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Research paper

Effects of choline on hemorheological properties and NO metabolism of human erythrocytes

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Abstract. *Purpose of the study:* To determine the effects of choline on red blood cell membrane properties and NO metabolism.
Material and methods: Aliquots of venous blood from eleven healthy subjects were incubated *in vitro* with choline concentrations 10^{-10} to 10^{-3} M. The following parameters were determined: erythrocyte deformability, aggregation and membrane lipid fluidity, plasma K^+, Na^+, Ca^{2+}, total blood haemoglobin and methemoglobin concentrations. Additionally, plasma and intra-erythrocyte nitrites concentrations were measured.
Results: Choline increases erythrocyte deformability at lower shear stresses, decreases erythrocyte aggregation, increases membrane lipid fluidity, and decreases of Na^+ plasma concentrations. We also find an increase of nitrites concentration both in the plasma and in the intra-erythrocyte compartment.
Conclusion: Choline induces changes on erythrocyte membrane properties, Na^+ plasma concentration, and NO metabolites concentrations.
Keywords: Choline, hemorheology, NO, nitrites

1. Introduction

Acetylcholine (ACh) has been shown to be present in human blood circulation (8.65 ± 1.02 pM) [1,2], being produced by T lymphocytes [3,4] and endothelial cells [5]. Milner et al. has suggested that production of acetylcholine by the endothelial cells, is improved by modifications in blood flow conditions and by hypoxia [5,6].

Previous *in vitro* studies of our group have shown that ACh decreases the erythrocyte aggregation, increases the erythrocyte deformability and decreases the haemoglobin affinity to oxygen [7]. The changes in hemorheologic properties of the erythrocyte, has physiological relevance, because they induce changes in blood viscosity, modulate the distribution of blood in the several vascular territories and tissue oxygenation [8–10]. Additionally (i) we have demonstrated that ACh induces changes on erythrocyte NO metabolism namely an increase of nitrites and nitrates and (ii) we and others have shown that within

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certain physiological concentrations, NO tend to enhance erythrocyte deformability and, at higher concentrations the opposite effect was verified [11–13]. Non-cholinergic physiological relevance of acetylcholine effects on membranes of endothelial cells, lymphocytes, and erythrocytes [14–16] has been questioned by the presence of acetylcholinesterase, the enzyme that hydrolyses acetylcholine to acetate and choline.

Choline is an essential nutrient founded in specialised fat molecules such as phosphatidylcholine (lecithin) which is a structural component of all human cell membranes. Plasma choline molecules could reach the inside cellular space by a Na^+/HCO_3^- co-dependent choline transport [17] and its plasma content was recently evaluated which could rise benefits in population based studies associated with chronic diseases [18].

Based on these facts the aim of the present study was to determine if choline induces changes on the hemorheologic properties of erythrocyte, plasma ion concentration and erythrocytes NO metabolism.

2. Materials and methods

2.1. Materials

Choline chloride and $ZnSO_4$ (Sigma Aldrich Química, SA); Griess reagents: [sulfanilamide, HCl, and N-(1-napthyl)-ethylenediamine (NEDD)] and 1,6-dihenyl-1,3,5-hexatriene (Molecular Probes).

2.2. Solutions

Choline (Ch) 10^{-3}, 10^{-5}, 10^{-7}, 10^{-8} and 10^{-10} M were prepared in distilled water.

2.3. Subjects

Eleven healthy Caucasian males [aged 30 ± 10 (SD)] gave their informed consent to participate in the study. All the subjects were students or workers at the Faculty of Medicine of Lisbon, with residence in Lisbon (Portugal) or Lisbon outskirts.

2.4. Experimental procedure

Ten millilitres of blood from each subject was collected from a forearm vein to tubes with heparin 10 IU (sodium heparin 5,000 IU/ml from B. Braun Medical Ld) for 1 ml of blood. After that, the blood was divided into six 1 ml aliquots and centrifuged at 11,000 rpm for 1 minute in Biofuge 15 centrifuge (Haraeus, Sepatech). Then, 10 μl of plasma was taken from each aliquot and replaced with the same volume of (i) Ch 10^{-8} M, (ii) Ch 10^{-6} M, (iii) Ch 10^{-5} M, (iv) Ch 10^{-3} M, (v) Ch 10^{-1} M. The remaining aliquot served as control. Aliquots with Ch final concentrations of Ch 0 M, Ch 10^{-10} M, Ch 10^{-8} M, Ch 10^{-7} M, Ch 10^{-5} M, Ch 10^{-3} M, were incubated at room temperature, for 15 minutes.

