Nitric oxide effects on human erythrocytes structural and functional properties – An *in vitro* study

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**Abstract.** NO is present in the blood at $10^{-7}$ M under physiological conditions, but at concentrations higher than $10^{-6}$ M during inflammatory disease states. The aim of this study was to characterize what are the effects of these different NO concentrations on erythrocyte structural and functional properties. Blood was collected from eleven healthy men and incubated with Spermine NONOate in order to expose it during incubation time to NO concentrations between $10^{-7}$ M and $10^{-3}$ M. We measured erythrocyte aggregation and deformability, membrane lipid peroxidation and fluidity, p50, hemoglobin, oxyhemoglobin, methemoglobin concentrations and plasma pH, pO$_2$, pCO$_2$, Na$^+$, K$^+$ and Ca$^{2+}$. When blood was exposed to NO $10^{-7}$ M erythrocyte deformability increase and p50 decrease. In presence of NO $10^{-5}$ M lipid fluidity and p50 decrease. When blood was exposed to NO $10^{-3}$ M methemoglobin concentration increase and erythrocyte deformability and p50 decrease but membrane fluidity and lipid peroxidation were similar to control. In conclusion, dependent of NO concentrations there is different effects on erythrocytes structural and functional properties.

1. Introduction

Nitric oxide is an endogenous mediator synthesized and released constitutively by vascular endothelial cells to underneath smooth muscle cells and to the blood [14]. Immunologic and inflammatory stimuli have been shown to induce the expression of an isoenzyme of NO synthase (iNOS) in endothelial cells and monocytes [34]. While NO is present in the blood at $10^{-7}$ M when produced constitutively under physiological conditions, it is present at concentrations higher than $10^{-6}$ M during inflammatory, infectious and degenerative disease states [7].

Erythrocytes are blood cells and thus exposed continuously to NO and its metabolites. The relative concentrations of NO and its metabolites, as well as the exact chemical nature of those are influenced by the “redox status” of the cells [2].

Some of NO produced by vascular endothelial cells may reach the erythrocytes, diffuse across erythrocyte membrane and react with either oxyhemoglobin to form nitrate and methemoglobin, or with hemoglobin to form nitrosylhemoglobin, or with 93-cysteine residue of the globin $\beta$-subunit to form S-nitrosohemoglobin [9,18,22].

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Eiserich et al. (1995) demonstrated that NO reacts directly with tryptophan and tyrosine radicals in amino acids, peptides and proteins [6]. Additionally, NO has the capacity to modulate the activity of proteins through reversible reaction with available functional groups, notably Fe and thiols. S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase promotes ADP-ribosylation of the enzyme and is associated with inhibition of its activity [31].

In the aqueous phase of plasma, NO may react with molecular oxygen to form nitrite, or with superoxide anion to form peroxynitrite \((\text{ONOO}^-)\). NO reacts with superoxide at an almost near-diffusion-limited rate of \(6.7 \times 10^9 \text{ M}^{-1} \text{s}^{-1}\) and formation of peroxynitrite will only occur where the appearance rate of NO is equal or lower than that of superoxide formation [8].

Peroxynitrite is a very reactive molecule interacting with cell membrane and other components. It was shown to oxidize glutathione and protein cysteine residues, potentially depleting cells of antioxidant defenses [28]. Peroxynitrite and/or peroxynitrous acid will also initiate the oxidation of unsaturated fatty acids [38]. Peroxynitrite also exhibits reactivity with protein tyrosine residues [13]. Peroxynitrite can react with other components in cells besides protein tyrosine residues, but these competing reactions may be minimized \textit{in vivo} because of the presence of CO\(_2\), which catalyzes peroxynitrite-mediated protein nitration [45].

Starzyk et al. (1997) have found that during an acute phase of lipopolysaccharide (LPS) action in rats, the excessive amount of NO originated leads to a decrease of erythrocyte deformability, and L-NAME (a NOS inhibitor) exerts a protective effect on LPS-impaired deformability [43]. Bateman et al. (2001) confirmed these data using aminoguanidine, an iNOS inhibitor which prevented the overproduction of systemic NO, accumulation of NO within erythrocytes and also prevented erythrocyte deformability in septic rats [1]. On the other hand, basal release of NO is important for maintaining erythrocyte deformability at the physiological range, because lowering basal NO levels with NOS inhibitors leads to impairment of erythrocyte deformability [43].

