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Hemorheological effects of sodium fluorescein in rats

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Abstract. Sodium fluorescein is widely used in clinical practice for the study of the retinal circulation by angiography. It has been reported several hemorheological and microvascular abnormalities induced by this compound. The aim of this work was to analyse the hemorheological effects of intravenous sodium fluorescein in an animal model. Twenty male 10–16 weeks-old Wistar rats were used, under systemic anaesthesia. The animals were divided in 2 groups of 10 each: (1) intravenous injection of sodium fluorescein (14 mg/kg of body weight) – Group NaF, (2) controls (injection of NaCl 0.9%) – Group CTRL. A blood sample was drawn by aortic puncture after 60 minutes and hemorheological parameters determined: hematocrit, hemoglobin, metahemoglobin, carboxyhemoglobin, plasma viscosity, erythrocyte deformability, membrane fluidity and acetylcholinesterase activity. In the Group NaF there was a 20% reduction of the AChE activity ($p < 0.05$) and an increase in PV ($p < 0.05$). Concerning hemoglobin status, a three-fold increase in COHb ($p < 0.001$) was shown. In conclusion, the NaF injection in the animal model produces hemorheological abnormalities similar to those reported in the human.

Keywords: Sodium fluorescein, plasma viscosity, erythrocyte membrane, membrane fluidity, carboxyhemoglobin, erythrocyte deformability, rats

1. Introduction

Sodium fluorescein (NaF) has been widely used as a contrast dye in clinical practice mainly for the investigation of the chorioretinal microcirculation. Several fluorescein derivatives are also used in fluorescence techniques for microcirculatory research. Sodium fluorescein (NaF) circulates in blood bound to plasma proteins (namely albumin) and the erythrocyte membrane [1], however, it does not penetrate into the erythrocyte [2]. Its presence in circulation has been linked to the occurrence of detrimental effects in blood homeostasis [3–5].

There is evidence that NaF interferes with the erythrocyte membrane [3]. In clinical setting, NaF angiography increases erythrocyte aggregation along with prolongation of retinal circulation time [4]. Sargento et al. [5] reported that, following NaF injection for retinal angiography in diabetic patients, whole blood viscosity increased, erythrocyte acetylcholinesterase (AChE) activity decreased, while higher erythrocyte aggregation was correlated with decreased erythrocyte deformability and increased membrane rigidity. Increased red blood cell carboxyhemoglobin and methemoglobin levels were also described. These observed modifications on blood rheology and properties of the erythrocyte membrane are likely to be linked to a detrimental effect on the microcirculatory blood flow [6].

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The objective of this work was to determine the hemorheologic effects of NaF in an animal experimental model.

2. Materials and methods

2.1. Animals

Male Wistar rats 10–16 weeks old ($n = 20$) were used. All animal experiments were performed according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and the European Union Directive 86/609/CEE. Surgical anesthesia was achieved with intramuscular urethane (Sigma, St. Louis, MO) 1.5 g/kg. A polyethylene catheter was placed in the right jugular vein for the intravenous injection of substances. Throughout the experiments the animals were allowed to breathe spontaneously, kept hydrated with the subcutaneous injection of 0.9% NaCl (3 ml/kg/h, while maintaining the body temperature with an heating lamp).

2.2. Hematologic counts

Leukocyte count (with differential granulocyte and mononuclear cell count), as well as hematocrit, erythrocyte count and mean corpuscular volume (MCV), were measured with a Cell Dyn 1600 hemocytometer (Abbott, Abbot Park, IL), using blood anticoagulated with K_3EDTA.

2.3. Erythrocyte membrane functional properties

These were assessed from blood anticoagulated with sodium heparin using the following parameters: erythrocyte membrane fluidity, erythrocyte acetylcholinesterase activity and the erythrocyte deformability.

(1) Erythrocyte membrane lipid fluidity was determined by means of fluorescence polarisation with two probes: (i) 1,6-biphenyl-1,3,5-hexatriene (DPH) and (ii) 1,4-(trimethyl)-phenyl-6-phenylhexa-1,3,5-triene (TMA-DPH), for hydrophobic and external polar region, respectively [7]. Increased values of fluorescence polarization are associated with lower probe molecules rotation within the membrane layer, meaning increased membrane rigidity. For both probes, it was used a Hitachi F3000 (Hitachi, Tochio, Japan) spectrofluoremeter. Both probes were obtained from Molecular Probes, Eugene, USA.

