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ARTICLE

Short-term effects of hemodiafiltration versus conventional hemodialysis on erythrocyte performance

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Abstract: Hemodiafiltration (HDF) is a renal replacement therapy that is based on the principles of diffusion and convection for the elimination of uremic toxins. A significant and increasing number of end-stage renal disease (ESRD) patients are treated with HDF, even in the absence of definite and conclusive survival and anemia treatment data. However, its effects on red blood cell (RBC) physiological features have not been examined in depth. In this study, ESRD patients under regular HDF or conventional hemodialysis (cHD) treatment were examined for RBC-related parameters, including anemia, hemolysis, cell shape, redox status, removal signaling, membrane protein composition, and microvesiculation, in repeated paired measurements accomplished before and right after each dialysis session. The HDF group was characterized by better redox potential and suppressed exovesiculation of blood cells compared with the cHD group pre-dialysis. However, HDF was associated with a temporary but acute, oxidative-stress-driven increase in hemolysis, RBC removal signaling, and stomatocytosis, probably associated with the effective clearance of dialyzable natural antioxidant components, including uric acid, from the uremic plasma. The nature of these adverse short-term effects of HDF on post-dialysis plasma and RBCs strongly suggests the use of a parallel antioxidant therapy during the HDF session.

Key words: end-stage renal disease, hemodiafiltration, hemodialysis, anemia, red blood cells, oxidative stress, stomatocytes, microvesicles, uric acid.

Résumé : L'hémodiafiltration (HDF) est un traitement de substitution rénal fondé sur les principes de diffusion et de convection pour l'élimination des toxines de l'urémie. Un nombre important et croissant de patients atteints d'insuffisance rénale terminale (IRT) sont traités par HDF, même en absence de données définitives et concluantes quant au taux de survie et au traitement de l'anémie. Cependant, son effet sur les caractéristiques physiologiques des globules rouges (GR) n'a pas été étudié en profondeur. Dans cette étude, nous nous sommes penchés sur des patients atteints d'IRT et subissant une HDF régulière ou une hémodialyse traditionnelle (HDt) quant aux paramètres liés aux GR, y compris l'anémie, l'hémolyse, la forme des cellules, l'état d'oxydoréduction, la signalisation du retrait de GR, la composition des membranes en protéines et la microvésiculation, dans des mesures appariées répétées avant et immédiatement après chaque séance de dialyse. Le groupe HDF était caractérisé par la présence d'un meilleur potentiel d'oxydoréduction et de l'inhibition de l'exovésiculation des globules sanguins par rapport au groupe HDt avant dialyse. Cependant, l'HDF était associée à une augmentation de l'hémolyse temporaire, mais aiguë, entraînée par le stress oxydatif, une signalisation de retrait des GR et une stomatocytose, probablement associées à la clairance effective des composants antioxydants naturels dialysables — y compris l'acide urique — du plasma urémique. La nature de ces effets indésirable à court terme de l'HDF sur le plasma et les GR après dialyse suggère fortement l'utilisation d'un traitement antioxydant en parallèle au cours de la séance d'HDF. [Traduit par la Rédaction]

Mots-clés : insuffisance rénale terminale, hémodiafiltration, hémodialyse, anémie, globules rouges, stress oxydatif, stomatocytes, microvésicules, acide urique.

Introduction

Accumulation of uremic toxins in the plasma contributes to chronic inflammation, endothelial dysfunction, amyloidosis, oxidative stress, and anemia in patients with end-stage renal disease (ESRD) (Georgatzakou et al. 2016). Hemodialysis (HD) is a renal replacement therapy that targets to the elimination of uremic toxins and water excess from blood. Conventional hemodialysis (cHD), which provides diffusive clearance of low molecular weight solutes, is the main modality of renal replacement therapy for ESRD patients worldwide. The more recently introduced hemodiafiltration (HDF), which uses a combination of convective and diffusive processes for solute removal, seems to have higher biocompatibility and efficiency in the clearance of uremic solutes across a wider molecular weight range, including middle-sized β_2-microglobulin, homocysteine, polyamines, and other molecules that influence the endothelial function (Penne et al. 2010). In addition, several studies have suggested that HDF may better control hemodynamic stability, inflammation and iron availability, redox status, erythropoiesis and erythropoiesis-stimulating agents resistance, and even mortality (probably dose-dependent on the achievement of a

Received 24 April 2017. Accepted 21 July 2017.

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critical convection volume), when compared with cHD (Maduell et al. 2013; Marcelli et al. 2016; Mercadal et al. 2016). However, the superiority of this renal replacement therapy compared with cHD is still a matter of debate, because data on the effectiveness of HDF on several clinical parameters, ranging from anemia to hard endpoints, are conflicting (Karamperis et al. 2005; Susantitaphong et al. 2013; Vilar et al. 2009). In fact, despite the extended (spanning more than 2 decades) clinical experience with both cHD and HDF, there are no conclusive evidence from large-scale prospective randomized controlled trials to validate the translatability of the current findings on clinical outcomes, including renal anemia (Ronco 2011; Więcek and Piecha 2015). In spite of this, there are still no studies reported, describing adverse outcomes to support that HDF is inferior to cHD in any relevant clinical parameters (Basile et al. 2017).

