Erythrocyte Membrane Fluidity Alterations in Sudden Sensorineural Hearing Loss Patients: The Role of Oxidative Stress

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Abstract

Introduction Sudden sensorineural hearing loss (SSNHL) involves an acute unexplained hearing loss, nearly always unilateral, that occurs over less than a 72-hour period. SSNHL pathogenesis is not yet fully understood. Cochlear vascular occlusion has been proposed as a potential mechanism of hearing damage and cochlear ischaemia has been related to alterations of cochlear microvessels. In addition, some researchers have focused their attention on the rheological alterations and blood hyperviscosity. Erythrocyte deformability plays a key role in determining blood viscosity, and it is critical to cochlear perfusion. It has been shown that oxidative stress-induced erythrocyte membrane fluidity alterations are linked to the progression of cardiovascular diseases.

Methods To determine whether erythrocytes from SSNHL patients show signs of oxidative stress, and whether this condition can modify the haemorheologic profile in these patients, we analysed haemorheologic profile and erythrocyte oxidative stress in 35 SSNHL patients and 35 healthy subjects, matched for age and sex. Fluorescence anisotropy was used to evaluate the fluidity of erythrocyte membranes.

Results Our results show a significant structural and functional involvement of erythrocyte membrane alterations in SSNHL, as well as elevated levels of membrane lipid peroxidation and intracellular reactive oxygen species (ROS) production. In addition, erythrocyte-derived ROS and erythrocyte lipid peroxidation positively correlated with whole blood viscosity and erythrocyte deformability. Moreover, in vitro experiments demonstrated that ROS display a key role in erythrocyte membrane fluidity.

Conclusion These findings indicate that erythrocyte oxidative stress plays a key role in the pathogenesis of SSNHL and pave the way to new therapeutic interventions.
Introduction

Sudden sensorineural hearing loss (SSNHL) is defined as a loss greater than 30 dB occurring in at least three contiguous frequencies over a period of less than 72 hours. The incidence of SSNHL is approximately 5 to 20 cases per 100,000 person-years; the vast majority of cases are unilateral and occur preferentially in the fourth decade of life, with equal sex distribution. To date, SSNHL pathogenesis is not fully understood. Several hypotheses have been proposed, such as viral infections, immune-mediated diseases and thrombotic and vascular events, but the results are conflicting. Cochlear vascular occlusion has been proposed as a potential mechanism of hearing damage and cochlear ischaemia has been related to alterations of cochlear microvessels. In addition, clinical studies have shown that SSNHL patients often present with systemic arterial hypertension, diabetes mellitus, dyslipidaemia, alone or associated with systemic sclerosis, and thromboembolic risk. Some researchers have focused their attention on the rheological alterations (high haematocrit [Hct] and plasma viscosity, high red blood cell [RBC] aggregation, reduced RBC deformability). Indeed, elevated blood viscosity was hypothesized to cause ischaemia at the labyrinthine artery level, leading to cochlear damage and organ of Corti dysfunction.

Erythrocyte aggregation is one of the principal determinants of blood viscosity at low shearing rates (slow flow). The slow circulatory rate and a high resistance to flow of the cochlear microcirculation create a favourable environment for the formation of erythrocyte aggregates. Very few studies investigated haemorheological profile in SSNHL patients. In a case control study carried out in SSNHL patients, we found an alteration in blood rheology associated with haemostatic changes; in addition, some papers report a beneficial role for aphaeresis treatment. Recently, an association of SSNHL with oxidative stress has been demonstrated; however, its contribution to haemorheological profile in SSNHL patients is still unknown. In the present study, we investigated erythrocyte oxidative stress and rheological alterations in 35 SSNHL patients and in 35 age- and sex-matched healthy subjects.

Materials and Methods

Thirty-five patients (22 males/13 females; age: 62 years [range: 52–72 years]) with a diagnosis of SSNHL referred to the Department of Audiology of Careggi University Hospital (Florence, Italy) were enrolled. All patients underwent complete audiological examination, complete history taking and general physical examination. The diagnosis of SSNHL was made by experienced audiologists by excluding other causes of sudden deafness such as viral, congenital, inflammatory, degenerative or traumatic. Thirty-five healthy subjects comparable for age and sex (22 males/13 females; age: 61 years [range: 51–72 years]), friends or partners of patients, who volunteered to undergo laboratory investigations were enrolled as control population. Exclusion criteria for patients and controls were any history of arterial or venous thrombotic disease or chronic inflammatory diseases, and rheumatic and neoplastic diseases. The presence of traditional cardiovascular risk factors was assessed on the basis of patient’s interview and hospital records. The subjects were classified as having hypertension according to the guidelines of European Society of Hypertension/European Society of Cardiology, or, if they reported taking antihypertensive medications, as verified by the interviewer. Diabetic subjects were defined according to the American Diabetes Association or on the basis of self-reported data (if confirmed by medication or chart review). Dyslipidaemia was defined according to the criteria of the 2013 ACC/AHA (American College of Cardiology/American Heart Association) guidelines. Current smoking status was determined at the time of physical examination.

