocyte decrease and both parameters recover to normal levels after Valsartan administration [33]. The plasma viscosity, blood viscosity and erythrocyte deformability follow the same profile of values variation during the systemic hypertension development and recovering [33].

Hemodynamic and biochemical and hemorheological measurements were done *in vivo* with humans committed with sepsis, in intensive care unit, during 24 h after admission before died. The data have showed, during those days, increase values of erythrocyte NO efflux, unequal blood flow and decrease microvascular blood flow index quantified the last two in the sub-lingual microcirculation by a probe tied to SidestreamDark Field (SDF) imaging [34].

The advance of the mathematical models to describe blood flow in microcirculation depends on data obtained with "*in vivo*" experimental animal models. This means that the approaches assumed need to consider the presence of erythrocytes. The goal is to have more data to support new vascular technologies.

3. Physiological erythrocytes properties- *ex vivo* studies of inflammatory diseases

The end of the acute inflammation by clearance of the injurious stimuli trigger tissues and organs recovering to normal physiological functions. In chronic inflammation, where the resolution phase was absent, progression of mononuclear cell infiltrates, angiogenesis and fibrosis install originating autoimmune, hypertension, diabetes mellitus and amyotrophic lateral sclerosis (ALS) diseases among others [35–39]. Systemic hypertension is associated with vascular inflammation, and sub clinical atherosclerosis and a risk factor of acute myocardial infarction [40]. It was verified in blood samples

taken from three different groups of patients status, namely hypercholesterolemia, hypertension and renal transplantation, that decreased erythrocyte deformability (ED) is associated with more ability to delivery NO [41]. Less ED, more resident time of erythrocytes in micro vessels and more mechanical compression able to signaling the NO efflux.

The hemorheological disturbances are correlated with cardiovascular risk factors and markers of inflammation being more pronounced in sub-classes of patients with autoimmune disease with metabolic syndrome [35]. Erythrocyte NO efflux is negatively associated with carotid intima-media thickness (IMT) and an independent predictor of carotid (IMT) [35]. It is shown decreased erythrocyte deformability, increased erythrocyte aggregation and blood viscosity in patients with systemic lupus erythematosus and rheumatoid arthritis [35].

It was observed that the erythrocytes of patients with the chronic inflammatory disease ALS have lower concentration of nitrite and nitrate content and rescue more NO verified by low level of it efflux [39]. The respiratory function of this patients with ALS change in inverse relationship with de bioavailability of NO in erythrocytes [39]. AChE enzyme activity of erythrocytes obtained from the ALS patients present higher values than health humans [39].

Patients with retinal vasculitis (capillary occlusions) presented increased erythrocyte rigidity, lower ED, which normalised after treatment and improvement of ocular fundus visualized by angiography [42]. In another ocular disease with decrease of blood perfusion and damage of optic nerve, named glaucoma patients have presented increased values of erythrocyte AChE, NO efflux and aggregation, lower ED and higher blood viscosity [43, 44]. In diabetic patients with high glycated haemoglobin and serum glycoproteins showed worse erythrocyte deformability higher the degree retinopathy development with more difficulties in blood perfusion in micro vessels [38].

In a follow-up study of 45 days of patients with accelerated phase of hypertension characterized by retinopathy of degree III and IV presented in the first day high values of erythrocyte AChE enzyme activity, erythrocyte rigidity (decrease deformability) plasma AChE, and plasma viscosity. At discharge the patients presented normal values of mean arterial pressure, higher erythrocyte aggregation, impairment of plasma viscosity but yet higher than the normal range values [36]. The ophthalmologic abnormalities ameliorate with vasoconstriction, papilledema, cotton-wool exudates and haemorrhagic impairments [36]. One initial study with erythrocytes from patients with arterial hypertension showed increased values of AChE [45]. This data raise the study of the kinetic characterization of the enzyme *in situ* and in a purified form evidenced that erythrocyte AChE is inhibited at higher ACh concentrations which means that its natural substrate act also as inhibitor [46]. It was evidenced that erythrocyte membrane enzyme AChE participates in the NO signal transduction, as described above and also is a biomarker of ALS, essential hypertension and Parkinson disease [39, 45, 47].

4. Conclusion

The erythrocytes bioavailability in NO quantified in blood samples during the acute phase, subclinical and chronic inflammations could be explained by a compensatory mechanism for balance the endothelial dysfunction present in those vascular diseases. Association between values of NO efflux and IMT, ED, respiratory function and microvascular flow index were identified as described above.

From all studies it will be necessary to test the feasibility of monitoring NO in healthy and patients with vascular diseases using a fiber probe-based on diffuse reflectance spectroscopy system. Diffuse reflectance systems have the flexibility to be cost-effective and portable for use in a clinical setting. Ignaro, (Nobel Prize in Physiology or Medicine in 1998) showed, by spectral analysis, that the binding of NO to oxygenated hemoglobin shift the yellow light to green one [48].

A small sensor could be developed for ear, fingers but not for application on sub-lingual microcirculation where is known an entero-salivary circulation of nitrate in humans [49].

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