Efficient purification of the blood of all relevant uremic toxins, including fluid and salt overload, remains the fundamental objective of all dialysis therapies. However, hematological and metabolic disorders (e.g., anemia, mineral bone disease, oxidative stress, etc.) that accompany renal failure need to be corrected as well as part of dialysis therapy itself. While red blood cell (RBC) performance as a function of cHD-associated parameters has been studied in ESRD in the past, showing exacerbation of oxidative, metabolic, and mechanical stress (Antonelou et al. 2011, 2014), the probable effects of HDF on RBC physiology have not been widely assessed. However, this is clinically relevant when considering that (*i*) the decreased RBC survival in the hostile, uremic environment (Vos et al. 2011) may be benefited by the clearance adequacy (Ayesh Haj Yousef et al. 2014) or further undermined by several dialysis-related stressful stimuli, through premature aging, eryptosis, and other molecular pathways (Abed et al. 2014; Georgatzakou et al. 2016); (*ii*) the results of large clinical trials pointed out that treatment of severe anemia in ESRD by erythropoiesis-stimulating agents seems to be more complex than initially appreciated (Więcek and Piecha 2015); and, finally, (*iii*) a significant and increasing number of ESRD patients are treated with HDF, even in the absence of definite and conclusive anemia treatment and survival data. Thus, the present study focused on differences observed in the RBC physiology and performance between HDF- and cHD-treated ESRD patients, which are probably related to the management of anemia secondary to ESRD.

Materials and methods

Subjects

Thirty-two ESRD patients responsive to standard doses of recombinant human erythropoietin supplementation (9225 ± 4781 IU/week, administrated intravenously at the end of the dialysis session) and twelve, age- and gender-matched, healthy subjects were studied in repeated paired tests (n = 3) accomplished before and right after dialysis. Sixteen patients had been under high-flux HDF and sixteen patients under low-flux cHD treatment at least for the last 3 months before examination (Table 1). Blood flow rate was 300 mL/min vs. 350 mL/min and dialysate flow rate was 500 mL/min vs. 700 mL/min during the cHD and HDF therapies, respectively. The transmembrane pressures in the dialyzers were 0–50 mm Hg vs. 160–230 mm Hg for the cHD and the HDF, respectively. The amount of ultrafiltration varied between patients from 2.5 to 3.5 L per session, but there were no significant differences in the amount of ultrafiltration between the HD and HDF group of patients under investigation. Moreover, there was no change in the dialysis machines (Gambro AK200 or Hospal Integra) and in the dialyzers or membranes used (highly biocompatible, synthetic membranes of polyamix or polyacrilonytrile provided by Gambro) during the time of study. ESRD patients were receiving food supplements (carnitine 6 g/week, B1 100 mg/week, B6 100 mg/week, and B12 1 g/week), as well as 100 mg/week ferric hydroxide sucrose complex and heparin (3050 ± 1690 IU/session) intravenously. The primary cause of renal failure was hypertensive nephropathy (n = 2), IgA nephropathy (n = 4), glomerulonephritis (n = 4), polycystic kidney disease (n = 2), nephrosclerosis (n = 2), nephrolithiasis (n = 2), and chronic renal failure of unknown etiology (n = 16). Patients were all clinically stable at the time (~3 months) of investigation, without clinically appreciated difference in the severity of cardiovascular disease between the HD and HDF groups. In addition, patients with diabetes mellitus, uncontrolled hypertension, active infections, and malignant, inflammatory, autoimmune, and hematological diseases, or under blood transfusion were excluded. The study was submitted to and approved by the Research Bioethics and BioSecure Committee of the Department of Biology at National and Kapodistrian University of Athens (NKUA). Investigations were carried out in accordance with the principles of the Declaration of Helsinki. The experiments were reviewed and approved by the NKUA's ethics review committee and informed consent was obtained from all blood donors prior to participating in this study.

Laboratory testing and immunoblotting of RBC membrane proteins

Hematological analysis, standard biochemical tests in the serum, and electrolyte estimation were performed by using automatic blood cell counter and analyzers, respectively. Plasma free hemoglobin levels, as an index of intravascular hemolysis, were calculated by the method of Harboe (1959). After a double centrifugation of plasma at 1000*g* for 10 min, supernatants were incubated for 30 min at 20 °C and absorbance was measured at 380, 415, and 450 nm. The formula $2 \times OD_{415} - OD_{380} - OD_{450}$ was used for the calculation of final OD.

Purified leukodepleted RBC fractions were prepared by the method of Beutler et al. (1976) and membranes were isolated by RBC lysis with hypotonic (5 mmol/L) sodium phosphate buffer (pH 8.0) containing protease inhibitors. Equal amounts of membrane protein (15 μg) were resolved in 10% SDS–PAGE gels, electrophoretically transferred onto nitrocellulose membranes, and immunoblotted against major membrane proteins (Antonelou et al. 2014). Subsequently, the membrane was incubated with the appropriate horseradish-peroxidase-conjugated secondary antibody and the immunoreactivity was visualized by enhanced chemiluminescence. The protein bands were quantified in units of intensity by using lengthwise scanning densitometry and an image-processing program (Gel Analyzer version 1.0; Biosure, Athens, Greece).

Redox status of plasma and RBCs

Total, uric acid-dependent, and uric acid-independent antioxidant capacity of plasma were measured by the ferric reducing antioxidant power assay (Benzie and Strain 1996), with and without uricase treatment (0.125 U/mL, for 20 min at 20 °C) (Duplancic et al. 2011). Briefly, plasma was incubated with freshly prepared working FRAP solution (containing 300 mmol/L acetate buffer pH 3.6, 10 mmol/L 2,4,6-tripyridyl-s-triazine in 40 mmol/L HCl, and 20 mmol/L FeCl3 in 10:1:1 ratio) and incubated for 4 min at 37 °C in a water bath. Absorbance was measured at 593 nm.

Intracellular accumulation of reactive oxygen species (ROS) with and without stimulation by 100 μmol/L *tert*-butyl hydrperoxide (tBHP) was detected fluorometrically (VersaFluor Fluorometer) by using the fluorescent probe CMH_2DCFDA (Invitrogen), as previously described (Antonelou et al. 2014).

