Effects of acetylcholine and SpermineNONOate on erythrocyte hemorheologic and oxygen carrying properties

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Abstract. *Purpose of the study:* To determine the effects of acetylcholine and SpermineNONOate – a NO donor – on RBC membrane and oxygen carrying properties. *Material and methods:* Aliquots of venous blood from eleven heathy subjects were incubated with ACh 10^{-3} M, ACh 10^{-5} M, SpermineNONOate 10^{-5} M and SpermineNONOate 10^{-4} M. The following parameters were determined: erythrocyte aggregation and deformability, hematocrit, plasma pH, osmolality, K^+, Na^+, Ca^{2+} concentrations, hemoglobin, oxyhemoglobin, carboxihemoglobin and methemoglobin concentrations, oxygen saturation of hemoglobin, oxygen and carbon dioxide parcial pressures and p50. *Results:* In presence of ACh there is an increase of erythrocyte deformability, decrease of erythrocyte aggregation, plasma pH, K^+ and Na^+ concentration, increase of Ca^{2+} concentration and p50. In presence of SpermineNONOate there an increase of erythrocyte deformability, plasma pH, decrease of Na^+ and Ca^{2+} concentration, increase of metHb concentration and decrease of p50. *Conclusion:* Acetylcholine and SpermineNONOate are able to induce changes on RBC membrane and oxygen carrying properties.

1. Introduction

Nitric oxide (NO), the "endothelium derived relaxing factor", has several cardiovascular functions, namely as a potent vasodilator [1], platelet aggregation inhibitor [2] and red blood cell (RBC) deformability modulator [3–6]. Even through the effects of NO on platelets and vascular smooth muscle have been established for some time, very little is known about its effects on the RBC.

Some authors found that NO can react with haemoglobin, forming nitrosohaemoglobin [8–13]. According to those authors, haemoglobin nitrosilation occurs preferentially at high oxygen pressure (pulmonary territory), whilst NO release from nitrosohaemoglobin is favoured when the oxygen pressure is low (capillary territory). These findings seem to substantiate the idea that NO may be somehow involved in the respiratory cycle. Other researchers found that, within certain physiological concentrations, NO tends to enhance RBC deformability and, when in concentrations outside those values, it has the opposite effect [5].

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Recent studies indicate that RBCs are able to synthesize their own NO from L-arginine, in a reaction catalysed by the enzyme NO synthase [14–16]. In addition, it has been shown that acetylcholine (ACh), known to enhance NO production on endothelial cells [17], also induces an increase of NO release from the RBC, independently of the presence of L-arginine (*submitted for publication*). It is important to refer the description by Tang et al. [18,19] of acetylcholine muscarinic receptors M1 in RBC.

Based on these facts it was hypothesised that ACh, by inducing the production of NO, could cause changes on the RBC membrane and oxygen carrying properties.

To test this hypothesis, venous blood was collected from human male subjects and was incubated *in vitro* in the presence or absence of ACh and a NO donor substance, SpermineNONOate. After the incubation period the following parameters were assessed: (i) RBC deformability, (ii) RBC aggregation, (iii) K^+, Na^+, and Ca^{2+} plasma concentration, (iv) pO_2, pCO_2, sO_2, p50 and pH, (v) haemoglobin, oxyhaemoglobin, deoxyhaemoglobin and methaemoglobin, (vi) haematocrit and (vii) plasma osmolality.

2. Materials and methods

2.1. Materials

Acetylcholine chloride (Sigma Aldrich Química SA); SpermineNONOate (Calbiochem).

2.2. Solutions

Acetylcholine 10^{-3} M and 10^{-1} M, prepared in distilled water, native pH SpermineNONOate 10^{-3} M and 10^{-2} M prepared in distilled water pH 12.

2.3. Subjects

Eleven healthy Caucasian males (aged 20 ± 3 (SD)).

All the subjects were students at the Faculty of Medicine of Lisbon, with residence in Lisbon (Portugal) or Lisbon outskirts. All the subjects gave their informed consent.