After the incubation period we evaluated (i) the erythrocyte hemorheological properties by determination of the erythrocyte deformability, the aggregation and membrane lipid fluidity, (ii) the ions plasma concentration namely K^+, Na^+, and Ca^{2+}, and (iii) the erythrocyte NO metabolites by determination of intra-erythrocyte and plasma nitrites and (iv) haemoglobin and methemoglobin concentrations.

2.4.1. Erythrocyte deformability

Erythrocyte deformability was determined using the Rheodyn SSD laser difractometer from Myrenne (Roetgen, Germany) [19], following the guidelines of the International Committee for Standardization in Haematology [20].

The Rheodyn SSD difractometer determines erythrocyte deformability by simulating the shear stresses exerted by the blood flow and vascular walls on the erythrocytes. 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 erythrocytes 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 (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:

$$\mathrm{EI} = \frac{\mathrm{lumA} - \mathrm{lumB}}{\mathrm{lumA} + \mathrm{lumB}} \times 100.$$

2.4.2. Erythrocyte aggregation

Erythrocyte aggregation was determined using the MA1 aggregometer from Myrenne (Roetgen, Germany) [21].

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 by to ways: (a) in stasis during 10 seconds, and (b) at shear rate of 4/s during 5 seconds, after dispersion of the blood sample.

2.4.3. 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. 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 ($\lambda_{ex} = 352$ 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. [22].

2.4.4. Plasma potassium (K^+), sodium (Na^+) and calcium (Ca^{2+}) plasma concentrations and blood haemoglobin (Hb) and methemoglobin (metHb) concentrations

Plasma K^+, Na^+, Ca^{2+} concentrations were determined with the ABL505 electrode system from Radiometer (Copenhagen, Denmark). Hb and metHb values were determined with the Osm3 Hemoximeter from Radiometer.

2.4.5. Measurement of nitrite concentration in intra erythrocyte compartment and plasma using Griess reaction method [23]

The blood aliquots were centrifuged at 11,000 rpm for 1 minute using the Centrifuge Biofuge 15 Heraeus®. After that the plasma was isolated from the pellets (packaged erythrocytes and buffy coat). Finally the buffy coat was discarded. Nitrites concentration was measured both in plasma and intra-erythrocyte compartment.

For plasma nitrite concentration measurement, plasma was diluted $1/4$ and desproteinizated with $ZnSO_4$ (30%). After centrifuged at $3000g$ for 15 minutes, the supernatant was incubated with Griess reagents, sulfanilamide, HCl, and N-(1-napthyl)-ethylenediamine (NEDD) according to Griess Reagent Kit instructions from Molecular Probes. The absorbance of samples was measured at 548 nm relative to reference sample (Griess reaction and deionized water), in spectrophotometer Spectronic® 20 Genesys™. The nitrite concentrations were determined by comparison with a calibration curve (1–100 μM) of sodium nitrite in deionized water.

Nitrite concentrations in intra-erythrocyte compartment were measured after submission of the pellet of each suspension to haemolysis and haemoglobin precipitation. Haemolysis was induced with distilled water and haemoglobin precipitation with cold ethanol and chloroform. After vortex agitation the mixture was centrifuged at $9600g$ for 1 minute using the Centrifuge Biofuge 15 Heraeus®. Clear supernatants were processed as explained above.

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 several aliquots. For every parameter all samples were tested against the control. Statistical significance was considered for values of $p < 0.05$.

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

3. Results

3.1. RBC 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 the blood was incubated in presence of choline there was a significant increase of erythrocyte deformability at low shear stress values (Fig. 1).

As shown in Fig. 1, RBC deformability at shear stress values of 0.3 Pa increased significantly in blood aliquots incubated with choline 10^{-10} M (1.99 ± 1.61), 10^{-8} M (2.34 ± 1.61), 10^{-7} M (2.12 ± 1.42), 10^{-5} M (2.65 ± 1.84) and 10^{-3} M (1.79 ± 1.07) relatively to the control values (1.67 ± 1.40; $p < 0.05$). The same was verified at shear stress values of 0.6 Pa $p \leqslant 0.05$. At higher shear stress values no significant changes were verified on RBC deformability in presence of choline.