Scanning electron microscopy and flow cytometry analysis of RBCs and microvesicles

Isolated RBCs were fixed by 2% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated in ascending ethanol series, and examined in a Philips SEM515 microscope after coating with gold–palladium (Tousimis Samsputter–2a, Rockville, Maryland, USA). RBC shape classification was performed by using standard criteria, as previously adopted (Antonelou et al. 2011).

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Table 1. Demographic, therapy-associated, and hematological characteristics of the patient cohorts.

	HDF patients (n = 16)		cHD patients (n = 16)		
	Pre-dialysis	Post-dialysis	Pre-dialysis	Post-dialysis	Controls (n = 12)
Clinical parameters					
Age (years)	64±11		72±7		60±14
Mass (kg)	75±17		65±15		60±10
HD treatment (months)	35±15		40±30		—
Epo dose (IU/week)	9100±5527		9350±4204		—
Biochemical parameters					
WBC (×10³/μL)	6.6±1.5	6.5±1.2	5.7±1.7	5.8±2.3	6.8±1.1
RBC (×10⁶/μL)	**3.59±0.18**	**4.10±0.35**†	**3.61±0.40**	**3.99±0.89**	4.77±0.56
Hemoglobin (g/dL)	11.7±0.3*	12.8±1.6†	**10.9±0.7***	11.8±1.0	14.2±1.4
Hematocrit (%)	36.1±1.0*	37.9±4.2*	**33.9±1.9***	**33.2±3.8***	43.1±4.6
MCV (fL)	**100.4±3.0**	93.0±6.0*,†	95.4±9.0	84.0±9.0*,†	93.2±5.4
MCH (pg)	32.6±1.3*	31.7±2.0	30.2±3.2*	29.6±4.4	32.5±2.2
MCHC (g/dL)	32.5±0.6*	34.1±1.3†	**31.2±0.6***	33.6±3.7	33.7±1.5
RDW (%)	**15.1±1.1***	14.1±1.7	**16.6±1.4***	**15.7±1.8**	12.8±0.9
PTH (pg/mL)	**195±65**	N/D	**235±129**	N/D	44±18
Urea (mg/dL)	**143±23**	32±11†	**123±27**	38±9†	31±12
URR (%)	78±6*		69±7*		—
Creatinine (mg/dL)	**8.6±1.2***	N/D	**6.7±2.0***	N/D	0.8±0.2
Uric acid (mg/dL)	**7.9±1.8***	N/D	**6.1±1.0***	N/D	4.2±1.2
Cholesterol (mg/dL)	148±29	N/D	145±36	N/D	145±18
Triglycerides (mg/dL)	150±60	N/D	146±42	N/D	132±29
HDL (mg/dL)	**45±11**	N/D	**43±11**	N/D	73±9
Calcium (mg/dL)	9.3±0.2	N/D	9.3±0.3	N/D	9.3±0.2
Phosphorus (mg/dL)	5.0±0.6	N/D	4.8±0.8	N/D	3.6±0.6
Potassium (mmol/L)	5.0±0.3	4.5±0.5	4.9±0.5	4.7±0.6	4.2±0.3
Sodium (mmol/L)	137±2	N/D	137±2	N/D	139±2
Fe (μg/dL)	72.4±17.4	N/D	66.5±11.5	N/D	108.9±15.4
Ferritin (ng/mL)	**914±436**	N/D	**831±487**	N/D	72±18
TIBC (μg/dL)	280±37*	N/D	**242±41***	N/D	348±27
Total proteins (g/dL)	7.1±0.3	N/D	7.1±0.3	N/D	7.4±0.5
Albumin (g/dL)	4.3±0.3	N/D	4.3±0.6	N/D	4.2±0.4
ALP (IU/L)	83±19	N/D	79±12	N/D	63±10
γGT (IU/L)	17±7	N/D	14±5	N/D	12±9
TAC (μmol/L Fe^{2+})	**1127±168**	**401±65**†	**1021±198**	**465±77**†	706±159
UAiAC (μmol/L Fe^{2+})	**449±90**	253±32†	**450±102**	261±53†	239±45
UAdAC (μmol/L Fe^{2+})	**678±104***	**147±47***,†	571±107*	**199±45***,†	467±126

Note: Values are presented as mean ± SD. Values in boldface type are hemodialysis (HD) or hemodiafiltration (HDF) vs. healthy subjects; *, HDF vs. HD; †, pre- vs. post- dialysis; $P < 0.05$. ALP, alkaline phosphatase; cHD, conventional hemodialysis; Epo, recombinant human erythropoietin; γGT, gamma-glutamyl transferase; HDL, high-density lipoprotein; MCV, mean corpuscular volume; N/D, not determined; PTH, parathormone; RBC, red blood cell; RDW, RBC distribution width; TAC, total antioxidant capacity; TIBC, total iron-binding capacity; UAdAC, uric acid-dependent antioxidant capacity; UAiAC, uric acid-independent antioxidant capacity; URR, urea reduction ratio; WBC, white blood cell. Parameter (conversion factor) SI unit: WBC count (1.0) 10^9/L; RBC count (1.0) 10^{12}/L; hemoglobin (10.0) g/L; hematocrit (0.01) proportion of 1.0; MCH (0.0621) fmol; MCHC (0.620) mmol/L; PTH (1.0) ng/L; urea (6.006) mmol/L; creatinine (88.4) μmol/L; uric acid (59.84) μmol/L; cholesterol (0.0259) mmol/L; triglycerides (0.0113) mmol/L; HDL (0.0259) mmol/L; calcium (0.25) mmol/L; phosphorus (0.323) mmol/L; Fe (0.179) μmol/L; ferritin (2.247) pmol/L; TIBC (0.179) μmol/L; total proteins (10.0) g/L; albumin (10.0) g/L); ALP (16.66) nmol/(s·L); γGT (16.66) nmol/(s·L).

