

## INFLUENCE OF FLUORIDE IONS ON $\text{Na}^+\text{-H}^+$ EXCHANGER ACTIVITY IN HUMAN RED BLOOD CELLS

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**Summary:** The influence of fluoride ion ( $\text{F}^-$ ) at concentrations of 0.25 and 2.5 mM on intracellular pH and  $\text{Na}^+\text{-H}^+$  exchanger (NHE) activity in human red blood cells was investigated. Erythrocytes from 15 healthy individuals were examined. We found that  $\text{F}^-$  caused a decrease in NHE activity and an increase in intracellular  $\text{H}^+$  ion concentration (decrease of intracellular pH).

**Keywords:** Erythrocytes, Fluoride, Intracellular pH, Red blood cells, Sodium-proton exchanger

### INTRODUCTION

Fluoride ions can inhibit or activate various functions in blood cells. Neutrophils affected by fluoride exhibit increased oxygen intake and production of superoxide anion along with decreased phagocytic ability.<sup>1</sup> At concentrations typical for fluorine intoxication,  $\text{F}^-$  (2.5 mM) (*in vitro* studies) has a toxic effect on clonogenicity of human progenitor cord blood cells<sup>2</sup> and bone marrow haematopoietic cells.<sup>3</sup>

Fluoride ion affects erythrocyte membrane transport systems, *e.g.* inhibits  $\text{K}^+\text{-Cl}^-$  co-transport<sup>4</sup> in erythrocytes suspended in hypoosmotic medium. Alterations in cation pump activity caused by fluoride occur as a direct inhibition of  $\text{Na}^+\text{-K}^+\text{-ATP-ase}$ .<sup>5</sup> This can be observed in erythrocyte shadows in people exposed to fluoride under both *in vitro* and *in vivo* conditions.<sup>6</sup> Fluoride ion *in vivo* decreases ATP concentration in erythrocytes.<sup>7</sup>

The aim of the present study was to evaluate the influence of fluoride on  $\text{Na}^+\text{-H}^+$  exchanger (NHE) activity in human red blood cells. The role of NHE is to trade extracellular  $\text{Na}^+$  for intracellular  $\text{H}^+$ , thereby protecting the cell from excessive acidification. It is also known that intracellular pH changes often initiate cell growth or changes in its functional status. In this way NHE may control cell proliferation and other cell functions such as secretion of hormones. Another important function of NHE is cell volume regulation and absorption of  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{HCO}_3^-$  in proximal nephron and intestine epithelial cells. NHE activity has been found in almost all investigated cell types. Being easy to obtain human erythrocytes are valuable for NHE research. Moreover alteration in the  $\text{Na}^+\text{-H}^+$  exchanger activity is considered one of the mechanisms contributing to the development of essential hypertension. In addition the relationship between erythrocyte  $\text{Na}^+\text{-H}^+$  ex-

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changer activity and a predisposition to nephropathy in patients with type 1 and type 2 diabetes has also been demonstrated.<sup>8</sup>

#### MATERIALS AND METHODS

Erythrocytes from 15 patients (6 men, 9 women), aged 23 to 53 years (mean age  $33 \pm 9$  years), were examined (Table 1). Subjects with diseases that may affect NHE activity (i.e., primary hypertension, diabetes mellitus and hyperthyreosis) and patients with positive family history of these diseases were excluded from the study. The protocol of the study was approved by the Bioethics Committee of the Pomeranian Academy of Medicine. All the patients provided written informed consent.

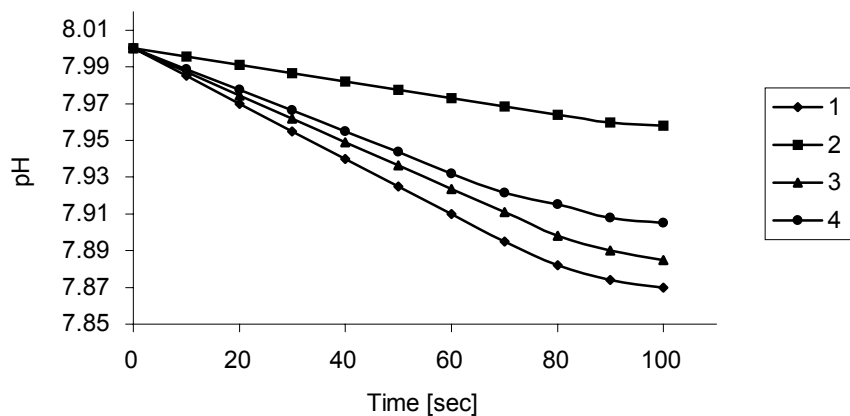
**Table 1.** The influence of Na<sup>+</sup>-H<sup>+</sup> exchanger (NHE) activity and intracellular pH

