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Original article

Effects of various osmolarity on human red blood cells in terms of potassium efflux and hemolysis induced by free radicals

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Abstract

Objectives: To study in an *in vitro* model of erythrocytes exposed to free radicals the effects of hyperosmolarity and hypoosmolarity on the induced potassium efflux and hemolysis.
Design setting: Erythrocytes were separated from plasma and suspended in 3 different phosphate buffer solutions (HYPO, ISO, and HYPER) containing, respectively, 100, 150 and 200 mmol/l of Na. Free radicals were generated from 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH). Potassium efflux (flame photometry) and hemolysis (Drabkin method) were measured. Measurements were expressed in % (versus total) and area under % versus time curves were calculated (% min). An ANOVA was used for statistical analysis.
Results: In presence of AAPH, hemolysis was significantly greater in HYPO (732.45 ± 40 % min) and lower in HYPER (578.97 ± 15 % min) as compared to ISO (608.30 ± 42 % min). Potassium efflux was significantly increased in HYPER (7508.04 ± 85 % min) as compared with HYPO (5308.74 ± 62 % min) and ISO (6561.39 ± 62 % min).
Discussion: Our data suggest that hyperosmolarity increases the resistance of the red blood cells when exposed to free radicals as shown by the decreased hemolysis. In such conditions, the potassium efflux analysis appears to be inappropriate to evaluate the free radical effects on erythrocytes.

Keywords: Erythrocytes; Oxidative stress; Sodium; Cell damage; Pro-oxidant

1. Introduction

Red cell tolerance toward an exposition to free radicals may be greatly modified by many environmental factors or molecules such as drugs [1], vitamin E [2] or free radicals scavenger [3]. In normal conditions, variations of osmolarity may interfere with the cell membrane polarization or function and induce cell shape changes [4–6] that may also modify the behavior of erythrocytes in presence of free radicals. However, very little is known on the effects of modification in natremia and osmolarity on red cells exposed to free radicals. We, therefore, conducted this *in vitro* experiment to precise this point.

2. Materials and methods

2.1. Oxidative stress

The 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) (Interchim, Montluçon, France) was used as a pro-oxidant to generate free radicals. AAPH has already been used for similar studies and it was found to produce alkoxyl free radicals in a time- and concentration-dependant manner [7,8]. At the concentration of 20 mM, AAPH induces

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a hemolysis that occurs roughly after 2 h of exposure, and for higher concentrations, hemolysis is observed earlier [7,9]. For low concentrations of AAPH, the potassium efflux was found to be an early indicator of the cell damage induced by the free radicals [10].

2.2. Erythrocyte preparation

Fresh red blood cells were obtained by vein puncture from 5 healthy volunteers and studied separately. Erythrocytes were separated from blood plasma by centrifugation (5 min at 1500 rev min^{-1}, at 4 °C) and washed 5 times in warm isotonic saline solution (NaCl 0.9%). The supernatant and buffy coat were carefully removed after each wash. Three different phosphate buffer solutions were prepared using Na_2PO_4H and $NaPO_4H_2$ solutions and various quantities of NaCl: HYPO contained 100 mM of Na (measured osmolarity was 176 mosm l^{-1}), ISO 150 mM of Na (measured osmolarity 272 mosm l^{-1}) and HYPER 200 mM of Na (measured osmolarity 357 mosm l^{-1}). The pH of the different buffer solutions was kept constant at 7.40.

After separation, erythrocytes were suspended in the phosphate buffer solutions, in order to get a 20% suspension, depending on the group they belong to (see below) and incubated at 37 °C.

2.3. Groups

For each experiment 6 groups were studied: incubation in absence of AAPH (HYPO−, ISO− and HYPER−) or in presence of 20 mM AAPH (HYPO+, ISO+ and HYPER+).

2.4. Potassium efflux measurement

At time = 0 and, every 30 min during 2 h, 0.4 ml of the suspension was drawn, added to 0.75 ml of saline solution and centrifuged at 1500 rev min^{-1} at 4 °C for 5 min. Supernatant of 0.25 ml was added to 4.75 ml of phosphate buffer and the extracellular concentration of potassium was measured by flame photometry (Flame Photometer 410, CIBA Corning, France). For each sample and at each time, the concentration of extracellular potassium was normalized, according to the concentration of potassium measured when total hemolysis was achieved by saponification (Triton X-100, BDH Chemicals Ltd., Poole, England) and was expressed in %. The evolution of the extracellular concentration of potassium versus time was studied. To quantify the total potassium efflux, areas under the concentration of extracellular potassium versus time curves (AUC) were calculated and expressed in % min [11].

2.5. Hemolysis

At time = 0 and at 30, 60, 90 and 120 min, 0.4 ml of the suspension was drawn, added to 0.75 ml of saline solution and centrifuged at 1500 rev min^{-1} at 4 °C for 5 min. Supernatant of 0.25 ml was added to 4.75 ml of phosphate buffer and all the forms of hemoglobin present in the extracellular medium were measured using the Drabkin method [12]. Measurements were not performed at 30 min because previous studies have shown that AAPH, at the concentration of 50 mM, induces hemolysis that occurs after 60 min. For each sample and at each time, the concentration of hemoglobin was normalized, according to the concentration of hemoglobin measured when total hemolysis was achieved by saponification (Triton $X^{®}_{100}$, DBH Chemicals Ltd., Poole, England) and was expressed in %. The evolution of the extracellular concentration of hemoglobin versus time was studied. To quantify the total hemolysis, areas under the concentration of hemoglobin versus time curves (AUC) were calculated and expressed in % min [11].

2.6. Statistical analysis

Data were expressed as mean ± S.D. For statistical analysis, an analysis of variance was used and followed by a Tukey test. A $p < 0.05$ was considered as significant.

3. Results

In the absence of AAPH, no significant potassium efflux or hemolysis was noted during the 2 h of experiment, and no difference was observed between the different types of buffer solutions (Table 1).

When erythrocytes were incubated with AAPH, an expected hemolysis occurred in all groups (Fig. 1). Hemolysis was significantly greater ($p < 0.05$) in HYPO+ and lower, but not significantly in HYPER+ as compared to ISO+ (Table 2). A potassium efflux was also observed during the 2 h of incubation (Fig. 2). The potassium efflux was significantly increased with higher osmolarity (Table 2).

4. Discussion and conclusion

When erythrocytes are incubated with AAPH, the induced potassium efflux and hemolysis are thought to involve an alteration of the red cell membrane [13] including many processes such as a modification of the band 3 protein [8,14] and lipid peroxidation [15]. Variations of extracellular sodium concentration induce modifications in erythrocytes volume and ion transport [4,5] that may modify the resistance of red cells exposed to free radicals. As hypernatremia and hyponatremia are frequent events in clinical fields involving an oxidative stress we decided to conduct this *in vitro* work to study the effects of various extracellular sodium contents and osmolarity

Table 1
Potassium efflux expressed in % (compared to total hemolysis induced by saponification) and hemolysis after 2 h of experiment in absence of AAPH

	HYPO−	ISO−	HYPER−
Efflux K+ (%)	6.18 ± 1.04	5.83 ± 0.98	5.92 ± 0.88
Hemolysis (%)	4.15 ± 0.87	4.27 ± 0.79	3.93 ± 0.68

No significant variations are noted as compared to T_0 and between the 3 groups.