All participants provided signed informed consent; the study was approved by the local ethics committee, and complies with the Declaration of Helsinki.

Blood Measurements

Blood samples were collected from the antecubital vein into evacuated plastic tubes (Vacutainer) in the morning, after an overnight fasting. Plasma samples were obtained by centrifuging blood at 2,000 × g for 10 minutes at 4°C. Complete blood cell count was measured by using the Sysmex XE-2100 haematology analyser (Sysmex, Kobe, Japan) and fibrinogen concentration was assessed according to the von Clauss method (Werfen, Bedford, Massachusetts, United States). Whole blood viscosity (WBV) was measured at 37°C using the rotational viscosimeter Physica MCR 301 (Anton Paar, Turin, Italy). WBV was analysed at 0.512 and 94.5/s shear rates. Measurements of RBC deformability were assessed using the Laser-Assisted Optical Rotational Cell Analyzer (LORCA; Mechatronics Instruments BV, AN Zwaag, the Netherlands). A suspension of RBC was mixed with polyvinylpyrrolidone (PVP) 360 solution, an isotonic viscous medium (PVP of 4%; molecular weight of 360 kDa; viscosity of 30 ± 2 mPa s), to obtain a final solution with a constant Hct of 0.2%. Using a Couette system composed of a glass cup and a precisely fitting bob, with a gap of 0.36 mm between the cylinders, the liquid solution was sheared and illuminated by a laser beam in order to obtain a diffraction pattern produced by the deformed cells. The elongation index (EI) is calculated as: $EI = (a - b)/(a + b)$, where a and b are, respectively, the length and width of the diffraction pattern. The geometry of the diffraction pattern is elliptical. Given a shear stress, the greater was RBC deformability, the higher was the EI. At 37°C, we obtained the shear stress–EI curves for nine consecutive shear stresses because human RBC deformability reaches a plateau at 50 Pa: 0.3, 0.53, 0.95, 1.69, 3.0, 5.33, 9.48, 16.87 and 30. For the analysis, we reported the EI obtained at 3.0 Pa as reported in the guidelines for haemorheological laboratory technique. Inter-assay variabilities for each shear stress were as follows: 51, 82, 3.9, 2.4, 1.7, 1.6, 1.1, 1.3, 1.7, 1.2, 1.4 and 1.2%. For the different haemorheological variables, intra-assay coefficients of variation were less than 1.6%, and inter-assay coefficients of variation were less than 4.5% (reference values in our laboratory).

Assessment of Erythrocyte ROS Generation and Lipid Peroxidation by Flow Cytometry

After collection, 3-μL ethylenediamine tetraacetic acid (EDTA) anticoagulated blood samples were re-suspended in 87 μL of RPMI (Roswell Park Memorial Institute medium) without...
serum and phenol red, and incubated with Anti-Human glycophorin A phycoerythrin (PE) (BD biosciences; 10 μL) at 37°C in the dark for 15 minutes, following manufacturer’s protocol. Next, the cells were centrifuged, the supernatant discarded and cells washed twice in phosphate buffer saline (PBS). To determine the level of intracellular reactive oxygen species (ROS) generation and lipid peroxidation, cells were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA; (2.5 μM, Life Technologies, Carlsbad, California, United States) and BODIPY 581/591 C11 (5 μM, Life Technologies) in RPMI without serum and phenol red for 15 minutes at 37°C, respectively. After labelling, cells were washed and resuspended in PBS and analysed immediately using a FACSCanto flow cytometer (Becton-Dickinson, San Jose, California, United States). The sample flow rate was adjusted to about 1,000 cells/s. For a single analysis, the fluorescence properties of 100,000 erythrocytes were collected. Data were analysed using BD FACSDiva software (Becton-Dickinson).

**Serum 8-Isoprostanes Assays**

Serum 8-isoprostanes were assessed by validated Enzyme immuno assay (EIA) assay method using 8-Isoprostane ELISA (enzyme-linked immunosorbent assay) Kit (Cayman Chemical, Ann Arbor, Michigan, United States) as previously reported. Intra-assay and inter-assay coefficients of variation were 5.5 and 4.8%, respectively.

**Red Cell Membrane Preparation**

Erythrocyte membranes were prepared by the method of Dodge with buffer modification. Erythrocytes were haemolyzed with 20 mM Tris-HCl buffer, pH 7.4, supplemented with 1 mM EDTA and 0.01% PMSF on ice for 15 minutes. The erythrocyte membranes were centrifuged at 20,000 × g for 5 minutes. The membranes were washed several times with the above-mentioned buffer until the ‘white ghost’ (haemoglobin-free) state was attained. All buffers were cooled to 4°C prior to use and the whole preparation procedure was conducted on ice. The protein concentration was estimated according to the method of Bradford. The concentration of protein in the sample was read from a calibration curve in the range 50–300 μg/mL using albumin from bovine serum as the standard.