These earlier studies support the idea that NO effects on cells and specifically on erythrocytes depends on its concentrations. The aim of this study was to characterize the effects of a range of NO concentrations between \(10^{-7} \text{ M}\) and \(10^{-3} \text{ M}\) on erythrocyte hemorheological properties (aggregation and deformability), membrane dynamic and structure (ions permeability, lipid peroxidation and membrane fluidity) and oxygen hemoglobin affinity.

2. Materials and methods

2.1. Materials

SpermineNONOate was purchased from Calbiochem. 1,6-diphenyl-1,3,5-hexatriene was purchase from Molecular Probes.

2.2. Subjects and blood collection

Blood was collected from a forearm vein of ten healthy caucasian males \((22 \pm 3\text{ years old})\). All the subjects were students at the Faculty of Medicine of Lisbon. All the subjects gave their informed consent. Blood was collected to tubes with heparin 10 UI/ml according to the guidelines for measurement of blood viscosity and erythrocyte deformability [16].
2.3. Experimental procedure

After blood collection, blood was divided into six aliquots each one with 2 milliliters and centrifuged at 9600g for 1 minute in a Biofuge 15 centrifuge (Heraeus, Sepatech). Then 40 µl of plasma was taken from each aliquot and replaced with the same volume of SpermineNONOate solutions in order to obtain SpermineNONOate final concentrations of 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M. The remaining aliquot served as control. All aliquots were incubated at 37°C for 40 minutes, based on previous information about half-life of SpermineNONOate [19]. As each molecule of SpermineNONOate donates two molecules of NO, and its half-life is of 39 minutes, at the end of the incubation time, samples were exposed to NO concentrations equal to initial SpermineNONOate concentrations. Based on these data what we know is the cumulative NO concentration but not the instantaneous one.

2.4. Membrane lipid fluidity

Membrane lipid fluidity was determined by measuring fluorescence anisotropy with 1,6-diphenyl-1,3,5-hexatriene (DPH) probe for hydrophobic zone of membrane [40]. Higher values of fluorescence anisotropy are related with lower fluidity. Ten microliters of packed erythrocytes was resuspended in phosphate buffer 155 mM with DPH 2.2 mM. The fluorescence anisotropy (\(\lambda_{ex} = 465\) nm, \(\lambda_{em} = 465\) nm) of a 1/10 dilution of last solution was measured using a spectrofluorometer Hitachi F3000 (Hitachi, Japan), according the method described by Shirilo et al. (1981).

2.5. Membrane lipid peroxidation

Malonyldialdehyde (MDA), and end product of fatty acid lipid peroxidation, like other aldehydes formed during lipid peroxidation, can react with thiobarbituric acid (TBA) to form a colored complex that has maximum absorbance at 532 nm. The TBA reactivity of erythrocytes was a measure of membrane lipid peroxidation and was determined by the Stocks and Dormandy modified method of TBA-reactivity [17]. In this procedure, 0.2 ml of packed erythrocytes were suspended in 0.8 ml of phosphate-buffered saline made up of 8.1 g NaCl, 2.302 g Na_2 HPO_4 and 0.194 g NaH_2 PO_4, per liter, pH 7.4. To this, 0.5 ml of 30% trichloroacetic acid was added. Tubes were vortexed and allowed to stand in ice for at least 2 h. Tubes were centrifuged at 670g for 15 minutes using the centrifuge Sorvall® TC6. One milliliter from supernatant of each tube was transferred to another tube and 0.075 ml of EDTA 1 M and 0.25 ml of TBA 1%–NaOH 0.05 nM were added. Tubes were mixed and kept in a 100°C water bath for 15 minutes. After that tubes were centrifuged 2 minutes at 18 000g using the centrifuge Biofuge 15 (Heraeus Sepatech). Absorbance was read at 532 nm in spectrophotometer Beckman Model 35 after tubes were cooled to room temperature. The MDA concentration was expressed as nmol/10^{10} erythrocytes. The erythrocyte counts were done in Abott cell-Dyn 1600.

2.6. Erythrocyte deformability

Erythrocyte deformability was determined using the Rheodyn SSD laser diffractometer from Myrenne (Roetgen, Germany).