RBC aggregation (Fig. 2) assessed *in vitro* during 5 s at shear rate of 4/s decreased significantly in presence of choline 10^{-10} M (8.1 ± 1.85, $p < 0.02$), 10^{-8} M (8.14 ± 1.88, $p < 0.03$) and 10^{-5} M (9.04 ± 1.52, $p < 0.01$) relatively to the control (9.68 ± 1.35). When RBC aggregation was assessed during 10 s of stasis, its values only decreased significantly in presence of choline 10^{-10} M (11.70 ± 2.49) relatively to control (13.22 ± 2.03, $p < 0.04$).

3.2. RBC membrane fluidity

Erythrocyte membrane lipid fluidity was assessed by measuring fluorescence anisotropy of DPH probe on erythrocyte membrane being the control value 0.314 ± 0.03 (au). As showed in Fig. 3 the mean fluorescence anisotropy remained approximately the same between the control aliquot and when blood was incubated with choline except for choline 10^{-10} M for which it was observed a significant decrease of fluorescence anisotropy to 0.286 ± 0.03 (au), $p < 0.05$. A decrease of fluorescence anisotropy of DPH

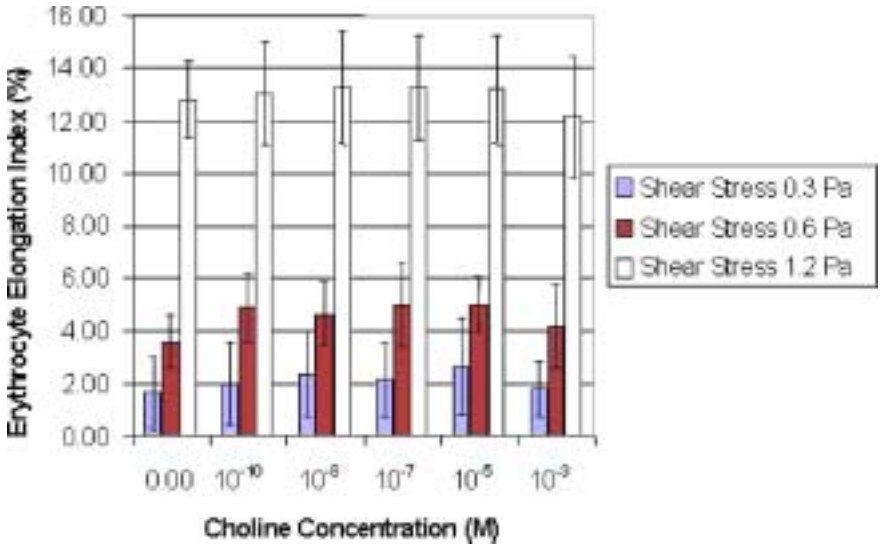

Fig. 1. Values (mean ± standard deviation) of the erythrocyte elongation index (%) at shear stress values between 0.3 and 1.2 Pa obtained after blood sample aliquots incubation without and with choline concentrations between 10^{-10} and 10^{-3} M during 15 minutes.

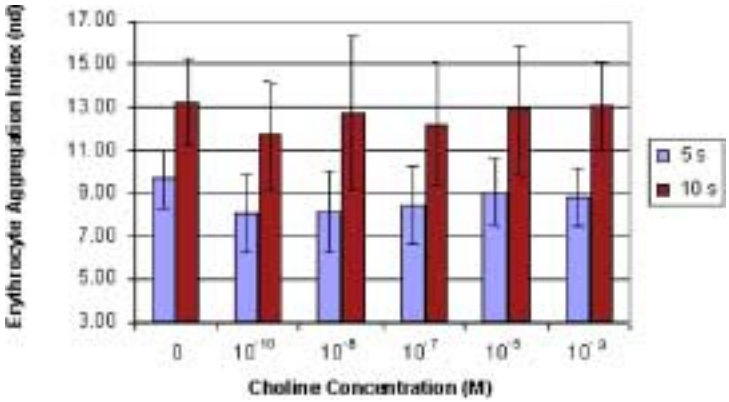

Fig. 2. Values (mean ± standard deviation) of erythrocyte aggregation index obtained with blood sample aliquots after incubation without and with choline concentrations between 10^{-10} and 10^{-3} M during 15 minutes.

probe means an increase of lipid fluidity of hydrophobic zone of the erythrocyte membrane. Although the value obtained with the choline concentration of 10^{-10} M was very similar to the choline concentration of 10^{-3} M, this last change was not statistical significant relatively to the control aliquot.