**Fluidity of Erythrocyte Membranes**

The fluidity of erythrocyte membranes was measured by means of fluorescence anisotropy using two fluorescent probes: 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4′-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) as previously reported. DPH is localized in hydrophobic region near the centre of the bilayer, while TMA-DPH is incorporated into the polar region of the erythrocyte membrane. Fluorescence anisotropy measurements were carried out with an LS-55 fluorescence spectrometer (PerkinElmer, Buckinghamshire, UK). The excitation and emission wavelengths were 348 and 426 nm, respectively. The cuvette holder was temperature controlled (37°C). Erythrocyte membranes were diluted with buffered saline to protein concentration of 100 μg/mL. Final concentration of fluorescent probes was 1 μM. The fluorescence anisotropy of probe X is defined as follows:

\[ r(X) = \frac{(I_{vv} - I_{vh} \cdot G)}{(I_{vv} + 2I_{vh} \cdot G)} \]

where \( I_{vv} \) and \( I_{vh} \) are the intensities of the fluorescence (in arbitrary units) emitted, respectively, parallel and perpendicular to the direction of the vertically polarized excitation light and \( G \) is the correction factor \((G = I_{vh}/I_{hh})\) for the optical system given by the ratio of the vertically to the horizontally polarized emission components when the excitation light is polarized in the horizontal direction. \( X \) represents DPH or TMA-DPH. According to Shinitzky, the fluorescence anisotropy values are inversely proportional to cell membrane fluidity. A high degree of fluorescence anisotropy represents a high structural order or low cell membrane fluidity.

**In Vitro AAPH-Induced Blood Oxidation**

To demonstrate the key role of oxidative stress in RBC alterations, an in vitro approach was also used. Blood samples (20 mL) from 12 control subjects were collected in Vacutainer tubes containing EDTA (0.17 mol/L). After collection, 20 mL of blood was incubated at 37°C for 1 hour in the presence of (25-, 50- and 100-mM final concentration) 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)—a water-soluble azo compound used extensively as a free radical generator—and the remaining 10 mL of blood was maintained at 37°C for 1 hour without AAPH treatment. Following this, all experiments were performed in treated and untreated samples. In addition, to demonstrate the essential role of oxidative stress in RBC membrane alterations, all the above experiments were also performed in the presence of the antioxidant Trolox (100 μM). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid) is a water-soluble analogue of the free radical scavenger α-tocopherol. Trolox has advantages over α-tocopherol, which is lipid soluble, because it can be incorporated in both the water and the lipid compartments of the cells.

**Confocal Microscopy Analysis**

Whole blood was mixed with the same volume of RPMI (Sigma-Aldrich, St. Louis, Missouri, United States) and gently laid on a histopaque-1077 (Sigma-Aldrich). The sample was then centrifuged at 450 × g for 20 minutes at 10°C. After separation, RBC were isolated and washed twice with RPMI 1640. Isolated RBC were incubated with H<sub>2</sub>DCFDA (2.5 μM; Life Technologies) and BODIPY 581/591 C11 (5 μM; Life Technologies) in RPMI without serum and phenol red for 15 minutes at 37°C. After labelling, cells were washed and re-suspended in PBS and immediately analysed using a Leica TCS SP5 confocal scanning microscope (Mannheim, Germany) equipped with an argon laser for fluorescence analysis. A series of optical sections (1,024 × 1,024 pixels) was taken through the cells using a Leica 63X objective and a 4X electronic zoom and then projected as a single composite image by superimposition.

**Statistical Analysis**

Statistical analysis was performed using the SPSS (Statistical Package for Social Sciences; Chicago, Illinois, United States) software for Windows (version 20.0). The nonparametric Mann–Whitney test for unpaired data was used for comparisons between single groups. The χ<sup>2</sup> test was used to test for
proportions. Correlation analyses were measured by using the Spearman correlation test. Partial correlation analyses adjusted for age, sex, cardiovascular risk factors, medications, leukocyte number and mean corpuscular volume were also performed. A logistic regression analysis was used to evaluate the risk of SSNHL according to the cardiovascular risk factors, the use of therapies, RBC ROS production, 8-isoprostanes and RBC membrane lipid peroxidation. For logistic regression analysis odds ratios (OR) and 95% confidence intervals (CI) are presented. Variables showing, at the univariate logistic regression analysis, an association with the disease (< 0.20) were introduced in the multivariate logistic regression model. Due to the collinearity problem, each variable, that is, RBC-derived ROS, RBC lipid peroxidation, DPH and TMA-DPH were added separately into the final regression model. In order to study the association between erythrocyte membrane viscosity and deformability, a linear regression analysis was performed by introducing age, sex, cardiovascular risk factors, medications, leukocyte number and mean corpuscular volume (MVC), RBC ROS production, 8-isoprostanes and RBC membrane lipid peroxidation into the linear regression model in SSNHL patients. A p-value of less than 0.05 was considered statistically significant.