2.4. Experimental procedure

To determine the effects induced by NO on RBC, ten millilitres of blood from each subject was collected from a forearm vein to tubes with heparin 10 UI/ml. After that, the blood was divided into six 1 ml aliquots and centrifuged at 12 000 rpm for 1 minute in Biofuge 15 centrifuge (Haraeus, Sepatech). Then, 20 μl of plasma was taken from each aliquot and replaced with the same volume of ACh 10^{-3} M, ACh 10^{-1} M, SpermineNONOate 10^{-3} M and SpermineNONOate 10^{-2} M. The remaining two aliquots served as controls. Aliquots with ACh final concentrations of 10^{-5} M and ACh 10^{-3} M, as well as their respective control aliquot, were incubated at 37°C for 5 minutes. Aliquots with SpermineNONOate final concentrations of 10^{-5} M and 10^{-3} M, as well as their respective control aliquot, were incubated at 37°C for 30 minutes. 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 the concentration of NO in the samples was the desired one.

2.4.1. RBC deformability

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

The Rheodyn SSD difractometer determines RBC deformability by simulating the shear stresses exerted by the blood flow and vascular walls on the RBCs. RBCs 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 RBCs to deform to ellipsoids and align with the fluid shear stress. If a laser beam is allowed to pass through the RBCs suspension a diffraction pattern appears on the opposite end. That diffraction pattern will be circular with resting RBCs, but becomes elliptical with deformed RBCs. The light intensity (lum) of the diffraction pattern is measured at two different points (A and B), equidistant from the centre of the image. The elongation index (EI), in percentage, is obtained according to the following formula:

$$EI = \frac{lum\,A - lum\,B}{lum\,A + lum\,B} \times 100.$$

2.4.2. RBC aggregation

RBC 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.

2.4.3. Potassium (K^+), sodium (Na^+) and calcium (Ca^{2+}) plasma concentration, oxygen pressure (pO_2), carbon dioxide pressure (pCO_2), pH, haemoglobin (Hb), oxyhaemoglobin (O_2Hb), carboxyhaemoglobin (COHb), methaemoglobin (metHb) and haemoglobin oxygen saturation values (sO_2)

Plasma pH, pO_2, pCO_2 and K^+, Na^+, Ca^{2+} concentrations were determined with the ABL505 electrode system from Radiometer (Copenhagen, Denmark).

Hb, O_2Hb, COHb and metHb values were determined with the Osm3 hemoximeter from Radiometer.

2.4.4. p50

p50 is the oxygen tension at half saturation (50%) of blood. High and low values indicate decreased and increased affinity of oxygen to haemoglobin, respectively.

This parameter is determined by ABL505 electrode system connected to Osm3 hemoximeter, both from Radiometer (Copenhagen, Denmark) connected to ABL505 calculates the value of p50 mathematically, based on the values of carboxyhaemoglobin, methaemoglobin (provided by the Osm3 hemoximeter) and the oxygen dissociation curve.

2.4.5. Haematocrit

The haematocrit was determined with the 4223 Centrifuge from ALC (Italy).

2.4.6. Plasma osmolality

Plasma osmolality was determined at the end of each assay. The aliquots were centrifuged at 12 000 rpm for 1 minute in the Biofuge 15 centrifuge from Haraeus. After that the plasma osmolality was determined with the Osmomat 0,30 Cryoscopic Osmometer from Gonotec (Berlin, Germany).

2.5. Statistical analysis

The results are presented as means $\pm$ standard deviation.

Student's t-test for paired observations was used to evaluate statistical significance of differences between the studied parameters. For every parameter all samples were tested against their respective control, and both controls were tested against each other. Statistical significance was considered for values of $P < 0.05$.

The statistical analysis was performed using the following software (for IBM PCs or compatible platforms): (i) SPSS 10 (SPSS Inc.), (ii) SYSTAT Version 9 (SPSS Inc.) and (iii) SigmaPlot 2000 version 6.0 (SPSS Inc.).

3. Results

3.1. RBC deformability

As show in Fig. 1, RBC deformability increased slightly but significantly in the aliquots incubated with ACh 10^{-5} M for low shear stress values (6 Pa) [37.0 ± 1.4 vs. 36.5 ± 1.6 (control), $P < 0.030$], and for higher shear stress values (30 Pa) [46.8 ± 3.5 vs. 45.8 ± 3.7 (control), $P < 0.041$]. RBC deformability in presence of acetylcholine 10^{-3} M has no significant changes relatively to control, despite a tendency to increase as we can observe from data analysis. It seems that increasing acetylcholine concentrations blunts this effect in RBC deformability.