Enumeration, phenotyping, and phosphatidylserine (PS) exposure on RBCs and microvesicles (MVs) were performed by multicolor flow cytometry using the phycoerythrin (PE)–annexin V apoptosis kit and FITC-conjugated anti-CD235 (BD Pharmingen), as previously described (Tzounakas et al. 2016). MVs were identified by size (<1 μm), exposure of RBC-specific markers (anti-CD235), and PS exposure (through annexin V binding, $AnnV^+$) in plasma isolated after a double 2500*g* spin of citrated blood at 20 °C.

Statistical analysis

All experiments were performed in triplicate. For statistical analysis, the Statistical Package for Social Sciences (IBM SPSS; version 22.0 for Windows IBM Corp., Armonk, New York, USA; administrated by NKUA) was used. Inter-group differences were evaluated by Student's *t* test or one-way ANOVA and Mann–Whitney analysis, as appropriate. A Bonferroni correction was used where needed. Pearson's and Spearman's tests were performed to assess correlation (*r*) between parameters following or not normal distribution profiles, respectively. Significance was accepted at $P < 0.05$.

Results

Overall, the ESRD patients were characterized by low RBC count and increased RBC distribution width (RDW). cHD patients exhibited lower hemoglobin (Hb 10.9 ± 0.7 g/dL), hematocrit (Hct 33.9 ± 1.9%), mean corpuscular hemoglobin concentration (MCHC 31.2 ± 0.6 g/dL), and total iron-binding capacity (TIBC 242 ± 41 μg/dL) values compared with patients under HDF, pre-dialysis. At the end of each HDF session, a significant amelioration of the RBC count and indexes (RBC count, Hb, MCV, MCHC, and RDW) was observed, whereas in cHD group only Hb and MCHC improved compared with the pre-dialysis levels (Table 1).

In respect to uremia, HDF patients exhibited significantly higher creatinine and uric acid (creatinine: 8.6 ± 1.2 mg/dL vs. 6.7 ± 2.0 mg/dL, $P = 0.023$; uric acid: 7.9 ± 1.8 mg/dL vs. 6.1 ± 1.0 mg/dL, $P = 0.009$) along with a trend for higher urea levels (143 ± 23 vs.

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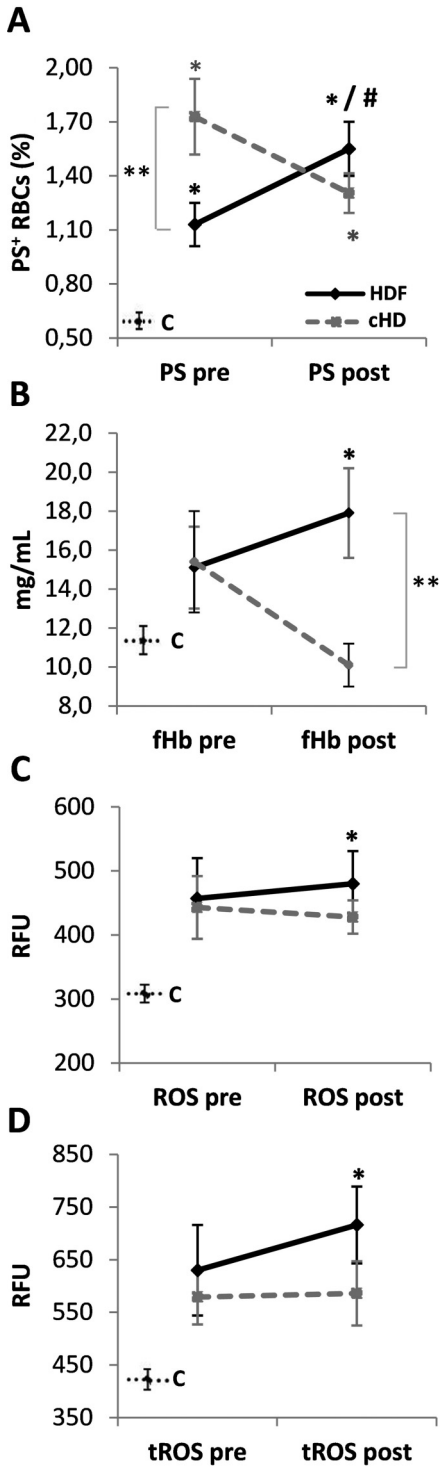
Fig. 1. Variation in red blood cell (RBC) and plasma characteristics. Percentage of PS+ RBCs (A; **, $P = 0.026$, and #, $P = 0.0005$), levels of plasma free Hb (intravascular hemolysis, B; **, $P = 0.008$), intracellular ROS (C), and tBHP-induced intracellular ROS (D), before (pre) and after (post) dialysis session. HDF, cHD: patients under hemodiafiltration (HDF) or conventional hemodialysis (cHD) treatment, respectively; C, healthy subjects (controls); RFU, relative fluorescence units. *, $P < 0.05$ vs. controls; **, $P < 0.05$ HDF vs. cHD; #, $P < 0.05$ pre- vs. post- dialysis session. Values are the mean ± SEM.



123 ± 27 mg/dL $P = 0.110$, respectively), compared with cHD patients, in spite of the better urea reduction ratio (78% ± 6% vs. 69% ± 7% $P = 0.007$ for HDF vs. cHD, respectively) (Table 1). High pre-dialysis levels of uric acid in HDF patients were reflected in the elevated uric acid-dependent antioxidant capacity of plasma, which decreased significantly post-dialysis in both groups (Table 1).