The Rheodyn SSD diffractometer determines RBC deformability by stimulating the shear stresses exerted by the blood flow and vascular walls on the erythrocytes [41]. Erythrocytes are suspended in a viscous medium and placed between a rotating optical disk and a stationary disk, where they are going to be subjected to well defined shear stresses, that forces the erythrocytes to deform to ellipsoids and
align with the fluid shear stress. If a laser beam is allowed to pass through the erythrocyte suspension a diffraction pattern appears on the opposite end. That diffraction pattern will be circular with resting erythrocytes, but becomes elliptical with deformed erythrocytes. The light intensity \((\text{lum})\) of the diffraction pattern is measured at two different points (A and B), equidistant from the center of the image. The erythrocyte elongation index (EEI), in percentage, is obtained according to the following formula:

\[
\text{EEI} (\%) = \frac{\text{lum}_A - \text{lum}_B}{\text{lum}_A + \text{lum}_B} \times 100.
\]

2.7. Erythrocyte aggregation

Erythrocyte aggregation was determined using the MA1 aggregometer from Myrenne (Roetgen, Germany). The MA1 aggregometer consists of a rotative cone-plate aggregometer, that disperses the sample by high shear stress (600/s), and a photometer that determines the extent of aggregation. The intensity of light, exerted by a light emitting diode, is measured after transmission through the blood sample using a photodiode. The aggregation was determined in stasis during 10 seconds, after dispersion of the blood sample [37].

2.8. \(\text{K}^+\), \(\text{Na}^+\), \(\text{Ca}^{2+}\) plasma concentrations, pH, oxygen pressure \((\text{pO}_2)\), carbon dioxide pressure \((\text{pCO}_2)\), hemoglobin (Hb), oxyhemoglobin \((\text{O}_2\text{Hb})\), carboxyhemoglobin \((\text{COHb})\), methemoglobin \((\text{MetHb})\), hemoglobin oxygen saturation \((\text{sO}_2)\) and oxygen tension at half saturation of blood \((\text{p}50)\)

Plasma pH, \(\text{pO}_2\), \(\text{pCO}_2\), \(\text{Na}^+\), \(\text{K}^+\) and \(\text{Ca}^{2+}\) concentrations were determined with the ABL505 electrode system from Radiometer (Copenhagen, Denmark). Hb, \(\text{O}_2\text{Hb}\), COHb, MetHb, and \(\text{sO}_2\) were determined with the Osm3 hemoximeter from Radiometer. \(\text{p}50\), an index of hemoglobin oxygen affinity, is determined by ABL505 electrode system connected to Osm3 hemoximeter, both from Radiometer. ABL505 calculates the value of \(\text{p}50\) mathematically, based on the values of carboxyhemoglobin, methemoglobin and the oxygen dissociation curve.

2.9. Statistical analysis

Data are presented as means \(\pm\) standard error. Student’s \(t\) test for paired observations was used to evaluate statistical significance of differences between the studied parameters. Statistical significance was considered for values of \(p < 0.05\).

The statistical analysis was performed using the following software SPSS 10 (SPSS Inc.).

3. Results

3.1. Effects of nitric oxide on erythrocyte membrane lipid fluidity and peroxidation (Fig. 1)

Membrane lipid fluidity was assessed by measuring fluorescence anisotropy of DPH probe on erythrocyte membrane being the control value \(0.284 \pm 0.009\) (au). When we incubate blood with SpermineNONOate \(10^{-7}\) M, for example, we know that blood was exposed to a total NO concentration of \(10^{-7}\) M. The same we suppose real to other concentrations of SpermineNONOate. As showed in Fig. 1, the mean fluorescence anisotropy remained approximately the same when blood was incubated with
Fig. 1. Malonyldialdehyde (MDA) concentration values and fluorescence anisotropy values obtained with DPH probe introduced on human erythrocytes membrane after incubation without and with SpermineNONOate (SpNO) concentrations between $10^{-7}$ M and $10^{-3}$ M during 40 minutes.

SpermineNONOate $10^{-7}$ M and $10^{-6}$ M. However when blood was incubated with SpermineNONOate $10^{-5}$ M, we observed a significant increase of fluorescence anisotropy to $0.308 \pm 0.005$ (au), $p < 0.04$. An increase of fluorescence anisotropy of DPH probe means a decrease of lipid fluidity of hydrophobic zone of erythrocyte membrane. Increasing the concentration of SpermineNONOate there was a decrease of fluorescence anisotropy, what means an increase of lipid fluidity to values similar to the control ones.