Results

The demographic and clinical characteristics of SSNHL patients and healthy subjects are reported in Table 1. SSNHL patients had hypertension, dyslipidaemia and diabetes mellitus more frequently than controls (Table 1).

Erythrocyte Oxidative Stress

Flow cytometry analysis was used to evaluate erythrocyte oxidative stress. As reported in Figure 1A, erythrocyte from SSNHL patients showed significantly higher ROS levels than healthy subjects (687 ± 346 vs. 283 ± 56; p < 0.0001). Erythrocyte membrane lipid peroxidation levels from SSNHL patients were significantly higher than those from the controls (1,631 ± 861 vs. 641 ± 155; p < 0.0001; Fig. 1A).

Among clinical characteristics, ROS production or lipid peroxidation did not differ between SSNHL patients with or without traditional cardiovascular risk factors.

Serum 8-Isoprostanes

Systemic oxidative stress was assessed by measuring the lipid peroxidation marker 8-isoprostanes in serum from patients and controls. As reported in Figure 1B, serum from SSNHL patients showed significantly higher 8-isoprostanes levels than that from healthy subjects (177.4 ± 43.9 vs. 81.2 ± 23.3; p < 0.001).

Red Blood Cell Membrane Anisotropy

Fluorescent probes were used to evaluate the fluidity of erythrocyte membranes. TMA-DPH and DPH, which are known to reflect membrane fluidity in the hydrophilic layer and in the hydrophobic core of the lipid bilayer, respectively,29,30 were applied. In Figure 1C, the analyses of fluorescence anisotropy (r) of the erythrocyte membrane are reported. DPH and TMA-DPH probes show significant differences between SSNHL patients and the control group (DPH: 0.2657 ± 0.0294 vs. 0.2593 ± 0.0147; p < 0.0001; TMA-DPH: 0.2878 ± 0.0298 vs. 0.2593 ± 0.0131; p < 0.0001). The change of fluorescent probes fluorescence anisotropy was rather small but was statistically significant. The effect was observed in the case of both fluorescent probes, which means that fluidity changes occurred on the surface of membranes as well as in the lipid core.

Haemorrhological Parameters

WBV both at 0.512 and 94.5/s shear rates was significantly different between patients and controls, (26.0 ± 4.0 vs. 19.8 ± 1.0; p < 0.0001 and 4.9 ± 0.5 vs. 3.80 ± 0.3; p < 0.0001, respectively; Fig. 1D). EI was significantly lower in patients than in controls (0.3609 ± 0.0284 vs. 0.4306 ± 0.0091; p < 0.0001; Fig. 1E).

Correlation between Investigated Parameters

As shown in Figure 2, erythrocyte-derived ROS significantly correlated with WBV analysed at a shear rate of 0.512/s ($r^2 = 0.3309; p = 0.0003$), with WBV analysed at a shear rate of 94.5/s ($r^2 = 0.2298; p = 0.0036$), with the EL ($r^2 = 0.4284; p < 0.0001$) and with the erythrocyte membrane anisotropy: with DPH ($r^2 = 0.6553; p = 0.01752$) and with TMA-DPH ($r^2 = 0.6577; p < 0.0001$). Erythrocyte membrane lipid peroxidation significantly correlated with WBV analysed at a shear rate of 0.512/s ($r^2 = 0.2469; p = 0.0024$), with WBV analysed at

Table 1 Clinical characteristics of study population

<table>
<thead>
<tr>
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<th>SSNHL patients (N = 35)</th>
<th>Controls (N = 35)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y, median (IQR)</td>
<td>62 (52–72)</td>
<td>61 (51–72)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Males/females, n</td>
<td>22/13</td>
<td>22/13</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>24 (68.6)</td>
<td>10 (28.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>Dyslipidaemia, n (%)</td>
<td>24 (68.6)</td>
<td>14 (40.0)</td>
<td>0.015</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>6 (17.1)</td>
<td>1 (2.9)</td>
<td>0.053</td>
</tr>
<tr>
<td>Smoking habit, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smokers</td>
<td>11 (31.4)</td>
<td>15 (42.9)</td>
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</tr>
<tr>
<td>Ex-smokers</td>
<td>16 (45.7)</td>
<td>10 (28.6)</td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>8 (22.9)</td>
<td>6 (17.1)</td>
<td></td>
</tr>
<tr>
<td>Antihypertensive drugs, n (%)</td>
<td>20 (57.1)</td>
<td>9 (25.7)</td>
<td>0.008</td>
</tr>
<tr>
<td>Hypoglycaemic drugs, n (%)</td>
<td>2 (5.7)</td>
<td>1 (2.9)</td>
<td>0.555</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>4 (11.4)</td>
<td>3 (8.6)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Antiplatelet drugs, n (%)</td>
<td>1 (2.9)</td>
<td>3 (8.6)</td>
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<tr>
<td>Oral anticoagulants, n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviations: IQR, interquartile range; n.s., not significant; SSNHL, sudden sensorineural hearing loss.
a shear rate of 94.5/s (r² = 0.2612; p = 0.0017), with the EI (r² = 0.4353; p < 0.0001) and with the erythrocyte membrane anisotropy: with DPH (r² = 0.5723; p < 0.0001) and with TMA-DPH (r² = 0.5337; p < 0.0001; – Fig. 3).