ESRD RBCs were more susceptible to PS externalization than healthy RBCs, independently of the dialysis technique applied. Yet, the percentage PS+ RBCs was significantly lower ($P = 0.026$) in HDF than in cHD patients before dialysis (1.13% ± 0.38% vs. 1.73% ± 0.67%, respectively) (Fig. 1A). Of note, dialysis method had a different impact on PS exposure on RBCs, because HDF increased it significantly (by 40%, $P = 0.031$) compared with the pre-dialysis levels, in contrast to the cHD (Fig. 1A). Likewise, HDF exhibited a negative effect on baseline hemolysis (increase from 15.1 ± 6.6 mg/dL to 17.9 ± 7.4 mg/dL, $P = 0.026$ vs. controls, Fig. 1B), intracellular ROS accumulation (from 457 ± 201 to 480 ± 162 relative fluorescence units, $P = 0.011$ vs. controls, Fig. 1C), and on exogenously-induced intracellular ROS levels (from 630 ± 273 to 717 ± 232 relative fluorescence units, $P = 0.009$ vs. controls, Fig. 1D), in striking contrast to a trend for amelioration (plasma free hemoglobin (fHb), Fig. 1B) or neutral effect (ROS, Figs. 1C and 1D) of cHD on those physiological features.

Examination of RBC shape revealed a trend for stomatocytic transformation in HDF RBCs pre-dialysis that was actively promoted by the dialysis session, resulting in significantly higher percentage of stomatocytes in HDF patients compared with the cHD patients post-dialysis (8.8% ± 2.6% vs. 6.2% ± 2.1%, respectively, $P = 0.023$) (Figs. 2A and 2B). Stomatocytosis was positively correlated with serum uric acid levels ($r = 0.810$, $P = 0.004$) but negatively with the percentage of PS+ RBCs ($r = -0.684$, $P = 0.043$) in HDF patients pre-dialysis.

As expected, the uremic plasma was further characterized by pathological accumulation of total and RBC-derived (R-MVs) circulating microvesicles (Fig. 2C, left panel). Both populations of extracellular vesicles were significantly lesser in the HDF (MVs: 9330 ± 4813 counts/μL; R-MVs: 1103 ± 693 counts/μL) compared with the cHD plasma (MVs: 21 853 ± 12 391 counts/μL; R-MVs: 2365 ± 1691 counts/μL) pre-dialysis ($P = 0.024$ and $P = 0.043$, respectively). Post-dialysis, the overall MVs population decreased to normal size (HDF: 4077 ± 2275 counts/μL; cHD: 5032 ± 3439 counts/μL), whereas the RBC-derived vesicles remained at high levels (HDF: 1214 ± 952; cHD: 1044 ± 627 vs. healthy subjects 130 ± 85 counts/μL) (Fig. 2C, right panel). In many patients treated by cHD, a significant reduction in R-MVs compared with the pre-dialysis levels was recorded; however, that reduction was not a statistically significant feature of cHD-group ($P = 0.080$) owing to high patient-to-patient variation.

Finally, the RBC membrane of HDF-treated patients was characterized by severe deficiency in aquaporin 1 (47% ± 7%, $P = 0.025$) and overexpression of Hsp70 (254% ± 31%, $P = 0.015$) compared with healthy subjects (100%), but also by significantly lower levels of stomatin compared with the membrane of cHD patients pre-dialysis (81% ± 11% vs. 126% ± 10%, respectively, $P = 0.009$). The RBC membrane of cHD-treated subjects was severely deficient in CD47 (46% ± 11%, $P = 0.004$) but over-expressed Glut 1 (251% ± 38%, $P = 0.016$) and IgGs (693% ± 211%, $P = 0.033$) molecules (Fig. 3A). Following an HDF session, increased membrane binding of oxidized/denatured Hb was observed (182% ± 68%, $P = 0.047$) (Fig. 3B bar graph and insert). cHD seemed to be associated with an overall reduction in the expression level of several membrane proteins, including stomatin (77% ± 9%, $P = 0.005$, Fig. 3B). In some cases, that reduction tended to regularity (as in the case of Glut 1) but in other, cHD leaded to protein deficiency (aquaporin 1, 62% ± 8%, $P = 0.044$) or just exacerbated an existing shortage (CD47, from 46% ± 11% to 23% ± 5%, $P = 0.010$).