(2) Erythrocyte AChE activity was determined by Kaplan's modified method [8]. Erythrocyte AChE is a membrane enzyme localised on the outer side of the membrane. This erythrocyte enzyme expresses membrane integrity [9] since its catalytic activity can be modulated both by extraglobular effectors and indirectly by membrane phospholipid interaction or cytoskeleton modulation; intrinsic membrane fluidity changes are reported to modify AChE activity [10].

(3) Erythrocyte deformability capacity was calculated using a Rheodyn SSD laser diffractometer (Myrenne, Roetgen, Germany). Twenty μl of whole blood were diluted in 2 ml dextran solution (Pharmacia; osmolality 0.300, and pH 7.4, viscosity 0.24 Poise). This suspension was introduced into a measuring chamber formed by two glass disks, one static and the other connected through a rotational arm to a synchronised step motor. A 1 mV helium–neon laser beam is passed through the blood suspension, and the diffraction pattern analysed at eight shear stress forces (0.30/0.60/1.20/3.0/6.0/12.0/30.0/60.0 Pa). In the static position at rest, the laser diffraction pattern is circular, becoming elliptic as the erythrocytes are deformed by the application of higher shear stress forces. Light intensity and the diffraction pattern were

measured in two orthogonal axes and the erythrocyte elongation index (EEI) calculated from the length (L) and width (W): EEI $= 100 \times (L - W)/(L + W)$ [11].

(4) Hemoglobin, carboxyhemoglobin and methemoglobin were determined with an Osm 3 Hemoximeter (Radiometer, Copenhagen, Denmark), using the parameters set for analysis of rat hemoglobin.

Plasma viscosity. Anticoagulated with K_3EDTA were centrifuged at 12000 r.p.m. for 1 minute, and the resulting plasma was collected for the determination of plasma viscosity with the Harkness method [12]. Additionally, plasma osmolality was assessed with an Osmomat 0.30 Cryoscopic Osmometer (Gonotec, Berlin, Germany).

Plasma proteins. The protein concentration in plasma was determined by the Biuret method. The electrophoresis of plasma proteins was then performed by the application of 10 μl of plasma in agarose gel (pH 8.8) in a semi-automated Hydrasis (SEBIA, Issy-les-Molineaux, France) system. After coloration with amido black, the density of protein bands was measured in Hyris densitometer (SEBIA, Issy-les-Molineaux, France).

2.4. Experimental protocol

A solution of NaF (Sigma, St. Louis, MO) 14 mg/ml in NaCl 0.9% was prepared. The rats were divided into two groups. In one group (Group NaF; $n = 10$) the animals received an intravenous bolus of NaF 14 mg/kg; this dose was chosen from previous works [13]. The other group of animals were controls (Group CTRL; $n = 10$), receiving an equivalent intravenous bolus of NaCl 0.9%. Sixty minutes after fluorescein/NaCl 0.9% intravenous administration the animals were killed by exsanguination from the abdominal aorta, and blood samples were collected and anticoagulated with K_3EDTA (2 mg/ml) or sodium heparin (25 U/ml), as adequate for further determinations.

2.5. Statistics

Results are presented as mean $\pm$ standard deviation. Analysis was performed using the Student group t-test. Significance was considered if $p < 0.05$.

3. Results

Hematologic counts. There was a non significative increase in the number of circulating leukocytes in Group NaF. Additionally, there was no variation of the leukocyte differential count. Both groups were similar concerning the erythrocyte count, hematocrit and MCV (Table 1).

Erythrocyte membrane functional properties. Erythrocyte membrane AChE activity decreased by 20% ($p < 0.05$) in the animals injected with NaF (Table 2 and Fig. 1). Erythrocyte membrane fluidity (hydrophobic and external polar) and erythrocyte deformability did not change significantly (Table 2 and Fig. 2, respectively).

Hemoglobin status. A three-fold increase ($p < 0.001$) in carboxyhemoglobin concentration was observed in Group NaF (Fig. 3 and Table 2). Methemoglobin did not change significantly between both groups of animals (Table 2).

Plasma viscosity/osmolality. In Group NaF an increase in plasma viscosity ($p < 0.05$) was found, whereas plasma osmolality did not change (Table 2).

Table 1

Hematologic counts (mean ± s.d.) from blood samples of sodium fluorescein (NaF) and control (CTRL) animals

	CTRL ($n = 10$)	NaF ($n = 10$)	p^*
Leukocytes (/μl)	5930 ± 1965	6172 ± 2214	NS
Polymorphonucleated leukocytes (%)	16.1 ± 11.9	8.3 ± 7.6	NS
Mononucleated leukocytes (%)	68.4 ± 16.0	78.0 ± 12.8	NS
Erythrocytes (10^6/μl)	7.45 ± 0.45	7.79 ± 0.65	NS
Hematocrit (%)	44.5 ± 2.2	45.6 ± 1.8	NS
MCV	59.80 ± 2.66	58.80 ± 3.16	NS

*p: significance level for comparison between groups.
Abbreviations: NS, non-significant; MCV, mean corpuscular volume.