The control values of MDA concentrations on erythrocytes after 40 minutes at $37^\circ$C was $3.01 \pm 0.21$ nmol/10$^{10}$ erythrocytes. Although there were not significant changes of lipid peroxidation in presence of different concentrations of SpermineNONOate the obtained profile was similar to that of membrane fluidity (DPH fluorescence anisotropy) as showed in Fig. 1.

3.2. Effects of nitric oxide on erythrocyte deformability and aggregation

The elongation index of erythrocytes measured at eight different shear stresses gives us an idea of erythrocyte deformability at different places of circulation. When blood was incubated in presence of SpermineNONOate $10^{-7}$ M there is a significant increase of erythrocyte deformability at high shear stress values (Table 1). On the other hand when blood was incubated with SpermineNONOate $10^{-3}$ M there is a significant decrease of erythrocyte deformability at low shear stress values.

The erythrocyte aggregation index with a control value of $14.32 \pm 0.66$ (nd) did not significantly change when blood was incubated with different SpermineNONOate concentrations.

3.3. Effects of nitric oxide on plasma concentrations of sodium, potassium and calcium and plasma pH (Table 2)

There is a slight but significative decrease of sodium, potassium and calcium concentrations in the presence of all SpermineNONOate concentrations studied ($p < 0.05$), except for SpermineNONOate $10^{-3}$ M in presence of which the increase of $K^+$ concentration verified is not significative.

pH is increased in presence of SpermineNONOate $10^{-7}$ M, is similar to control for SpermineNONOate $10^{-6}$ M but significantly increased for higher SpermineNONOate concentrations.
Values of erythrocyte elongation index (%) at shear stress values between 0.3 Pa and 60 Pa after *in vitro* incubation of blood aliquots in absence and presence of different concentrations of SpermineNONOate (SpNO)

<table>
<thead>
<tr>
<th>Shear stress values</th>
<th>Control</th>
<th>SpNO 10^{-7} M</th>
<th>SpNO 10^{-8} M</th>
<th>SpNO 10^{-9} M</th>
<th>SpNO 10^{-10} M</th>
<th>SpNO 10^{-11} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 Pa</td>
<td>2.61 ± 0.43</td>
<td>2.58 ± 0.44</td>
<td>2.55 ± 0.40</td>
<td>2.63 ± 0.47</td>
<td>1.94 ± 0.34$^4$</td>
<td>2.08 ± 0.30</td>
</tr>
<tr>
<td>0.6 Pa</td>
<td>4.40 ± 0.31</td>
<td>4.19 ± 0.55$^2$</td>
<td>4.18 ± 0.52</td>
<td>4.23 ± 0.43</td>
<td>4.48 ± 0.48</td>
<td>3.83 ± 0.30$^3$</td>
</tr>
<tr>
<td>1.2 Pa</td>
<td>12.46 ± 0.77</td>
<td>11.86 ± 0.59</td>
<td>11.99 ± 0.74</td>
<td>11.82 ± 0.73</td>
<td>11.74 ± 0.73</td>
<td>11.66 ± 0.67$^5$</td>
</tr>
<tr>
<td>3.0 Pa</td>
<td>26.93 ± 0.80</td>
<td>26.63 ± 0.65</td>
<td>26.71 ± 0.82</td>
<td>26.12 ± 0.95</td>
<td>26.48 ± 0.79</td>
<td>26.20 ± 0.75</td>
</tr>
<tr>
<td>6.0 Pa</td>
<td>37.27 ± 0.89</td>
<td>37.17 ± 0.78</td>
<td>37.17 ± 0.83</td>
<td>35.97 ± 1.47</td>
<td>37.27 ± 0.93</td>
<td>37.19 ± 0.74</td>
</tr>
<tr>
<td>12 Pa</td>
<td>44.82 ± 1.42</td>
<td>45.22 ± 1.18</td>
<td>44.82 ± 1.23</td>
<td>43.55 ± 1.84</td>
<td>44.98 ± 1.28</td>
<td>45.27 ± 1.07</td>
</tr>
<tr>
<td>30 Pa</td>
<td>48.02 ± 1.84</td>
<td>48.96 ± 1.60$^1$</td>
<td>48.43 ± 1.60</td>
<td>46.94 ± 1.99</td>
<td>48.65 ± 1.67</td>
<td>48.82 ± 1.42</td>
</tr>
<tr>
<td>60 Pa</td>
<td>47.18 ± 1.89</td>
<td>48.45 ± 1.58$^1$</td>
<td>47.78 ± 1.72</td>
<td>46.37 ± 2.06</td>
<td>47.91 ± 1.61</td>
<td>47.81 ± 1.45</td>
</tr>
</tbody>
</table>

1$p < 0.04$; 2$p < 0.001$; 3$p < 0.01$; 4$p < 0.05$.