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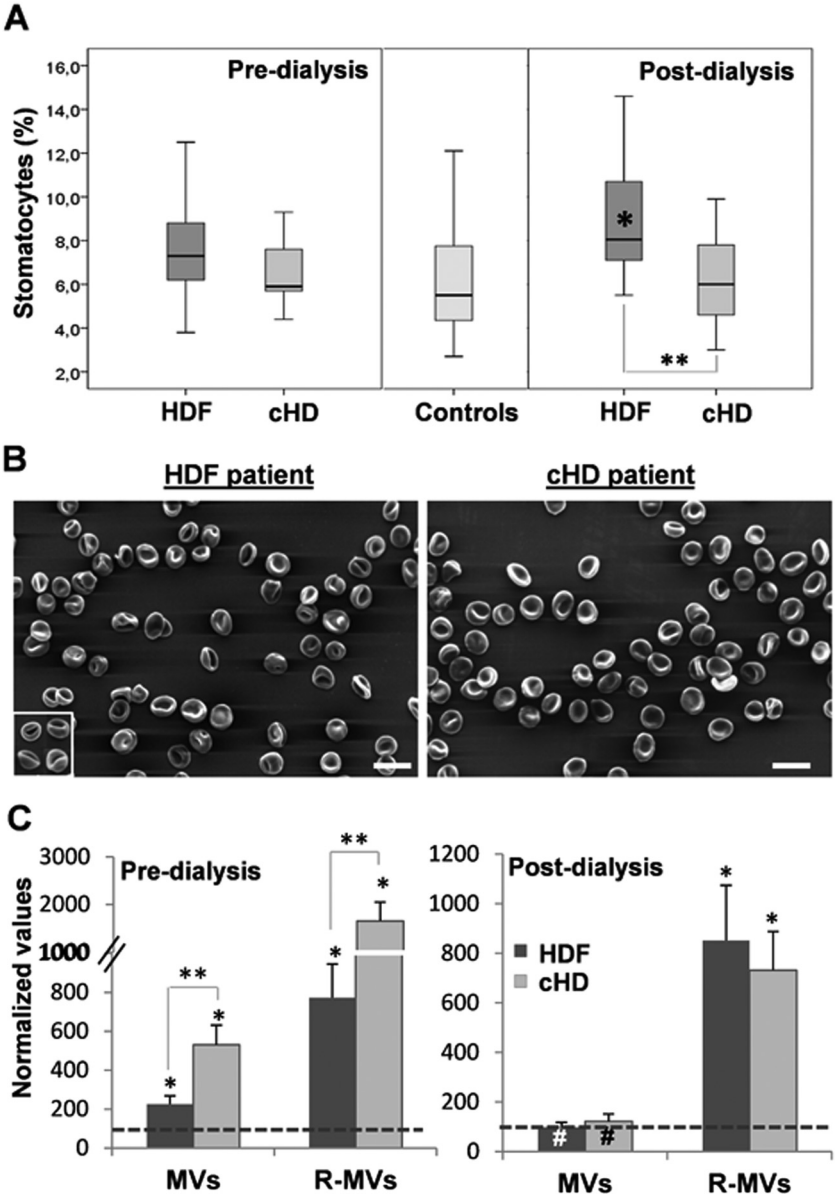
Fig. 2. Red blood cell (RBC) morphology and membrane exovesiculation in end-stage renal disease. (A) Box plots showing variation in stomatocytosis in hemodiafiltration (HDF) and conventional hemodialysis (cHD) patients compared with healthy controls (*, $P = 0.049$; **, $P = 0.023$). (B) Representative scanning electron micrographs of RBCs from patients under HDF or cHD treatment, after the dialysis session. Insert: typical stomatocytic shape transformations. Scale bars = 10 μm. (C) Flow cytometry analysis of total (MVs) and RBC-derived (R-MVs) microvesicles in the uremic plasma collected before (**, $P = 0.010$ for MVs and $P = 0.049$ for R-MVs) and after dialysis, following normalization to healthy plasma (100%, dotted line). Values are the mean ± SEM. *, $P < 0.05$ vs. control; **, $P < 0.05$ HDF vs. cHD; #, $P < 0.05$ pre- vs. post-dialysis session.



Discussion

Reduced lifespan and pathologic modifications in circulating RBCs arising from various types of uremic and dialysis-induced stresses, contribute to anemia and other hematological complications in ESRD. While cHD has been studied in respect to its probable effects on RBC morphology and physiology (Antonelou et al. 2011, 2014), whether and to what extent HDF represents a stress factor for blood cells, or whether it is more "friendly" for them as a renal replacement therapy compared with the cHD, is largely unknown. The present comparative study assessed an array of functionally important physiological features of circulating RBCs in patients regularly treated by either HDF or cHD.

According to the analysis, greater urea reduction ratio was recorded in patients treated by HDF compared with cHD, in spite of their worse basal toxin levels. HD adequacy is expected to have a beneficial effect on anemia. As a probable result of the better clearance of uremic compounds, which are supposed to suppress bone marrow's response to erythropoietin (Locatelli and Del Vecchio 2003) and to trigger inflammatory responses (Rossi et al. 2014), HDF patients exhibited on average better RBC indexes and lower levels of inflammation markers, such as TIBC and RDW, compared with the cHD patients before dialysis. Variability in RBC volume (RDW index) and low TIBC have been widely associated with protein-energy wasting, inflammation, poor quality of life, cardiovascular events, and mortality in HD patients, in both prospective and retrospective observational studies (Bross et al. 2009; Lippi et al. 2009; Vashistha et al. 2016). On a largely similar clinical ground of renal disease, time on dialysis, dose of and responsive-

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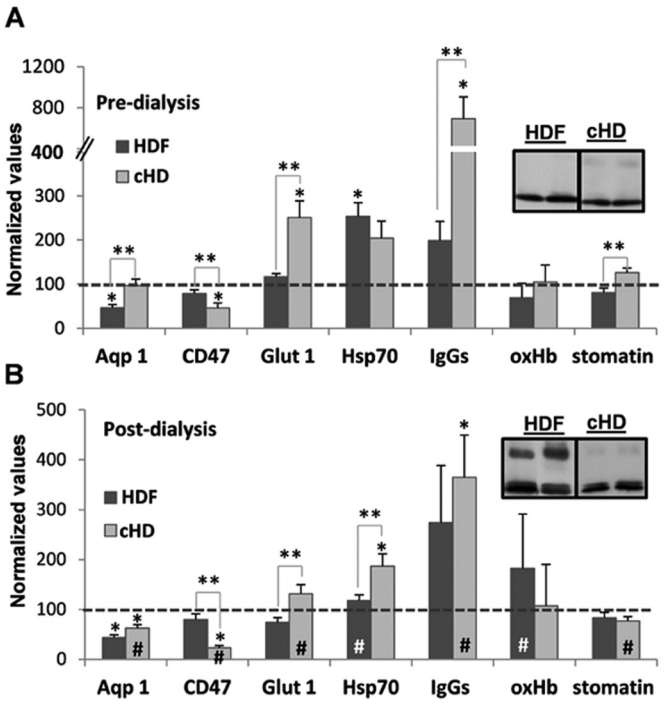
Fig. 3. Variation in the protein composition of the red blood cell (RBC) membrane in end-stage renal disease patients, before (A) and after (B) the dialysis session, following normalization to control (100%, dotted line). Values are the mean ± SEM. *, $P < 0.05$ vs. control; **, $P < 0.05$ hemodiafiltration (HDF) vs. conventional hemodialysis (cHD); #, $P < 0.05$ pre- vs. post- dialysis session. oxHb, oxidized/denatured Hb. Insert images: representative immunoblots showing increased oxidized/denatured Hb species in HDF-RBC membrane isolated immediately post-dialysis.



ness to recombinant human erythropoietin, vitamin supplementation, and co-morbidities in cHD and HDF patient groups, our results are rather consistent with studies reporting beneficial effects of chronic HDF over cHD in ESRD, thus continuing the controversy in the field (Bowry and Gatti 2011; Locatelli et al. 2012; Schneider et al. 2012; Susantitaphong et al. 2013).