Table 2

Hemorheological parameters (mean ± s.d.) determined after sodium fluorescein (NaF) or NaCl control (CTRL animals) endovenous administration

	NaF ($n = 10$)	CTRL ($n = 10$)	p^*
AChE (UI/mg Hb)	15.70 ± 4.11	20.00 ± 5.16	$p < 0.05$
DPH (n.d.)	0.288 ± 0.069	0.287 ± 0.061	NS
TMA-DPH (n.d.)	0.314 ± 0.029	0.312 ± 0.035	NS
Hemoglobin (g/dl)	16.00 ± 1.16	15.22 ± 0.89	NS
Carboxyhemoglobin (g/dl)	1.39 ± 0.70	0.43 ± 0.22	$p < 0.001$
Methemoglobin (g/dl)	1.09 ± 0.20	1.15 ± 0.08	NS
Plasma viscosity (mPa.s.)	1.09 ± 0.05	1.04 ± 0.03	$p < 0.05$
Plasma osmolality (mOsm)	0.354 ± 0.042	0.355 ± 0.034	NS

*p: significance level for comparison between groups.
Abbreviations: NS, non-significant; AChE, acetylcholinesterase; DPH and TMA-DPH, DPH and TMA-DPH fluorescence polarization, respectively (higher values mean decreased membrane fluidity); n.d., no dimension.

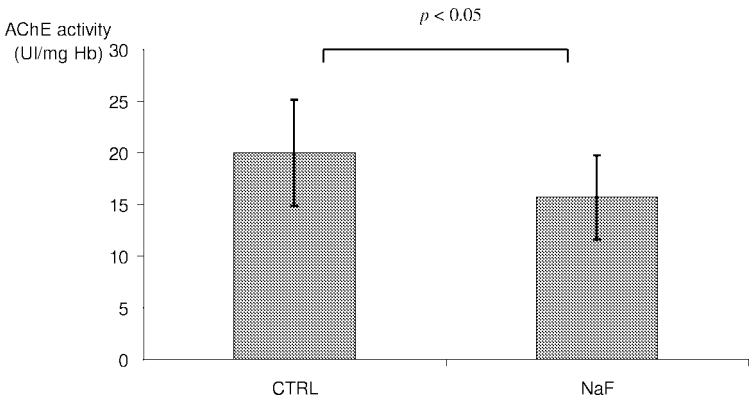

Fig. 1. Erythrocyte AChE activity values (mean ± s.d.). Group NaF showed a decreased activity of the enzyme compared to controls (15.70 ± 4.11 *versus* 20.00 ± 5.16; $p < 0.05$). NaF, fluorescein group ($n = 10$); CTRL, control group ($n = 10$).

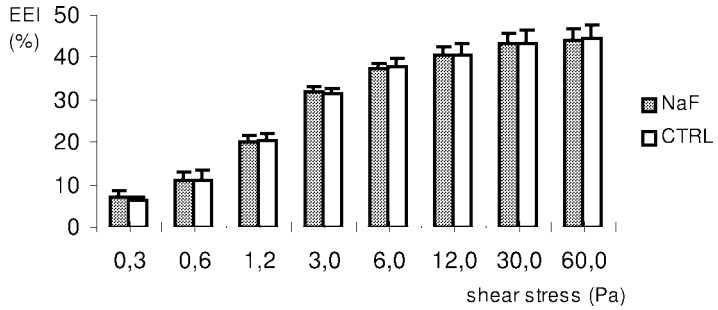

Fig. 2. Erythrocyte deformability measured by the erythrocyte elongation index (EEI). No differences were found between both groups. NaF, fluorescein group ($n = 10$); CTRL, control group ($n = 10$).

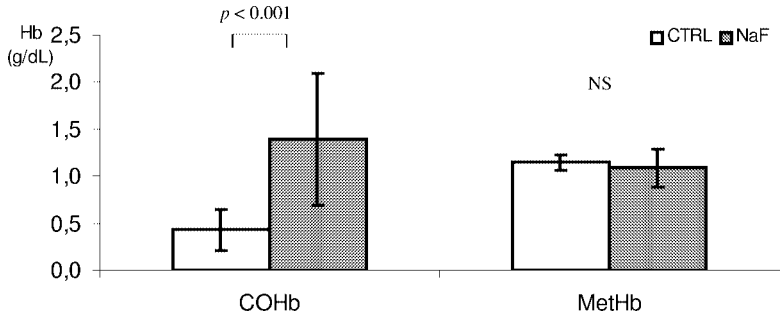

Fig. 3. Carboxyhemoglobin (COHb) and methemoglobin (MetHb) values (mean ± s.d.). A NaF induced a three fold increase in carboxyhemoglobin concentration (1.39 ± 0.70 *versus* 0.43 ± 0.22; $p < 0.001$). NaF, fluorescein group ($n = 10$); CTRL, control group ($n = 10$); NS, non-significant.