In aliquots incubated with SpermineNONOate (Fig. 2), RBC deformability increased significantly relatively to the control only for higher stress values (12, 30 and 60 Pa). In presence of SpermineNONOate
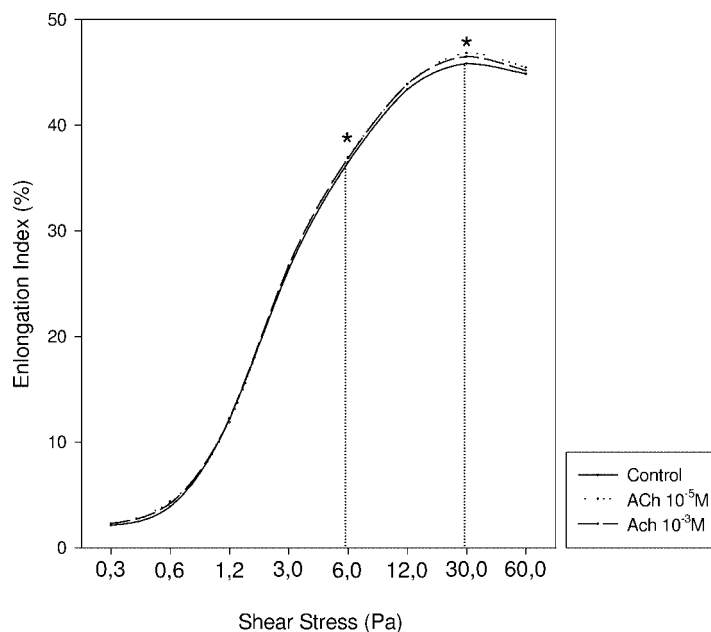

Fig. 1. Effect of ACh on the RBC deformability. * Indicates statistical difference of RBC deformability in aliquots incubated with ACh 10^{-5} M, as compared to control [37.0 ± 1.4 vs. 36.5 ± 1.6, $P < 0.030$ (6 Pa); 46.8 ± 3.5 vs. 45.8 ± 3.7, $P < 0.041$ (30 Pa)].
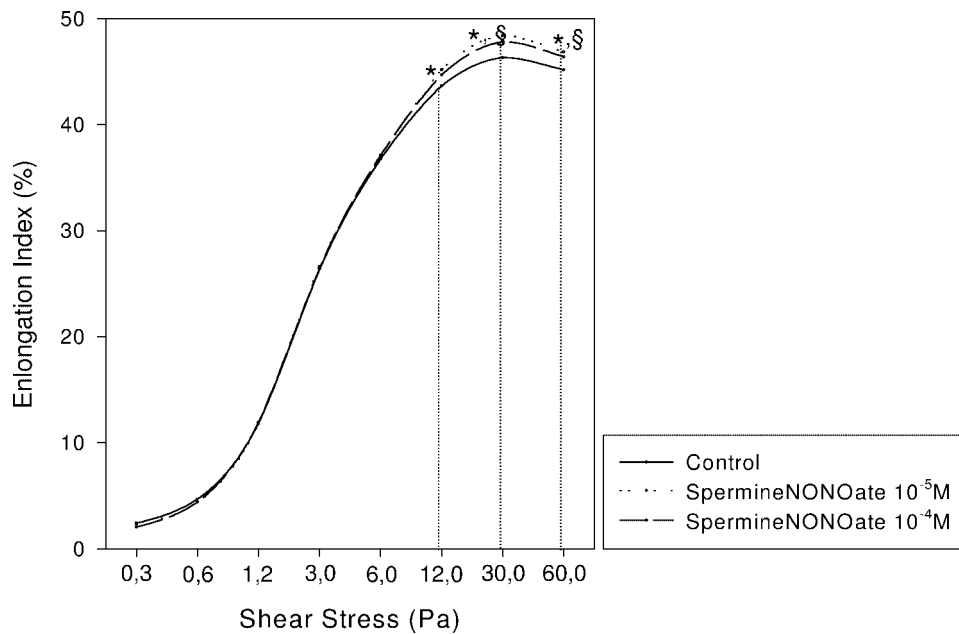
Fig. 2. Effect of SpermineNONOate on the RBC deformability. * Indicates statistical difference of RBC deformability in aliquots incubated with SpermineNONOate 10^{-5} M, as compared to control [45.17 ± 3.3 vs. 43.66 ± 3.4, $P < 0.012$ (12 Pa); 48.38 ± 4.5 vs. 46.32 ± 4.4 $P < 0.008$ (30 Pa); 46.85 ± 4.2 vs. 45.16 ± 4.6, $P < 0.016$ (60 Pa)]. § Indicates statistical difference of RBC deformability in aliquots incubated with SpermineNONOate 10^{-4} M, as compared to control [47.81 ± 4.4 vs. 46.32 ± 4.4, $P < 0.023$ (30 Pa); 46.38 ± 4.3 vs. 45.16 ± 4.6, $P < 0.019$ (60 Pa).]