At RBC level, low levels of intravascular hemolysis, irreversible RBC shape modifications, and intracellular oxidative stress were detected in patients treated by either cHD or HDF. However, the HDF-treated patients exhibited lower percentage of PS^+ circulating RBCs and MVs and almost normal protein composition of the RBC membrane compared with the cHD patients pre-dialysis. PS exposure on RBC surface, a common finding in ESRD RBCs (Abed et al. 2014; Bonomini et al. 1999), is a sign of eryptosis (namely, of premature suicidal RBC death) and a potent recognition signal leading to accelerated erythrophagocytosis and, thus, susceptibility to anemia. Moreover, accumulation of MVs has been implicated in inflammation and coagulation (including intraglomerular coagulation) issues in uremic patients (Ando et al. 2002; He et al. 2016) and, consequently, a lower vesiculation rate might be associated with the previously reported better endothelial function (Jia et al. 2016) and reduced cardiovascular mortality rates in HDF- vs. cHD-treated patients (Daugirdas 2016; Maduell et al. 2013). Finally, at RBC membrane level, the RBCs of the HDF-treated patients were only characterized by over-expression of Hsp70 (a molecular chaperone protective for membrane peripheral proteins (Biondani et al. 2008)) and deficiency in aquaporin 1 (the membrane water channel responsible for changes in RBC volume in response to the tonicity of the medium). Aquaporin-1 deficiency might be associated with the effects of erythropoietin on cell water regulation (Rentsch et al. 2006) and (or) with the stomatocytic transformation of RBCs, as discussed below. Regarding the RBC membrane of the cHD-treated patients, it was characterized by several disturbances, including over-accumulation of plasma IgGs, overexpression of glucose transporter 1, and sharp deficiency in the "marker-of-self" CD47. These modifications are indicative of (*i*) metabolic and probably oxidative stress, as glucose transporter 1 mediates the transport of both glucose and dehydroascorbic acid molecules into RBCs (Montel-Hagen et al. 2008), as well as (*ii*) susceptibility of cHD-RBCs to opsonization (IgGs binding) and erythrophagocytosis, because a severe deficiency of the membrane in CD47 may induce RBC clearance through the signal regulatory protein alpha inhibitory receptor in macrophages (Lutz and Bogdanova 2013).

The effective elimination of uremic compounds by the HDF brings stressful, though temporary, challenges to the RBCs of ESRD patients, which undermine their functional capacity soon after the dialysis session. Indeed, post-HDF, the uric acid-dependent antioxidant capacity of plasma fell to very low levels compared with the plasma post-cHD, as a result of the better clearance of the dialyzable uric acid. Nevertheless, apart from being a uremic toxin, the end product of purine metabolism in humans is a potent natural antioxidant factor, which protects both plasma (in the antioxidant capacity of which it contributes by 35%–65%) and cells from oxidative damages through its free radical scavenging capacity (Assis et al. 2015; Tzounakas et al. 2015). Of note, either as an indicator of better nutritional status or not, low uric acid has been proposed as a mortality risk factor in HD patients (Beberashvili et al. 2015) and, more recently, it was found that RBC life span (which is a significant measure of and (or) contributor to anemia) in dialysis patients is positively correlated with levels of uric acid and blood urea nitrogen (Ma et al. 2017). The high clearance potential of HDF probably results in the acute elimination of additional dilute factors that also function in strengthening the antioxidant defense reactions in plasma, including albumin (Ahrenholz et al. 2004), vitamins (Morena et al. 2002), and trace elements (Prodanchuk et al. 2013). Trace elements are cofactors for many antioxidant enzymes of blood (Chan et al. 1998), albumin is a main antioxidant protein of plasma contributing to its free-radical trapping capacity, whereas hydrophobic vitamin C helps vitamin E to work in lipid detoxification.

Thus, while in general, dialysis therapy per se seems to negatively affect the systemic redox status and the RBC antioxidant defenses (Poulianiti et al. 2016), HDF in particular, seemed to be associated with an additional temporary acute oxidative stress. The effective elimination of "waste" natural antioxidant factors from the uremic plasma by the HDF is manifested in the pathological levels of ROS accumulation and susceptibility to oxidative stimuli that were exclusively observed in HDF-RBCs right after the completion of the dialysis session. Oxidative stress of that origin, was further highlighted by the accumulation of oxidized/denatured Hb to the RBC membrane, the acute increase in PS externalization, the increased intravascular hemolysis (Quaye 2015) and, probably, by the appearance of transient stomatocytosis in HDF-treated patients. Eryptosis, which is strongly associated with oxidative stress and ceramide formation in uremia, is further triggered by the dialysis procedure (Abed et al. 2014), whereas deficiency in reduced glutathione has been related to higher risk of hemolysis in ESRD (Weinstein et al. 2000). The tripeptide of reduced glutathione is an important antioxidant molecule that can easily pass through filters' pores, especially in HDF, where convection reinforces the removal of substances. The release of free, extracellular Hb and its degradation products in post-dialysis plasma is expected to induce oxidative stress in HDF blood, as a part of a toxic feedback loop (Quaye 2015). Post-HDF RBC hemolysis and PS exposure, a marker of oxidative stress for RBCs (Mandal et al. 2002), might also represent adverse effects of that temporary increase in oxidative stimuli. Appearance of stomatocytes, which represent reversible RBC modifications, has been also related