As shown in – Fig. 4, serum 8-isoprostanates significantly correlated with WBV analysed at a shear rate of 0.512/s (r² = 0.1624; p = 0.0164), with WBV analysed at a shear rate of 94.5/s (r² = 0.1929; p = 0.0083), with the EI (r² = 0.4353; p < 0.0001) and with the erythrocyte membrane anisotropy: with DPH (r² = 0.1856; p = 0.0098) and with TMA-DPH (r² = 0.1571; p = 0.0184).

– Fig. 5A showed that DPH fluorescence anisotropy significantly correlated with WBV analysed at a shear rate of 0.512/s (r² = 0.3067; p = 0.0006), with WBV analysed at a shear rate of 94.5/s (r² = 0.2306; p = 0.0035), with the EI (r² = 0.3167; p = 0.0004), TMA-DPH fluorescence anisotropy significantly correlated with WBV analysed at a shear rate of 0.512/s (r² = 0.2843; p = 0.0010), with WBV analysed at a shear rate of 94.5/s (r² = 0.2543; p = 0.0020), with the EI (r² = 0.3045; p = 0.0006; – Fig. 5B).

No correlation between ROS production or lipid peroxidation and traditional risk factors, platelet volume, WBC, MCH, MCHC, RDV, platelet count and fibrinogen level were found.

Regression Analyses
At partial correlation analyses, RBC oxidative stress-related parameters (ROS production and lipid peroxidation) and the systemic oxidative status (serum isoprostanes) were significantly related with RBC membrane rigidity (DPH and TMA-DPH), WBV and RBC deformability (RBC EI) also after controlling for age, sex, cardiovascular risk factors, medications, leukocyte number and MCV (– Table 2). In particular, RBC ROS production is significantly related to DPH (r = 0.786; p < 0.001), TMA-DPH (r = 0.753; p < 0.001), WBV 0.512/s (0.430; p < 0.001), WBV 94.5/s (0.403, p < 0.001) and RBC EI (r = -0.607; p < 0.001); RBC lipid peroxidation is significantly related to DPH (r = 0.676; p < 0.001), TMA-DPH (r = 0.616; p < 0.001), WBV 0.512/s (0.496; p < 0.001), WBV 94.5/s (0.433; p < 0.001) and RBC EI (r = -0.553; p < 0.001); moreover, serum isoprostanes are significantly related to DPH (r = 0.677; p < 0.001), TMA-DPH (r = 0.571; p < 0.001), WBV 0.512/s (0.578; p < 0.001), WBV 94.5/s (0.553; p < 0.001) and RBC EI (r = -0.773; p < 0.01).

In – Table 3, univariate and multivariate logistic regression analyses for SSNHL are shown. At multivariate logistic regression analysis (– Table 3), after adjustment for several potential confounders, hypertension, diabetes, erythrocyte-derived ROS, erythrocyte lipid peroxidation, DPH and TMA-DPH and serum 8-isoprostanes remained significantly associated with SSNHL (p < 0.05).

ROS-Induced RBC Membrane Alterations
To demonstrate the key role of oxidative stress in RBC alterations, we performed an in vitro approach by treating blood samples from 12 healthy subjects with the free radical generator AAPH (– Fig. 6) in a dose-dependent manner. First, confocal microscopy analysis clearly demonstrated that AAPH-treated cells exhibited a dose-dependent increase in erythrocyte ROS production (– Fig. 6A) and lipid peroxidation (– Fig. 6B). As expected, Trolox (a water-soluble analogue of vitamin E) treatment reverted this effect (– Fig. 6A, B). In addition, confocal
microscopy revealed that AAPH-treated erythrocytes displayed crenated shapes with membrane cytoplasmic blebbing compared to untreated erythrocytes characterized by the usual biconcave disc appearance. These data were also confirmed by FACS (fluorescence-activated cell sorting) analysis (Fig. 6C). In particular, AAPH treatment significantly increased intracellular ROS production with respect to untreated cells at all considered concentrations (25 mM: 645 ± 113 vs. 298 ± 26; p < 0.0001; 50 mM: 1,010 ± 128 vs. 298 ± 26; p < 0.0001; 100 mM: 2,808 ± 576 vs. 298 ± 26; p < 0.0001). Lipid