10^{-5} M we observed an increase of RBC deformability for shear stress values of 12 Pa [45.17 ± 3.3 vs. 43.66 ± 3.4, $P < 0.012$]; 30 Pa [48.38 ± 4.5 vs. 46.32 ± 4.4, $P < 0.008$] and 60 Pa [46.85 ± 4.2 vs. 45.16 ± 4.6, $P < 0.016$]. In presence of SpermineNONOate 10^{-4} M RBC deformability increased significantly for shear stress values of 30 Pa [47.81 ± 4.4 vs. 46.32 ± 4.4, $P < 0.023$] and 60 Pa [46.38 ± 4.3 vs. 45.16 ± 4.6, $P < 0.019$]. According these results SpermineNONOate, or NO effects on RBC deformability are not concentrations dependent for the concentrations studied and are more pronounced than acetylcholine effects. However NO seems to affect RBC deformability only for higher shear stress values (corresponding to large blood vessels) and acetylcholine to have a small effects either at high or low shear stress values.

3.2. RBC aggregation

RBC aggregation decreased significantly only in the aliquots incubated with ACh 10^{-3} M, as compared to the control [13.0 ± 3.4 vs. 13.6 ± 3.7, $P < 0.025$] (Fig. 3).

The aliquots incubated with SpermineNONOate did not show any significant differences of RBC aggregation, relatively to the control.

3.3. K^+, Na^+, Ca^{2+} plasma concentrations

Potassium plasma concentration decreased, relatively to the control, in the aliquots incubated with ACh 10^{-5} M [3.850 ± 0.172 vs. 4.00 ± 0.194 mmol/l, $P < 0.002$], and in the aliquots incubated with
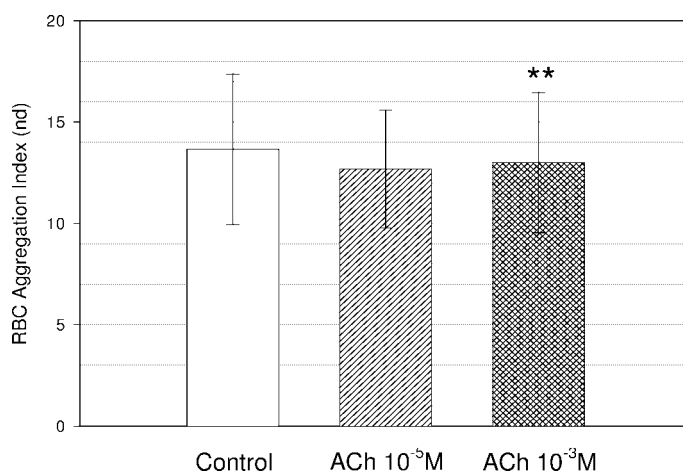
Fig. 3. Effect of ACh on the RBC aggregation index. ** Indicates statistical difference of RBC aggregation in aliquots incubated with ACh 10^{-3} M, as compared to control [13.0 ± 3.4 vs. 13.6 ± 3.7, $P < 0.025$].