Table 3
Values (mean ± s.d.) of the plasma protein concentration in sodium fluorescein (NaF) and control (CTRL) animals

	CTRL ($n = 5$)	NaF ($n = 7$)	p
Total protein (g/dl)	52.3 ± 1.6	50.6 ± 2.1	NS
Albumin (g/dl)	24.8 ± 0.27	24.2 ± 1.3	NS
α1-globulin (g/dl)	12.2 ± 1.01	12.2 ± 0.8	NS
α2-globulin (g/dl)	3.1 ± 0.5	3.1 ± 0.3	NS
$\beta\gamma$-globulin (g/dl)	8.9 ± 0.6	8.2 ± 0.4	NS
γ-globulin (g/dl)	3.2 ± 1.0	2.9 ± 0.9	NS

*p: significance level for comparison between groups.

Plasma proteins. There was no difference in total protein concentration or in the plasma protein electrophoresis pattern (Table 3).

4. Discussion

In previous reports NaF angiography was associated with several disturbances on the microcirculatory blood flow and on the hemorheological profile of diabetic and non-diabetic patients [3–5]. With this experimental protocol, we tried to further assess several microvascular effects of NaF using an animal model.

The increase in plasma viscosity following administration of NaF determined in the present work could be an important contributing factor for the already identified increase in whole blood viscosity [5] and might explain the previously described increase in retinal circulation time [4]. In association with blood hematocrit, plasma viscosity is an essential variable of blood rheology at high shear rates. It depends mainly on the protein composition of plasma, being directly related with mass and shape of long and asymmetric macromolecules, such as fibrinogen and other acute phase proteins (immunoglobulins and α2-macroglobulin) [14]. Since the increase in plasma viscosity was obtained only one hour after NaF injection, it is highly unlikely that it was caused by an increased production of these proteins; the presented data on plasma protein electrophoresis support this view. Nonetheless, as NaF circulates in blood mostly associated at the surface of plasma proteins [1], we hypothesise that NaF might increase the spatial interactions between plasma proteins at the molecular level, originating larger sized macromolecular complexes, and thus increasing plasma viscosity. Other determinant of plasma viscosity is plasma osmolality, which measures the total number of particles present in plasma, independently of its molecular weight; however, our experimental results ruled out plasma osmolality as a cause for increased plasma viscosity.

In the group of animals injected with NaF there was a decrease of erythrocyte AChE activity, which has already been reported in an human model [3,5] and could be related with: (i) membrane loss or fragmentation, which might be corroborated by the three-fold increase in carboxyhemoglobin concentration (as a marker of sudden hemoglobin degradation [15]), however is not supported by the unchanged erythrocyte count and MCV; (ii) direct interference of NaF with the enzyme, since it is able to crosslink with proteins [16] and interfere with the activity of several erythrocyte membrane proteins [17,18]; (iii) modulation of enzyme activity by interaction with membrane phospholipids, in view of the fact that fluorescein-modified proteins are able to interact and combine with fatty acids [19]. On the other hand, these erythrocyte membrane changes might be related to the systemic inflammatory response induced by NaF [20].

The three-fold increase in carboxyhemoglobin concentration, reproducing previous results [3,5] is a significative finding that must be interpreted cautiously and thoroughly analysed in future experiments. Previous results suggesting that NaF is excluded from erythrocyte cytosol, remaining in association with its membrane [2], are not in favour that a direct interaction of NaF with hemoglobin occurs. Also, as mentioned above, the data is against the possibility of an erythrocyte membrane loss or fragmentation to be the cause for this increase. However, and whatever is its cause, this increase in carboxyhemoglobin (making up for approximately 8.7% of total hemoglobin) might represent an accessory factor in microvascular dysfunction following the injection of NaF, by interfering with oxygen exchanges in the microcirculation.

In conclusion, NaF injection in the present animal model induces some of the previously described hemorheological abnormalities in retinal fluorescein angiography in diabetic and non-diabetic patients. The presented results are in favour that NaF interferes with the erythrocyte membrane and probably with the microcirculatory blood flow, what requires further investigation.

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