**Fig. 2** Correlation analysis among erythrocyte reactive oxygen species (ROS) production, whole blood viscosity (WBV; analysed at 0.512 and 94.5/s shear rates), erythrocyte deformability (elongation index) and erythrocyte membrane fluidity (using 1,6-diphenyl-1,3,5-hexatriene [DPH] and 1-[40-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene [TMA-DPH] probes) in SSNHL (sudden sensorineural hearing loss) patients. Erythrocyte ROS production positively and significantly correlated with WBV analysed at 0.512 and 94.5/s shear rates (p < 0.001) and with erythrocyte membrane fluidity (using DPH and TMA-DPH probes; p < 0.001). Erythrocyte ROS production inversely correlated with RBC deformability (EI; p < 0.0001).

**Fig. 3** Correlation analysis among erythrocyte membrane lipid peroxidation, whole blood viscosity (WBV; analysed at 0.512 and 94.5/s shear rates), erythrocyte deformability (elongation index [EI]) and erythrocyte membrane fluidity (using 1,6-diphenyl-1,3,5-hexatriene [DPH] and 1-[40-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene [TMA-DPH] probes) in SSNHL (sudden sensorineural hearing loss) patients. Erythrocyte membrane lipid peroxidation positively and significantly correlated with WBV analysed at 0.512 and 94.5/s shear rates (p < 0.01) and with erythrocyte membrane fluidity (using DPH and TMA-DPH probes; p < 0.0001). Erythrocyte membrane lipid peroxidation inversely correlated with red blood cell deformability (EI; p < 0.0001).
peroxidation also increased in a dose-dependent manner (25 mM: 1,227 ± 193 vs. 779 ± 44; p < 0.0001; 50 mM: 1,874 ± 103 vs. 779 ± 44; p < 0.0001; 100 mM: 4,278 ± 407 vs. 779 ± 44; p < 0.0001). Moreover, a significant increase in fluorescent probes fluorescence anisotropy in oxidized samples was found (Fig. 6D). The fluorescence anisotropy values are inversely proportional to cell membrane fluidity. High levels of fluorescence anisotropy indicate a high structural order or low cell membrane fluidity. In particular, erythrocyte membrane fluidity of the hydrophobic region of the bilayer (DPH probe) positively and significantly correlated with WBV analysed at 0.512 and 94.5/s shear rates (p < 0.01) and inversely correlated with erythrocyte deformability (EI; p < 0.001).
bilayer (DPH probe) decreased in AAPH-treated samples (25 mM: 0.2578 ± 0.0068 vs. 0.2198 ± 0.0047; p < 0.0001; 50 mM: 0.2953 ± 0.0111 vs. 0.2198 ± 0.0047; p < 0.0001; 100 mM: 0.3773 ± 0.0150 vs. 0.2198 ± 0.0047; p < 0.0001). This effect was similar into the polar region of the erythrocyte membrane (TMA-DPH probe; 25 mM: 0.2805 ± 0.0109 vs. 0.2527 ± 0.0081; p < 0.0001; 50 mM: 0.3128 ± 0.0085 vs. 0.2527 ± 0.0081; p < 0.0001; 100 mM: 0.3774 ± 0.0129 vs. 0.2527 ± 0.0081; p < 0.0001). Importantly, this effect was reversed by Trolox treatment, demonstrating the key role of oxidative stress in altering membrane rigidity. As shown in Figure 6E, a significant difference in WBV—94.5 s was observed between AAPH-treated and untreated blood samples from healthy subjects. At 0.512/s shear rate, increasing values were observed in a dose-dependent manner (25 mM: 24.37 ± 1.16 vs. 20.70 ± 0.93; p < 0.0001; 50 mM: 27.43 ± 1.27 vs. 20.70 ± 0.93; p < 0.0001; 100 mM: 33.94 ± 1.69 vs. 20.70 ± 0.93; p < 0.0001). Similar results were obtained at 94.5/s shear rate (25 mM: 4.6 ± 0.3 vs. 4 ± 0.2; p < 0.0001; 50 mM: 5.0 ± 0.3 vs. 4 ± 0.2; 100 mM: 5.3 ± 0.4 vs. 4.4 ± 0.8; p < 0.0001).