Table 1
Values of Na^+, K^+ and Ca^{2+}

	Control	ACh		Control	SpermineNONOate	
		10^{-5} M	10^{-3} M		10^{-5} M	10^{-4} M
Na^+ (mM)	138.64 ± 2.42	$135.0 \pm 2.10^*$	$134.55 \pm 1.92^*$#	138.45 ± 2.11	137.64 ± 2.06	$137.64 \pm 2.06^*$
K^+ (mM)	3.98 ± 0.19	3.83 ± 0.18	$3.73 \pm 0.17^*$#	3.95 ± 0.19	3.87 ± 0.23	3.74 ± 0.38
Ca^{2+} (mM)	1.09 ± 0.06	1.09 ± 0.06	$1.12 \pm 0.05^*$	$1.13 \pm 0.06^{**}$	$1.04 \pm 0.06^*$	$1.04 \pm 0.07^*$

$^+$ Indicates statistical difference as compared to control ($p < 0.05$).
# Indicates statistical difference as compared to aliquots with ACh 10^{-5} M.
** Indicates statistical difference between the control aliquots incubated for 30 minutes and the control aliquots incubated for 5 minutes ($p < 0.05$).

ACh 10^{-3} M [3.750 ± 0.158 vs. 4.00 ± 0.194, $P < 0.001$] (Table 1). No significant differences for this parameter were observed in the aliquots incubated with SpermineNONOate.

Sodium plasma concentrations decreased in the aliquots incubated with ACh 10^{-5} M [135.00 ± 2.10 vs. 138.64 ± 2.42 mmol/l, $P < 0.001$] and in the aliquots incubated with ACh 10^{-3} M [134.55 ± 1.92 vs. 138.64 ± 2.42 mmol/l, $P < 0.001$] (Table 1). In the aliquots incubated with SpermineNONOate 10^{-5} M, sodium plasma concentration also decreased relatively to the control [137.64 ± 2.06 vs. 138.45 ± 2.11 mmol/l, $P < 0.006$]. However the effect of SpermineNONOate is slighter than the acetylcholine one.

Calcium plasma concentration increased in the aliquots incubated with ACh 10^{-3} M [1.1191 ± 0.0509 vs. 1.0982 ± 0.0574 mmol/l, $P < 0.017$] (Table 1). Dissimilar to what happened in the aliquots incubated with ACh 10^{-3} M, a significant decrease of calcium plasma concentration was observed in the aliquots incubated with SpermineNONOate 10^{-5} M [1.0429 ± 0.0716 vs. 1.1264 ± 0.0605 mmol/l, $P < 0.003$], as well as in the aliquots incubated with SpermineNONOate 10^{-4} M [1.0455 ± 0.0601 vs. 1.1264 ± 0.0605 mmol/l, $P < 0.001$] (Table 1).

3.4. pO_2, pCO_2, sO_2, $p50$ and pH

pO_2 values decreased in the aliquots incubated with SpermineNONOate 10^{-5} M, relatively to the control [41.020 ± 8.657 vs. 50.714 ± 11.511 mmHg, $P < 0.008$], and in the aliquots incubated Sper-

Table 2

Values of haemoglobin, oxyhaemoglobin, carboxyhaemoglobin, methaemoglobin, haemoglobin oxygen saturation, haematocrit, plasma pO_2, pCO_2, p50, pH and osmolality

	Control	ACh		Control	SpermineNONOate	
		10^{-5} M	10^{-3} M		10^{-5} M	10^{-4} M
pO_2 (mmHg)	52.188 ± 13.415	56.688 ± 11.446	49.400 ± 11.268	50.714 ± 11.511	$41.020 \pm 8.657^*$	$46.300 \pm 10.672^*$
pCO_2 (mmHg)	29.578 ± 3.892	30.889 ± 2.336	$34.611 \pm 2.851^*$	30.975 ± 4.062	30.850 ± 4.012	$27.388 \pm 3.549^*$
p50	20.75 ± 2.23	21.02 ± 2.45	$23.84 \pm 3.51^*$	20.22 ± 3.14	19.88 ± 3.41	$18.48 \pm 2.81^*$
pH	7.480 ± 0.041	7.469 ± 0.020	$7.405 \pm 0.026^*$	7.464 ± 0.026	7.534 ± 0.038	7.543 ± 0.038
Hb (g/dl)	14.945 ± 1.593	14.727 ± 1.725	14.836 ± 1.600	15.240 ± 1.724	$14.233 \pm 1.400^*$	$14.840 \pm 1.774^*$
O_2Hb (%)	87.173 ± 9.419	87.091 ± 8.555	88.273 ± 6.962	86.770 ± 10.227	$84.083 \pm 14.212^*$	85.520 ± 9.221
COHb (%)	0.618 ± 0.387	0.645 ± 0.375	0.636 ± 0.335	0.640 ± 0.384	0.833 ± 0.423	0.690 ± 0.401
MetHb (%)	1.055 ± 0.144	1.073 ± 0.135	1.027 ± 0.179	1.120 ± 0.132	1.217 ± 0.133	1.790 ± 0.296§§
SO_2 (%)	88.682 ± 9.780	88.654 ± 8.727	89.664 ± 7.159	88.360 ± 10.527	85.867 ± 14.600	90.590 ± 9.449
Ht (%)	46.30 ± 2.83	46.10 ± 2.88	45.70 ± 2.26	45.80 ± 3.16	$45.57 \pm 3.31^*$	$44.50 \pm 3.34^*$
Osm (osmol/kg)	0.308 ± 0.002	$0.302 \pm 0.003^*$	0.307 ± 0.004	0.308 ± 0.003	0.309 ± 0.002	0.307 ± 0.003