3.3. Effects of choline on plasma and intra-erythrocyte nitrites and methaemoglobin concentration

Figure 4 presents the total nitrites concentration determined in blood aliquots as described in Material and methods section. We decided to present the total nitrite concentrations because it was verified a direct proportionality between plasma and intra-erythrocyte concentrations.

Excluding the blood aliquot incubated in the presence of choline 10^{-10} M, we verified a direct proportionality between choline concentration in blood aliquots and nitrite concentrations measured, i.e.,

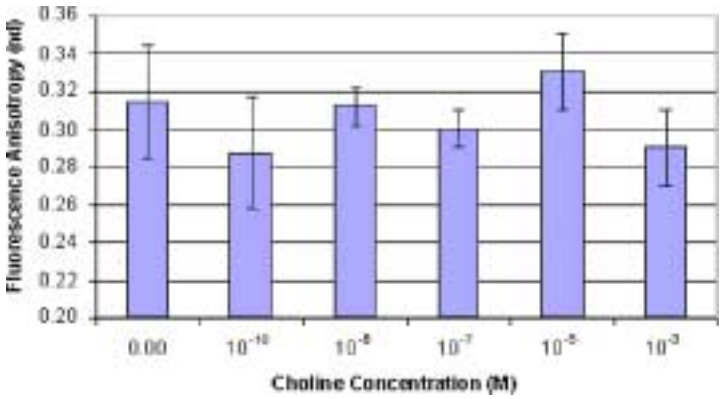

Fig. 3. Values (mean ± standard deviation) of fluorescence anisotropy values obtained with DPH probe introduced on human erythrocytes membrane after incubation without and with choline concentrations between 10^{-10} and 10^{-3} M during 15 minutes.

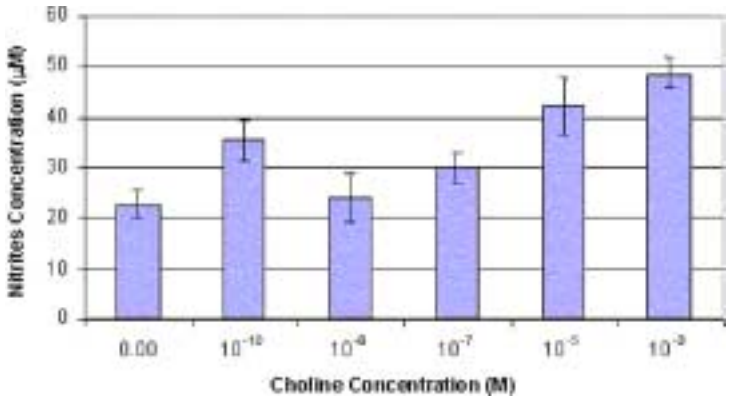

Fig. 4. Values of total nitrites concentrations determined in plasma and intra-erythrocyte compartment of blood aliquots incubated during 15 minutes without and with choline 10^{-10} to 10^{-5} M.

Table 1

Values of concentration of plasma Ca^{2+}, K^+, Na^+, and blood haemoglobin (Hb) and methemoglobin (MetHb) obtained after incubation without and with choline concentrations between 10^{-10} and 10^{-3} M during 15 minutes