**Table 2** Partial correlation coefficients between erythrocyte oxidative stress-related parameters (ROS production and lipid peroxidation), serum isoprostanes and erythrocyte membrane rigidity (1,6-diphenyl-1,3,5-hexatriene [DPH] and 1-[40-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene [TMA-DPH]), whole blood viscosity (WBV) and erythrocyte deformability (erythrocyte elongation index [EI]) after controlling for age, sex, cardiovascular risk factors, medications, leukocyte number and mean corpuscular volume.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DPH (RFU)</th>
<th>RBC ROS (RFU)</th>
<th>RBC membrane lipid peroxidation (RFU)</th>
<th>Serum isoprostanes (pg/mL)</th>
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<tr>
<td></td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td>0.786; p &lt; 0.001</td>
<td>0.676; p &lt; 0.001</td>
<td>0.616; p &lt; 0.001</td>
<td>0.677; p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>0.753; p &lt; 0.001</td>
<td>0.430; p &lt; 0.001</td>
<td>0.496; p &lt; 0.001</td>
<td>0.578; p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>0.403; p = 0.001</td>
<td>0.433; p &lt; 0.001</td>
<td>0.553; p &lt; 0.001</td>
<td>0.733; p &lt; 0.001</td>
</tr>
<tr>
<td>RBC E1</td>
<td>r = −0.607; p &lt; 0.001</td>
<td>r = −0.553; p &lt; 0.001</td>
<td>r = −0.733; p &lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3** Univariate and multivariate logistic regression analyses for SSNHL patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Univariate analysis (controls, n = 35; patients, n = 35)</th>
<th>Multivariate analysis (controls, n = 35; patients, n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI) p-Value</td>
<td>OR (95% CI) p-Value</td>
</tr>
<tr>
<td>Age (y)</td>
<td>0.99 (0.96–1.04) 0.931</td>
<td>–</td>
</tr>
<tr>
<td>Gender (F vs. M)</td>
<td>1.00 (0.38–2.64) 1.00</td>
<td>–</td>
</tr>
<tr>
<td>Hypertension</td>
<td>5.46 (1.96–15.18) 0.001</td>
<td>3.18 (1.45–22.42) 0.036</td>
</tr>
<tr>
<td>Dyslipidaemia</td>
<td>3.27 (1.22–8.75) 0.018</td>
<td>–</td>
</tr>
<tr>
<td>Diabetes</td>
<td>7.03 (0.80–61.87) 0.079</td>
<td>–</td>
</tr>
<tr>
<td>Smoking habit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex-smokers vs. non-smokers</td>
<td>2.76 (0.94–8.17) 0.067</td>
<td>1.65 (0.12–22.14) 0.706</td>
</tr>
<tr>
<td>Smokers vs. non-smokers</td>
<td>2.30 (0.63–8.39) 0.206</td>
<td>2.51 (0.08–77.11) 0.599</td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td>3.85 (1.40–10.59) 0.009</td>
<td>1.46 (0.20–10.67) 0.710</td>
</tr>
<tr>
<td>Hypoglycaemic drugs</td>
<td>2.06 (0.18–23.83) 0.563</td>
<td>–</td>
</tr>
<tr>
<td>Statins</td>
<td>1.38 (0.29–6.66) 0.158</td>
<td>1.95 (0.17–22.70) 0.629</td>
</tr>
<tr>
<td>Antiplatelet drugs</td>
<td>3.19 (0.32–32.24) 0.326</td>
<td>–</td>
</tr>
<tr>
<td>RBC-derived ROS for each 10-unit increase</td>
<td>1.47 (1.20–1.80) &lt;0.001</td>
<td>1.61 (1.17–2.22) 0.004</td>
</tr>
<tr>
<td>RBC lipid peroxidation for each 100-unit increase</td>
<td>1.75 (1.27–2.39) 0.001</td>
<td>1.75 (1.19–2.56) 0.004</td>
</tr>
<tr>
<td>8-Isoprostanes for each 10-unit increase</td>
<td>3.00 (1.69–5.42) &lt;0.001</td>
<td>6.88 (1.18–40.09) 0.032</td>
</tr>
<tr>
<td>DPH for each 100-unit increase</td>
<td>4.75 (2.18–10.35) &lt;0.001</td>
<td>7.82 (21.89–32.38) 0.005</td>
</tr>
<tr>
<td>TMA-DPH for each 100-unit increase</td>
<td>1.86 (1.33–2.61) &lt;0.001</td>
<td>1.89 (1.31–2.73) 0.001</td>
</tr>
</tbody>
</table>

**Note:** Analyses were adjusted for age, sex, cardiovascular risk factors, medications, leukocyte number and mean corpuscular volume.
When EI (►Fig. 6F) was measured (25 mM: 0.39/C6 0.02 vs. 0.44/C6 0.01; p < 0.0001; 50 mM: 0.37/C6 0.02 vs. 4/C6 0.2; p < 0.0001; 100 mM: 0.29/C6 0.03 vs. 4/C6 0.2; p < 0.0001), however, in all conditions, Trolox treatment was able to revert these effects. Collectively, these data demonstrate that oxidative stress has a key role in RBC membrane alterations.