* Indicates statistical difference as compared to control ($P < 0.05$).

mineNONOate 10^{-4} M [46.300 ± 10.672 vs. 50.174 ± 11.511 mmHg, $P < 0.005$] (Table 2). ACh incubated aliquots did not show any statistical difference relatively to the control for this parameter.

pCO$_2$ values increased in ACh 10^{-3} M incubated aliquots l [34.611 ± 2.851 vs. 29.578 ± 3.892 mmHg, $P < 0.002$] (Table 2). On the other hand, SpermineNONOate 10^{-4} M incubated aliquots evidenced a decrease of pCO$_2$ values [27.388 ± 3.549 vs. 30.975 ± 4.062 mmHg, $P < 0.015$].

p50 values increased in the aliquots incubated with ACh 10^{-3} M, relatively to the control [23.8388 ± 3.5127 vs. 20.7538 ± 2.2330 mmHg, $P < 0.030$] (Table 2). Contrary to what was observed in the aliquots incubated with ACh 10^{-3} M, those incubated with SpermineNONOate suffered a decrease of p50 values, as compared to the control [18.4857 ± 2.8058 vs. 20.2271 ± 3.1414 mmHg, $P < 0.030$].

Values of pH decreased in the aliquots incubated with ACh 10^{-3} M [7.405 ± 0.026 vs. 7.480 ± 0.041, $P < 0.001$] (Table 2). In the aliquots incubated with SpermineNONOate 10^{-5} M, pH values increased relatively to the control [7.534 ± 0.038 vs. 7.464 ± 0.026, $P < 0.002$] (Table 2). Similar results were obtained in the aliquots incubated with SpermineNONOate 10^{-4}, where pH values also increased relatively to the control [7.543 ± 0.038 vs. 7.464 ± 0.026, $P < 0.001$] (Table 2).

3.5. Haemoglobin, oxyhaemoglobin, carboxyhaemoglobin and methaemoglobin

Hb concentration decreased relatively to the control in the aliquots incubated with SpermineNONOate 10^{-5} M [14.233 ± 1.400 vs. 15.240 ± 1.724 g/dl, $P < 0.007$], and in the aliquots incubated with SpermineNONOate 10^{-4} M [14.840 ± 1.774 vs. 15.240 ± 1.724 g/dl, $P < 0.004$] (Table 2). In the aliquots incubated with ACh, no statistical difference of Hb concentration was observed, when compared against the control. It is important to refer that the 30 minute control.

Oxyhaemoglobin decreased relatively to the control in the aliquots incubated with SpermineNONOate 10^{-5} M [84.083 ± 14.212 vs. $86.770 \pm 10.227\%$, $P < 0.047$] (Table 2). There were no alterations, relatively to the control, of O$_2$Hb values in the ACh incubated aliquots.

In the aliquots incubated with SpermineNONOate 10^{-4} M, metHb values increased relatively to the control [1.790 ± 0.296 vs. $1.120 \pm 0.132\%$, $P < 0.001$] (Table 2). When compared against the control, no significant differences were observed for metHb values in the aliquots incubated with ACh.

Neither ACh nor SpermineNONOate induced any changes on COHb and sO_2 values.

In both aliquots incubated with SpermineNONOate, haematocrit values decreased relatively to the control (Table 2). No statistical difference, relatively to the control, was observed in the aliquots incubated with ACh.