	[choline] (M)					
	0	10^{-10}	10^{-8}	10^{-7}	10^{-5}	10^{-3}
$[Ca^{2+}]$ (mM)	1.17 ± 0.02	1.17 ± 0.01	1.17 ± 0.02	1.16 ± 0.01	1.16 ± 0.03	1.16 ± 0.02
$[K^+]$ (mM)	3.84 ± 0.20	3.83 ± 0.13	3.83 ± 0.16	3.85 ± 0.11	3.84 ± 0.13	3.79 ± 0.11
$[Na^+]$ (mM)	139.27 ± 1.90	138.45 ± 1.97	138.73 ± 1.74	138.09 ± 2.17	137.73 ± 2.20	137.55 ± 2.07
pH	7.40 ± 0.03	7.40 ± 0.02	7.40 ± 0.03	7.41 ± 0.03	7.40 ± 0.03	7.41 ± 0.02
MetHb (%)	1.63 ± 0.16	1.58 ± 0.14	1.60 ± 0.16	1.60 ± 0.14	1.59 ± 0.11	1.60 ± 0.08

there was an increase of nitrite concentrations with choline concentrations ($p < 0.05$). In the presence of choline 10^{-10} M there was also a significant increase of nitrite concentrations ($p < 0.05$) but higher than that one verified in the presence of choline 10^{-8} M and 10^{-7} M.

No significant changes were verified on methemoglobin percentage as showed in Table 1.

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

As shown in Table 1, sodium plasma concentrations decreased when blood aliquots were incubated in presence of choline 10^{-10} M (138.45 ± 1.98, $p < 0.03$), 10^{-7} M (138.09 ± 2.17, $p < 0.005$), 10^{-5} M (137.73 ± 2.19, $p < 0.009$) and 10^{-3} M (137.54 ± 2.07, $p < 0.002$) relatively to the control (139.27 ± 1.90). Potassium and calcium plasma concentrations did not change significantly in presence of choline relatively to the control blood aliquot.

4. Discussion

The results of this study showed that choline induces changes in human erythrocytes haemorheological properties (erythrocyte aggregation and deformability), lipid membrane fluidity and plasma sodium concentration. Additionally, choline induces significant changes on plasma and intra-erythrocyte nitrites concentration.

Erythrocyte aggregation and deformability are two close-related properties that influence blood viscosity in different places in vascular system [8]. Under normal blood flow, erythrocyte aggregation occurs predominantly in post-capillary venules where there is a decrease in vascular shear stress. Erythrocyte deformability is a property of erythrocytes that is essential to their passage in narrowed capillaries with lower diameters than erythrocytes. Lower erythrocyte deformability ability and increased erythrocyte aggregation tendency has been described in patients with essential hypertension [24,25] and abnormal haemorheological profile has been associated with cardiovascular disease and events development [26, 27]. Both erythrocyte deformability and aggregation depend on a normal compliance of erythrocyte membrane [28] and are properties influenced by the membrane structural and dynamic features [29]. For example, Mohandas [30] reported an association between erythrocyte deformability at lower shear stress and membrane lipid fluidity which is an index of order and rate of phospholipids movement in the bilayer. Evidence of the association between those two parameters has been observed also in erythrocyte of patients with alcoholic liver disease [31] and also in healthy volunteers submitted to a enriched omega-3 polyunsaturated fatty acid diet [32].

For the first time as far as we know in our *in vitro* study we verified that choline 10^{-10} M decreases erythrocyte aggregation and increases deformability (at lower shear stress), when present in aliquots blood samples of healthy humans (Figs 1, 2). Also choline 10^{-10} M induces an increase of lipid membrane fluidity verified by lower values of fluorescence anisotropy measured by DPH probe (Fig. 3) relatively to the control, which could be one factor among others that could contribute to the observed increased erythrocyte deformability in accordance with Mohandas statement [30]. However, for the other blood samples aliquots incubated in presence of choline concentrations namely 10^{-8} to 10^{-3} M there was no erythrocyte membrane fluidity changes relatively to the control, which support the importance of other factors despite the phospholipid fluidity. Among the parameters that influence erythrocyte aggregation and deformability we can consider the structural and functional of the cytoskeleton proteins [30,33]. However, the mechanism by which choline interferes either with membrane fluidity or cytoskeleton proteins are unknown. As mentioned before choline 10^{-10} M induce decreased erythrocyte aggregation and increased erythrocyte deformability allowing the red blood cells easily change reversible its shape likely as previously achieved in other *in vitro* study when, the ester of choline the acetylcholine was added to blood samples [7]. Both acetylcholine and choline have in common a quaternarium ammonium ion in its structure formula, being the first the natural substrate of the red blood cell membrane bound enzyme acetylcholinesterase, and the second its product of hydrolysis and also a reversible inhibitor [16,

34]. We could rise the hypothesis that both compounds besides its different physiological purposes could participate in similar transmembrane signalling process inducing perturbations which simultaneously the disaggregating forces overcame the aggregation forces and facilitated the erythrocyte deformability. An opposite effect was described in incubation medium of erythrocyte suspensions loaded with dextran where a similar mechanism was advanced [35].