Subject	Sex	Age	NHE activity [mmol/l cell/hr]			pH		
			F <sup>-</sup> [mmol/L]			F <sup>-</sup> [mmol/L]		
			0	0.25	2.5	0	0.25	2.5
1	F	27	7.938	6.502	5.292	7.39	7.34	7.31
2	F	27	13.230	9.450	7.182	7.42	7.36	7.28
3	F	27	20.280	10.096	5.301	7.34	7.28	7.28
4	M	28	7.500	5.906	5.632	7.37	7.33	7.30
5	F	27	9.935	6.503	5.335	7.44	7.33	7.32
6	M	29	5.286	4.540	9.828	7.43	7.34	7.33
7	F	29	4.809	5.504	4.069	7.32	7.26	7.25
8	M	28	7.798	6.872	5.995	7.29	7.25	7.24
9	M	43	6.952	7.472	10.147	7.45	7.37	7.37
10	M	29	9.923	11.207	10.008	7.30	7.24	7.22
11	F	44	7.180	10.156	7.708	7.32	7.21	7.17
12	M	43	9.067	7.316	7.100	7.37	7.23	7.24
13	F	23	5.576	6.460	3.641	7.35	7.26	7.22
14	F	40	11.926	9.306	6.031	7.42	7.38	7.37
15	F	53	8.958	8.388	7.885	7.39	7.29	7.28

Blood was collected in 10-mL test tubes containing EDTA. The following red blood cell parameters were assessed: MCV (mean corpuscular volume), MCHC (mean corpuscular haemoglobin concentration), and MCH (mean corpuscular haemoglobin). Blood was centrifuged and plasma and buffy coat were aspirated. The cells were washed three times at 37°C with solution containing 150 mM NaCl, 5mM HPO<sub>4</sub> at pH 7.4 (PBS – phosphate buffer solution).

Erythrocytes (hematocrit – 0.15) were suspended and periodically shaken in PBS solution containing 0.25 or 2.5 mM NaF solution and incubated at 37°C for 30 min. The cells were then centrifuged at 1850 g for 10 min at 4°C

and washed three times with PBS solution. Assessment of the red blood cell parameters was made and intracellular pH and NHE activity according to Orlov's method were measured.<sup>9</sup> NHE activity in red blood cells was quantified as amiloride-sensitive H<sup>+</sup> efflux from acid-loaded cells. Packed (Ht 0.85) erythrocytes (200  $\mu$ L) were suspended in 3.8 mL of a solution containing 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM KCl and 10 mM glucose. The medium was then acidified (pH 6.35-6.45) by 0.2 M HCl solution in 150 mM NaCl. To inhibit the anion exchanger as a main pathway of pH intracellular regulation, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, 200  $\mu$ M, Sigma-Aldrich) was added 10 minutes later. After the pH was adjusted to 7.95-8.00 by 0.05 M NaOH solution in 150 mM NaCl, the medium acidification rate (pH) was measured at 10 second intervals for 60 second. Measurements were performed with a pH-meter (Orion 920 A) with and without amiloride (N-amidino-3,5-diamino-6-chloropyrazinecarboxamide) (0.5 mM, Sigma-Aldrich), an inhibitor Na<sup>+</sup>-H<sup>+</sup> exchanger (Figure).



**Figure.** Kinetics of H<sup>+</sup> efflux from human erythrocytes in the absence (1) and presence of inhibitors: amiloride (2) 0.25 mmol/L NaF (3) and 2.5 mmol/L NaF (4).

The rate of Na<sup>+</sup>-H<sup>+</sup> exchange was determined as  $(\Delta\text{pH}_1 - \Delta\text{pH}_2) \cdot b \cdot \text{m}^{-1} \cdot \text{t}^{-1} \cdot \text{c}^{-1}$ , where  $\Delta\text{pH}_1$  and  $\Delta\text{pH}_2$  are initial rates of the medium acidification in the presence and absence of amiloride,  $b$  is the buffer capacity of the incubation medium (determined before each experiment by titration with NaOH and

HCl),  $m$  is the volume of erythrocytes [ $2 \times 10^{-4}$ L],  $t$  is time in seconds, and  $c$  is hematocrit of the packed erythrocytes – about 0.85 L/L).

Evaluation of intracellular pH was carried out with a Beckman microelectrode and by a pH meter (Orion 920 A) after erythrocyte hemolysis in distilled water (100  $\mu$ L packed RBC + 400  $\mu$ L distilled water). Statistical analysis was performed with ANOVA and Wilcoxon's test. The results of ANOVA enabled us to use Wilcoxon's pair test.<sup>10</sup>

## RESULTS

The experiments were performed at pH 7.4 with results shown in Tables 1 and 2. The mean erythrocyte intracellular pH was 7.36, and after incubation in solution containing fluoride at 0.25 and 2.5 mM concentrations it decreased to 7.29 and 7.28 ( $p = 0.011$ ), respectively. Changes of intracellular pH were not related to fluoride concentration (no statistically significant differences between pH<sub>i</sub> in erythrocytes incubated in 0.25 and 2.5 mM fluoride). Erythrocyte parameters (mean corpuscular haemoglobin concentration [MCHC], mean corpuscular haemoglobin [MCH], and mean corpuscular volume [MCV]) did not change after incubation of erythrocytes in fluoride solutions.