3.6. Plasma osmolality

Plasma osmolality decreased when compared to the control in the aliquots incubated with ACh 10^{-5} M [0.302 ± 0.003 vs. 0.308 ± 0.005 osmol/kg, $P < 0.001$] (Table 2). In the aliquots incubated with SpermineNONOate no significant difference, relatively to the control, was observed.

4. Discussion

This study shows that either acetylcholine or SpermineNONOate (a NO donor) induce changes on the RBC membrane and oxygen carrying properties. Furthermore, we also found that some of the changes induced by SpermineNONOate differ from those induced by acetylcholine. Despite small changes in some parameters have no clinical relevance, when they have statistical significance we considered them important to rise hypotheses about the mechanism of action of a particular substance.

Evidence that the RBC membrane properties were altered by the presence of NO (originated either by ACh or SpermineNONOate) is that the erythrocyte deformability, aggregation index , K^+, Na^+ and Ca^{2+} plasma concentration in aliquots incubated with ACh and SpermineNONOate showed significant differences when compared with their respective controls (Figs 1, 2 and 3).

The samples incubates with ACh 10^{-3} M showed a decrease of erythrocyte aggregation index when compared to the control [13.000 ± 3.454 vs. 13.655 ± 3.719, $p < 0.021$], and the samples incubated with ACh 10^{-5} M suffered an increase of the RBC deformability index for shear stress values of 6 and 30 Pa (Fig. 1). The samples incubated with SpermineNONOate exhibited an increase of the RBC deformability index for shear stress values of 12, 30 and 60 Pa (Fig. 2). Similar results, using SIN-1 as a NO donor, were obtained by Starzyk in his experiments with rats [7].

RBC aggregation and deformability are two close related phenomena but happen in different places in vascular system [20]. Under normal blood flow, erythrocyte aggregation happens in post-capillary venules where there is a decrease in vascular shear stress, facilitating the forward blood flow. Erythrocyte deformability is a property of erythrocytes that is essential to their passage in narrowed capillaries. Both processes depends on a normal compliance of RBC membrane. Any factor inhibiting the normal fluidity of cytoplasm (e.g., osmotic dehydration or chemical changes in haemoglobin) or any factor increasing the viscosity of membrane (e.g., haemoglobin binding, spectrin cross-linking) will interfere with deformability of RBC and aggregate formation [21].

NO is a highly reactive gas interacting with various constituents of RBC membrane. The half life of NO in blood has been estimated to be 0.46 ms [22]. The major biological end products of NO metabolism are nitrite (NO_2^-) and nitrate (NO_3^-). During metabolism NO_2^-/NO_3^-, NO can undergo a series of reactions that generate reactive nitrogen species (nitroxyl anion, nitrous oxide, nitrosyl cation, dinitrogen trioxide, nitrogen dioxide, dinitrogen tetraoxide, peroxynitrite and nitril cation). NO reacts with superoxide ($O_2^{-\cdot}$) to form peroxynitrite, that is a highly reactive species with a half-life of 1.0 second at 37°C and pH 7.4 that reacts with proteins, lipids, carbohydrates through oxidation and nitration mechanism. NO and its metabolites can stimulate lipid peroxidation. Oxidant metabolites of NO can deplete enzymatic and low molecular weight cellular antioxidants, reducing the likelihood of the termination of free

radical lipid propagation reactions. In contrast, when the concentrations of NO exceeds that of $O_2^{-\cdot}$, lipid peroxidation can be inhibited by NO, with a concurrent formation of nitrated lipid radical termination products [23]. As referred in last paragraph, changes in membrane viscosity can determine changes in erythrocyte aggregation and deformability.

The plasma concentration of K^+, Na^+ and Ca^{2+} also suffered significant alteration relatively to the control, when incubated with either of the used effectors. These changes in plasma ion concentration may result from changes in transport mechanisms which function is to maintain hydroelectrolytic equilibrium in blood cells. When compared against the control, the samples incubated with ACh evidenced a decrease of K^+ ($p < 0.002$) and Na^+ ($p < 0.001$) plasma concentrations, while Ca^{2+} plasma concentration increased ($p < 0.017$) (Table 1). On the other hand, the aliquots incubated with SpermineNONOate showed no significant changes for K^+ plasma concentration, while Na^+ and Ca^{2+} plasma concentrations decreased relatively to the control ($p < 0.006$ and $p < 0.003$, respectively). Intracellular K^+ and Na^+ concentrations are regulated by Na^+/K^+ ATPase that maintains high intracellular K^+ concentration and high extracellular Na^+ concentration. Despite the reference by some authors [24] to inhibition by NO of Na^+/K^+ ATPase activity, in our study there is no evidence of this fact because the changes in Na^+ and K^+ plasma concentration in presence of acetylcholine are in the same direction (decrease of extracellular concentrations). The reactions of NO with membrane biomolecules described in paragraphs above can induce changes in transport mechanisms that justify these changes in plasma ion concentrations.