Values of plasma concentrations of Na$^+$, K$^+$, Ca$^{2+}$, pH, pO$_2$ and pCO$_2$, and blood hemoglobin, oxyhemoglobin and methemoglobin concentrations after *in vitro* incubation of blood aliquots in absence and presence of different concentrations of SpermineNONOate (SpNO)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SpNO 10^{-7} M</th>
<th>SpNO 10^{-8} M</th>
<th>SpNO 10^{-9} M</th>
<th>SpNO 10^{-10} M</th>
<th>SpNO 10^{-11} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Na$^+$] (mmol/l)</td>
<td>139.9 ± 0.5</td>
<td>137.8 ± 0.5$^1$</td>
<td>138.2 ± 0.4$^1$</td>
<td>138.2 ± 0.5$^1$</td>
<td>137.7 ± 0.7</td>
<td>137.7 ± 0.8$^1$</td>
</tr>
<tr>
<td>[K$^+$] (mmol/l)</td>
<td>3.64 ± 0.08</td>
<td>3.51 ± 0.07$^1$</td>
<td>3.48 ± 0.06$^1$</td>
<td>3.51 ± 0.07$^1$</td>
<td>3.52 ± 0.07$^1$</td>
<td>3.73 ± 0.08</td>
</tr>
<tr>
<td>[Ca$^{2+}$] (mmol/l)</td>
<td>1.16 ± 0.02</td>
<td>1.13 ± 0.02$^1$</td>
<td>1.14 ± 0.02$^1$</td>
<td>1.14 ± 0.02$^1$</td>
<td>1.14 ± 0.02$^1$</td>
<td>1.12 ± 0.02$^1$</td>
</tr>
<tr>
<td>pH</td>
<td>7.34 ± 0.01</td>
<td>7.36 ± 0.01$^1$</td>
<td>7.34 ± 0.01</td>
<td>7.35 ± 0.01$^1$</td>
<td>7.37 ± 0.01$^1$</td>
<td>7.38 ± 0.02$^1$</td>
</tr>
<tr>
<td>pO$_2$ (mmHg)</td>
<td>41.79 ± 1.95</td>
<td>42.59 ± 1.97</td>
<td>43.50 ± 2.20$^2$</td>
<td>43.87 ± 2.54$^2$</td>
<td>42.08 ± 2.29</td>
<td>39.87 ± 3.14</td>
</tr>
<tr>
<td>pCO$_2$ (mmHg)</td>
<td>47.58 ± 1.27</td>
<td>43.56 ± 1.13$^1$</td>
<td>46.23 ± 1.04</td>
<td>45.16 ± 1.33$^2$</td>
<td>43.21 ± 0.76$^1$</td>
<td>43.93 ± 1.60$^2$</td>
</tr>
<tr>
<td>[Hb] (g/dl)</td>
<td>14.27 ± 0.30</td>
<td>14.33 ± 0.24</td>
<td>14.39 ± 0.27</td>
<td>14.22 ± 0.34</td>
<td>14.26 ± 0.28</td>
<td>14.33 ± 0.27</td>
</tr>
<tr>
<td>O$_2$Hb (%)</td>
<td>74.92 ± 2.53</td>
<td>77.29 ± 1.92</td>
<td>76.76 ± 2.20</td>
<td>77.83 ± 2.60</td>
<td>76.38 ± 2.65</td>
<td>72.67 ± 3.58</td>
</tr>
<tr>
<td>MethHb (%)</td>
<td>1.48 ± 0.06</td>
<td>1.49 ± 0.05</td>
<td>1.48 ± 0.04</td>
<td>1.58 ± 0.06</td>
<td>1.8 ± 0.13$^2$</td>
<td>4.97 ± 1.44$^2$</td>
</tr>
</tbody>
</table>

1$p < 0.001$; 2$p < 0.03$; 3$p < 0.005$.

3.4. Effects of nitric oxide on pO$_2$ and pCO$_2$ (Table 2)

PO$_2$ values increased in the aliquots incubated with SpermineNONOate 10$^{-5}$ M and 10$^{-6}$ M, relatively to control ($p < 0.001$). pCO$_2$ values decreased in the aliquots incubated with SpermineNONOate 10$^{-7}$, 10$^{-8}$, 10$^{-9}$ and 10$^{-3}$ M ($p < 0.001$).