After incubation with fluoride, the mean NHE activity in erythrocytes decreased by 15% from 9.09 mmol H<sup>+</sup>/L cell/hr to 7.71 mmol H<sup>+</sup>/L cell/hr for 0.25 mM F<sup>-</sup> concentration, and by 25.8% to 6.74 mmol H<sup>+</sup>/L cell/hr for 2.5 mM F<sup>-</sup> concentration ( $p = 0.047$ ).

**Table 2.** The influence of F<sup>-</sup> on NHE activity, intracellular pH, and erythrocyte parameters. Statistical characteristics are shown below the entries

F <sup>-</sup> [mmol/L]	NHE [mmol H <sup>+</sup> /L cell/hr]	pH	MCV [fL]	MCH [fmol]	MCHC [mmol/L]
0	9.09 ± 3.88	7.36 ± 0.052	89.18 ± 3.38	1.87 ± .07	21.07 ± 0.27
0.25	7.71 ± 1.95 p* = 0.069	7.29 ± 0.055 p = 0.001	89.27 ± 2.63 NS	1.87 ± 0.07 NS	21.25 ± 0.2 NS
2.5	6.74 ± 2.06 P† = 0.047	7.28 ± 0.56 p = 0.001	89.09 ± 3.12 NS	1.89 ± 0.08 NS	21.25 ± 0.2 NS

Values are means ± SD; \* comparison between control and 0.25 mM F<sup>-</sup>;

† comparison between control and 2.5 mM F<sup>-</sup>.

### DISCUSSION

The present study showed that 0.25 mM and 2.5 mM F<sup>-</sup> have a significant influence on intracellular pH and NHE (Na<sup>+</sup>-H<sup>+</sup> exchanger) activity. The intracellular pH decrease in erythrocytes exposed to F<sup>-</sup> confirms the theory about F<sup>-</sup> transfer through cellular membrane as HF molecules, but not in ionised form.<sup>11</sup> This process is opposed to the pH gradient – cells with intracellular pH of 7.36 incubated in a medium at pH of 7.4 in the presence of F<sup>-</sup> changed to an intracellular pH of 7.28.

One of the effects of transmembrane fluoride transfer as HF is that the toxic F<sup>-</sup> doses have less influence on organisms with alkalosis than on organisms with acidosis.<sup>11</sup>

In contrast to our studies, Kolosowa's research<sup>12</sup> on the influence of F<sup>-</sup> on NHE activity showed no changes of NHE activity in basal conditions and significant increase in NHE activity in hyperosmotic conditions. Increase in NHE activity was found in muscle cells in hyperosmotic conditions, but F<sup>-</sup> was used in 10 mM concentration (4 times higher than in our research), and any increase in NHE activity due to shrinking of the cells in hyperosmotic conditions was not taken into account.<sup>13</sup>

In our study, we did not find any increase in cell volume from F<sup>-</sup> concentrations below 2.5 mM.

Transmembrane sodium-ion proton exchange is an electroneutral reaction, requiring the presence of ATP. NHE is an example of secondary active transport, where energy derived from the Na<sup>+</sup> gradient gained by the Na<sup>+</sup>/K<sup>+</sup>-ATPase secondarily drives the exchange of extracellular Na<sup>+</sup> for intracellular H<sup>+</sup>.<sup>14</sup>

A high ATP concentration is a known factor essential for normal function of not only NHE but other cation exchangers as well.<sup>15</sup> Other authors report inhibition of NHE activity in cell cultures after ATP elimination.<sup>16,17</sup>

One of the negative effects of F<sup>-</sup> influence on organisms is the alteration of cell energetic processes. Compared to controls, erythrocytes from aluminium factory workers show a 50% decrease in ATP.<sup>18</sup> Erythrocytes from people living in areas polluted with fluorine compounds, unexposed to their direct influence, also show decrease in ATP.<sup>19</sup> Lower ATP concentrations in erythrocytes were also found in animals fed with fodder from regions polluted with fluoride compounds (aluminium factories).<sup>20</sup>

Our results also suggest that inhibition of NHE activity by F<sup>-</sup> is due to a decrease in ATP concentration in the cell and that this intracellular pH decrease results from transmembrane transfer of fluoride in the form of hydrogen fluoride.

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