Choline transport systems previously described in the erythrocyte [17] support the entrance of choline into the erythrocyte in a symport way with sodium ions and HCO_3^- where it can either interact with cytoskeleton proteins or the polar head phospholipids, and or be incorporated in membrane phospholipids, or even be oxidised, as described in other cell types to betaine [36]. Decreased values of extracellular sodium ions concentration were obtained in the blood samples aliquots incubated in presence of choline 10^{-10} to 10^{-3} M when compared with the values in its absence. Besides no influx measurement was performed in the present study the choline uptake could occur and may be cotransport with the choline influx. So if the presence of choline inside the erythrocyte was assumed it could be responsible directly or indirectly in the mechanisms that contributed to the increased deformability obtained in our study which deserve further investigation.

Intracellular K^+ and Na^+ concentrations are regulated by Na^+/K^+ ATPase that maintains high intracellular K^+ concentration and high extra-cellular Na^+ concentration [37]. Increased Na^+ intra-erythrocyte concentrations as a consequence of decreased Na^+ plasma concentration could affect the Na^+/K^+ ATPase however no repercussions in plasma K^+ ion concentrations neither in plasma pH was observed (Table 1). This did not means absence of occuring changes in others ions transport systems present in erythrocyte membranes [38–40]. So the decreased plasma sodium level could contribute to the impaired erythrocyte aggregation by lowering plasma osmolality which has been described as an influent parameters [41].

Additionally, choline can interact with muscarinic receptors at neuronal and cardiac level [42,43] and for the erythrocyte membrane has been described the existence of M1 and M3 muscarinic receptor agonist properties [44]. One of these studies refers that choline induces a sustained plateau of increased intracellular calcium and stimulates nitric oxide production. Although these type of studies were not conducted yet in erythrocytes, their conclusions could be hypothetically extrapolated for erythrocytes but no changes of the plasma calcium ion concentration was verified in our work in any blood samples aliquots incubated in presence of choline when in relation to the control aliquot. Otherwise significantly increased levels of NO metabolite nitrites were obtained in all blood samples aliquots in presence of choline regarding its absence (Fig. 4). Previous work performed with erythrocyte suspensions showed that at presence of choline 10^{-6} M and 10^{-5} M the values of nitrite concentrations were higher than in its absence [45]. The changes obtained in nitrite content induced by the presence of choline occurred with erythrocyte rheological properties improvement rising the hypothesis of a nitric oxide dependent mechanism existence. The effect of the leptin on the increased membrane lipid fluidity was explained by via a nitric oxide metabolism [46].

Nitric oxide (NO) is a highly reactive gas interacting with various constituents of erythrocyte membrane [47] and also with haemoglobin where it binds to the haeme group and also with the cysteine 93 on the itself β-chain [48]. The major biological end products of NO metabolism are nitrite (NO_2^-) and nitrate (NO_3^-) the last one obtained from the NO interaction with oxyhemoglobin, with conversion into methemoglobin. NO reacts with superoxide anion ($O_2^{-\bullet}$) to form peroxynitrite, that gives either nitrate or reacts with proteins, lipids, carbohydrates through oxidation and nitration mechanisms. NO and its metabolites can stimulate lipid peroxidation [49]. In contrast, when the concentrations of NO exceed that

of O_2^-, lipid peroxidation can be inhibited by NO, with a concurrent formation of nitrated lipid radical termination products [44,50].

5. Conclusion

In conclusion, this study showed that choline is able to induce changes on the erythrocyte at membrane level and at NO metabolism, namely impaired and increased erythrocyte aggregation and deformability respectively as well as increasing the level of nitrites.

These results are important, because, they help to understand the effects of cholinergic substances on blood rheological properties, that may be an important step and another contribution for understanding the blood circulation physiology and pathology. By other side, we have a lot of drugs that act changing the production of acetylcholine, and choline and perhaps, these results could improve the knowledge of its action at the circulatory level.

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