3.5. Effects of nitric oxide on methemoglobin concentration (Table 2) and p50 value (Fig. 2)

The methemoglobin concentration remained approximately similar to control value (1.48 ± 0.04%) when blood was incubated with SpermineNONOate 10$^{-7}$ M to 10$^{-4}$ M but had a large increase when blood was incubated with SpermineNONOate 10$^{-3}$ M (4.97 ± 1.44%, $p < 0.04$).

The oxygen pressure corresponding to half saturation of hemoglobin (p50) traduces the affinity of hemoglobin to oxygen. The p50 value of control aliquot was 25.56 ± 0.49 mmHg. When blood was incubated with SpermineNONOate 10$^{-7}$ M the p50 value decreases (24.70 ± 0.39 mmHg, $p < 0.04$), but with SpermineNONOate 10$^{-6}$ M, the p50 value is similar to control aliquot. For SpermineNONOate concentrations 10$^{-5}$, 10$^{-4}$ and 10$^{-3}$ M the p50 value decreases relatively to control with a progressive profile ($p < 0.02$) (Fig. 2).
4. Discussion

The results presented here show that nitric oxide has different effects on erythrocyte structural and functional properties depending of its concentration in the blood.

When blood aliquots were incubated with SpermineNONOate concentrations of $10^{-7} \text{M}$ there was a slight increase of erythrocyte deformability relatively to control aliquots for high shear stress values (30 and 60 Pa) but no significative changes were verified on membrane lipid fluidity and lipid peroxidation. These last results support the hypothesis that in this in vitro model at this SpermineNONOate concentrations, there is scarce formation of peroxynitrite, which after protonation ($\text{pK}_a = 6.8$) forms peroxynitric acid that generates by homolytic and heterolytic cleavage the highly reactive radicals $\text{OH}^-$, $\text{NO}_2^-$ and $\text{NO}_2^+$, capable of oxidizing the membrane lipids [3]. Supposing that NO reaction with superoxide could not be the major route, it can react with $\text{O}_2$ forming nitrite, with plasma proteins forming nitrosothiols like S-nitrosoalbumin, with erythrocyte membrane proteins and reacts with deoxy- and oxyhemoglobin [20,24,42,47]. The slight increase of erythrocytes elongation index mentioned above can be induced by S-nitrosylation of membrane skeleton proteins modulating erythrocyte deformability. If this was the case, S-nitrosylation would modify the cytoskeleton proteins interactions in order to increase the erythrocyte deformability. However the effect of S-nitrosylation of erythrocyte cytoskeleton proteins was not studied yet. Based on our data that when NO is present in concentrations around $10^{-7} \text{M}$ there is no relevant changes on membrane lipid fluidity and peroxidation and an increase of erythrocyte deformability we hypothesize that these conditions could facilitate erythrocyte passage in narrow capillaries and then tissue oxygenation. However shear stress values of 30 and 60 Pa are very increased relatively to physiologic values of about 10 Pa in capillaries where erythrocyte deformability is determinant for normal blood flow [30]. On the other hand tissue oxygenation is also modulated by oxygen–hemoglobin affinity [25] assessed in this study by p50 value which decrease relatively to control value when blood was incubated with SpermineNONOate $10^{-7} \text{M}$, returning to values similar to control for SpermineNONOate $10^{-6} \text{M}$. A decrease of p50 means an increase of hemoglobin–oxygen affinity and then a decrease of oxygen delivery to tissues [29]. This decrease of p50 can be justified by the increase of plasma pH and decrease of pCO$_2$ verified when blood aliquots were incubated with SpermineNONOate $10^{-7} \text{M}$ (Table 1). According with these data, Puscas et al. [35] previously described
inhibition of erythrocyte carbonic anhydrase I by nitric oxide which consequently decrease the pCO₂ and subsequently increase the pH. In what concerns methemoglobin formation there is no changes comparing blood aliquots incubated with SpermineNONOate 10⁻⁷ M, 10⁻⁶ M and control aliquot.