In what concerns plasma pH, the aliquots incubated with ACh 10^{-3} M showed a decrease relatively to control ($p < 0.001$), whilst the aliquots incubated with SpermineNONOate showed an increase ($p < 0.002$) (Table 2). The decrease of pH in the aliquots incubated with ACh 10^{-3} M may be the result of acetate formation as a result of ACh hydrolysis by acetylcholinesterase (present unless in erythrocytes and lymphocytes membrane [25–28]). Based on decrease of Na^+ and K^+ plasma concentrations and pH decrease (increase of H^+ plasma concentration), we can hypothesise a change in activity of $K^+(Na^+)/H^+$ exchanger previously described in erythrocytes [29]. On the other hand Petrov and Lijnen [30] described the regulation of human erythrocyte Na^+/H^+ exchange by soluble and particulate guanylate cyclase. The same authors [31] describe the activation by protein kinase C and subsequently by calcium of erythrocyte Na^+/H^+ exchange in men which is according with characterisation of M1 muscarinic receptors in RBC by Tang et al. [18,19] in which ligand–receptor interaction triggers and increase of intracellular calcium and cGMP. Additionally, various authors [32–34] characterise the protein kinase C in erythrocyte.

The increase of pH in aliquots incubated with SpermineNONOate can be attributed to the fact of original SpermineNONOate solution added to the blood was buffered at pH 12 for means of storing. In the other hand, Puscas et al. [35] describe erythrocyte carbonic anhydrase I inhibition by nitric oxide which consequence is a decrease of pCO_2 and subsequently an increase of pH.

In presence of acetylcholine we observe an increase of pCO_2 in blood samples, which can be related with respective pH decrease. Both factors can explain the observed increase of p50 (decrease of haemoglobin affinity to oxygen). However is well known that NO interacts with haemoglobin, either in deoxygenated or oxygenated forms [22]. This fact can induce some changes in its affinity to oxygen. Kosaka et al. [36], refer NO as an enhancer of oxygen release from erythrocytes to peripheral tissues by breaking or stretching the heme iron–proximal histidine bond in the alpha subunit of haemoglobin while maintaining oxygen binding capacity.

In presence of SpermineNONOate we observe an increase of pH, which can explain the decrease of pCO_2 and both explain the decrease of p50 (increase of haemoglobin affinity to oxygen). The decrease of pO_2 can be explained by this last change. The decrease of p50 in presence of NO donor is in accordance with work of Hrinczenko et al. [37] which describe that DEANO and CysNO (NO donors) also

caused an increase of haemoglobin affinity to oxygen and caused significant methaemoglobin formation. In our work we also observe a significant increase of methaemoglobin concentration in presence of SpermineNONOate 10^{-4} M. Kelm and Yoshida [22] described that methaemoglobin formation results from reaction of NO with HbO_2. This fact is according with decrease of concentration of HbO_2 in aliquots incubated with SpermineNONOate.

The decrease of haematocrit and haemoglobin in aliquots incubated with SpermineNONOate can be attributed to haemolysis despite of no macroscopic observation of it. However, the NO reactions with membrane biomolecules can justify a low level of haemolysis.

5. Conclusion

In conclusion, this study showed that both ACh and SpermineNONOate are able to induce changes on the RBC membrane and oxygen carrying properties. Acetylcholine decreases the blood pH and increases pCO_2, p50 and pO_2. In other hand, SpermineNONOate increases blood pH and decreases pCO_2, p50, pO_2 and methaemoglobin formation. These results lead us to speculate about different reaction between NO produced intrinsically (acetylcholine) and the extracellular NO released by a donor (SpermineNONOate).

Acknowledgements

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