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with various oxidative and inflammatory states (Gyawali et al. 2015). Stomatocytes are generally considered as the morphological change adopted by erythrocytes during their response to oxidants (Vota et al. 2013). Notably, in our patients, the stomatocytic transformation of RBCs was positively correlated with serum uric acid levels and seemed to be a feature of the HDF group in many ways: HDF-RBCs were characterized by lower membrane expression of stomatin and its membrane protein partners (Rungaldier et al. 2013) aquaporin 1 and glucose transporter 1, compared with the cHD-RBCs, and, moreover, stomatocytosis was triggered by HDF and not by cHD. HDF-associated membrane-perturbing cationic factors might also induce a stomatocytic transformation of RBC morphology through preferential accumulation in and, therefore expansion of, the inner (relative to the outer) leaflet of the lipid bilayer (Reinhart and Chien 1987). Stomatocytosis is expected to affect cation permeability and intrinsic deformability of the membrane, as well as RBC aggregation and filterability in the microcirculation of HDF-treated ESRD group post-dialysis (Chabanel et al. 1987; Reinhart and Chien 1986).

Moreover, uremia and dialysis-associated stresses trigger exovesiculation in blood cells (Boulanger et al. 2007). Our patients (especially those of the cHD group) were characterized by overaccumulation of MVs to plasma, compared with healthy subjects. Dialysis lowered the mixed population of plasma MVs (where MVs of platelet, RBC, leukocyte, and endothelial origin are found) to normal levels, but it had minor effects on RBC-derived MVs, especially in the HDF group, suggesting dialysis-triggered neovesiculation of RBC membrane. To support this assumption, a trend for lower post-dialysis R-MVs levels was observed in cHD-treated patients compared with the pre-dialysis levels (despite at no statistically significant point due to high inter-patient variability), in whom post-dialysis oxidative stress (an established trigger of vesiculation in RBCs) remained at normal levels, in contrast to the HDF-treated patients. Moreover, the increased stomatocytic transformation seen in HDF-RBCs post-dialysis is expected to inhibit exovesiculation of the membrane (Schreier et al. 2000).

Although the time-course evaluation of this effect needs further studies of HDF-treated patients post-dialysis, our results suggest that application of HDF should be combined with antioxidant protection of blood during the dialysis or soon after it, to mitigate the increases in intravascular hemolysis, RBC removal signaling, and exovesiculation. Oral or intravenous supplementation of reduced glutathione, L-carnitine, and vitamin C has been shown to improve lifespan, metabolism, and other physiological properties of RBCs (Candan et al. 2002; Usberti et al. 1997). In addition, intravenous infusion of reduced glutathione or vitamin C soon after dialysis by vitamin-E-coated membranes was shown to improve anemia and ameliorate erythropoietin requirements in HD patients (D'Arrigo et al. 2017; Usberti et al. 2002; Yang et al. 2006).

To our knowledge, this study is the first to evaluate the effects of HDF on overall RBC biology and performance in ESRD patients. However, there are several limitations. First, the patient population was relatively small and, thus, some measurements that obviously differed between groups but exhibited high inter-patient variation were not found to be statistically significant. To overcome that limitation, we tried to construct as much as possible homogeneous patient groups (in terms of demographic and clinical data) and, moreover, we performed repeated paired measurements (at least 3 per patient) before and soon after the dialysis session to increase the statistical power of the study. Second, direct inflammation markers such as high-sensitivity C-reactive protein and interleukins were not measured and, thus, our results cannot be directly compared with those of other studies reporting the inflammation state of cHD- or HDF-treated ESRD patients on the basis of those classic measurements. Instead, alternative but also widely recognized markers of inflammation (RDW, TIBC) were used. Third, despite the fact that the 2 groups of patients did not differ significantly at baseline, patient's age and total time on HD treatment were not completely balanced between them, as a result of efforts to construct as possible as larger groups of clinically "equal" (in respect to comorbidities, dose of and responsiveness to erythropoiesis-stimulating agents, etc.) patients. Thus, a possible interference of those variables with our results on differences between cHD and HDF cannot be excluded. Fourth, the pre – post treatment values of free Hb, MVs, and other variables were not adjusted for the amount of ultrafiltration because we had only data for the average amount of ultrafiltration per patient and not for each treatment individually. Despite that, the amount of ultrafiltration exhibited very little intra-patient variation between sessions and no statistical difference between the groups. Finally, evaluation of the intracellular antioxidant activity in HDF vs. cHD RBCs and time-course recording of the RBC performance in HDF patients post-dialysis were not performed and, thus, the time when the redox status is balanced in those patients cannot be estimated. The potential short- and long-term adverse or beneficial effects of HDF on the RBCs must be tested by controlled clinical trials before recommendations can be made for clinical practice.

Conclusions

Taken together, similar low levels of baseline intravascular hemolysis, irreversible RBC shape modifications, and intracellular oxidative stress were detected in clinically stable ESRD patients treated by cHD or HDF. RBC removal signaling (PS, CD47, IgGs, etc.) and membrane vesiculation, which might underlie susceptibility to anemia, inflammation, thrombosis, and endothelial damages in ESRD, were lower in the RBCs of HDF-treated patients compared with cHD counterparts. The beneficial effects of HDF on RBCs are diluted by a temporary acute oxidative stress that undermines their functional capacity soon after the dialysis session, as a probable result of "over-eliminating" dialyzable natural antioxidant factors, including uric acid, from the uremic plasma.

Conflict of interest statement

The authors declare that there is no conflict of interest associated with this work.

Acknowledgements

We thank all the patients and healthy blood volunteers; we are extremely grateful to Vasileia Stoupa, Head Nurse in the Chronic Hemodialysis Centre "Ionion", for kindly organizing blood donations in a way that exhibited high professional standards, responsibility, and consistency along with excellent collaboration skills.

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