Additionally, another mechanism may be hypothesized to explain data referred above. When NO concentration is lower than or equal to that of superoxide anion it has been postulated that there could be formation of peroxynitrite anion [12]. The reason why there is no changes in lipid peroxidation and consequently membrane fluidity may be the reaction between peroxynitrite and CO₂ forming a highly reactive nitrosoperoxocarbonate (ONOOCO₂⁻) [36]. Since it is less stable than ONOO⁻, it has a much lower diffusion distance which limit lipid oxidation and nitrination. Besides decomposing before reaching the site of lipid reaction it may not be able to diffuse into hydrophobic compartments due to its negative charge [26]. This mechanism is supported by O’Donnel et al. studies in which CO₂ inhibits both oxidation and nitrination of linoleate in cholate-solubilised micelles [33]. In our study we observed a decrease of CO₂ concentrations when blood was incubated with SpermineNONOate 10⁻⁷ M, possibly due to its reaction with ONOO⁻.

When blood was incubated in presence of SpermineNONOate 10⁻⁵ M there was a significant decrease of lipid fluidity of hydrophobic zone of erythrocyte membrane and a non-significant increase of lipid peroxidation (Fig. 1). The decrease of lipid fluidity is probably due to the increase of lipid peroxidation. The increase of lipid peroxidation may be caused by increased production of peroxynitrite anion from the reaction between NO and superoxide anion. Studies using pure lipids (linoleic acid, phosphatidyl choline liposomes, cholesteryl linoleate and free cholesterol) have shown that ONOO⁻ can cause formation of several lipid oxidation products including, conjugated dienes malonyldialdehyde, lipid peroxides, lipid hydroxides, F2-isoprostanes and oxyysterols [33]. These reactions may justify the observed decrease of membrane lipid fluidity, although no changes were verified for erythrocyte deformability. When blood was incubated with SpermineNONOate 10⁻⁵ M there was a decrease of p50 similar to that occurred with SpermineNONOate 10⁻⁷ M, but the CO₂ concentration was similar to control. However metHb slightly increase and it is known that methemoglobin shift the oxygen dissociation curve to the left which means decrease of p50 and increase of oxygen–hemoglobin affinity [11,27].

When blood was incubated with SpermineNONOate 10⁻⁴ M and 10⁻³ M there was a non-significant concentration dependent decrease of lipid peroxidation comparing with SpermineNONOate 10⁻⁵ M. The same was verified with membrane lipid fluidity that increased. When blood aliquots were incubated with SpermineNONOate 10⁻³ M the membrane fluidity and lipid peroxidation were similar to control blood aliquots. These profile may be justified by NO decreasing lipid peroxidation. It was reported that when concentration of NO exceeds that of superoxide anion, lipid peroxidation can be inhibited by NO with a concurrent formation of nitrated lipid radical termination products [39]. However at this concentration there was a significant decrease of erythrocyte deformability at low shear stress values. Additionally, for these concentrations of NO there was a significant and concentration dependent increase of methemoglobin formation and consequently a decrease of p50 values. The formation of methemoglobin is derived from reaction of NO with oxyhemoglobin with formation of methemoglobin and nitrate anion [20]. The high accumulation verified only for the higher concentrations of NO may be justified by the generation of methemoglobin up to ability of methemoglobin reductase to reduces it back to reduced hemoglobin. These changes in oxygen dissociation curve may be particularly important in pathological conditions like sepsis where there is an abnormality of tissue oxygenation and levels of NO are equal or higher than 10⁻⁵ M [4,10,32,44]. Despite the maldistribution of blood flow [5] it is verified a decrease in oxygen extraction by compromising oxygen delivery [15]. In clinical and experimental models of sep-
sis [21,23,46] it has been verified a significant increase of membrane lipid viscosity (decrease of fluidity) and decrease of erythrocyte deformability.

5. Conclusion

In conclusion, depending of NO concentrations there is different effects mediated by NO or its metabolites. At lower concentrations of $10^{-7}$ M and $10^{-6}$ M NO do not change membrane lipid fluidity and peroxidation and increases erythrocyte deformability which in vivo could facilitate erythrocyte passage in narrow capillaries. At NO $10^{-5}$ M there is decrease of membrane fluidity, increase of lipid peroxidation and decrease of p50 which in vivo could decrease oxygen delivery to tissues by decreasing oxygen dissociation from hemoglobin. At NO $10^{-3}$ M there is an increase of methemoglobin and decrease of oxygen dissociation from hemoglobin and of erythrocyte deformability which could impair tissue oxygenation joining to hypotension state verified in vivo with these NO concentrations.

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References

[14] R. Mesquita et al. / Nitric oxide effects on human erythrocytes properties