**Discussion**

Thirty years ago, a hypercoagulable state has been proposed to be involved in the local hyperviscosity syndrome, causing an impairment in cochlear microcirculation. In SSNHL, whose pathogenesis still remains unknown, blood hyperviscosity has been described and associated with
Erythrocyte deformability.\textsuperscript{9,34} Furthermore, our previous work\textsuperscript{9} has reported in subjects with SSNHL the association/positive correlation between blood hyperviscosity and haemostatic changes.

A balance shifting towards increased oxidative stress in SSNHL has been recently reported,\textsuperscript{12} but no study, so far, has investigated the possible interplay among SSNHL, erythrocyte oxidative stress and blood viscosity. For the first time, our data show an increase in erythrocyte ROS production and membrane lipid peroxidation in SSNHL patients compared to control subjects; interestingly, these parameters correlate with erythrocyte deformability and WBV in these patients.

Oxidative stress, which is defined as an imbalance between ROS production and intracellular antioxidant systems, has been proposed as a risk factor for microcirculation injury.\textsuperscript{34,35} In addition, we confirmed an increased level of serum 8-isoprostanes as already reported in the plasma of SSNHL patients.\textsuperscript{12,36}

Erythrocyte deformability is crucial for maintaining normal circulation. The cochlea is provided with a terminal capillary bed and is not able to form collateral vessels that can restore blood flow in ischemic regions. Moreover, cochlear cells are particularly susceptible to blood flow reduction and occlusions in this area can lead to cochlea ischaemia.\textsuperscript{37} Decreased erythrocyte deformability impairs microcirculatory perfusion leading to hypoxia and endothelial dysfunction.

The high abundance of iron and haemoglobin and lack of nucleus and organelle make erythrocytes highly susceptible to oxidative stress. Therefore, an increase in oxidative stress is a probable driving force for enhanced erythrocyte membrane alterations.

Fig. 6 (Continued)
to oxidative stress. Moreover, erythrocyte membranes are composed of highly polyunsaturated fatty acids that make the membrane markedly susceptible to oxidation resulting in membrane bilayer structure alterations, decreased membrane fluidity and cell membrane damage. Studies have revealed that lipid peroxidation severely affects biomembranes. It induces disturbance of fine structures, alteration of integrity, fluidity, permeability and functional loss of biomembranes and also modifies low-density lipoprotein (LDL) to pro-atherogenic and pro-inflammatory forms, and generates potentially toxic products. Hence, lipid peroxidation is the major determinant of the decrease in membrane fluidity. However, the oxidative stress-mediated mechanisms that alter membrane functions are unknown. Our results point out that erythrocyte membrane fluidity is significantly altered in SSNHL patients compared to controls, suggesting that erythrocyte membranes are stiffer and less fluid in SSNHL patients.

Shear rate, shear stress and blood viscosity have been correlated with erythrocyte membrane fluidity and, in vivo, shear forces might participate to the control of erythrocyte membrane fluidity. It is well known that erythrocytes are highly flexible, capable of extreme changes in their shape and might adapt their membranes to blood flow conditions. For the first time, in SSNHL patients we found an inverse correlation between erythrocyte deformability and membrane lipid peroxidation, confirming that lipid peroxidation can exert a deep impact on membrane structure. Another important finding is the significant and inverse correlation between erythrocyte ROS production and Ei in SSNHL patients.

Interestingly, the results of the statistical analyses indicate that erythrocyte-derived ROS and erythrocyte lipid peroxidation still remained significantly associated with SSNHL and positively correlated with erythrocyte membrane viscosity and deformability even after adjustment for several potential confounders (including hypertension and diabetes). In fact, in patients with diabetes, increased internal membrane viscosity resulting from glycosylation leads to decreased erythrocyte deformability and also the severity of hypertension is associated with erythrocyte aggregation and deformability. Moreover, our in vitro experiments clearly demonstrate the key role of oxidative stress in RBC alterations.

This study is not without limitations. For instance, the use of a control group comprising healthy volunteers may have resulted in higher OR. As SSNHL patients and healthy subjects were likely from different source populations, the studied variables may have followed a different population distribution to that from which patients originally originated. Moreover, our findings were obtained using a small sample size and will require verification in a larger cohort, while the retrospective nature of the study precludes definitive conclusions as to the role of alterations in SSNHL onset, as all laboratory parameters were recorded at a single time point post-SSNHL. Nevertheless, the results reported here indicate significant structural and functional membrane alterations as well as elevated levels of membrane lipid peroxidation and intracellular ROS production in erythrocytes from SSNHL patients. In conclusion, it can be suggested that ROS-induced erythrocyte alterations play a role in SSNHL pathogenesis and might represent a new therapeutic target.

What is known about this topic?

- Sudden sensorineural hearing loss (SSNHL), characterized by an acute unexplained hearing loss that occurs over less than a 72-hour period, has an unclear pathogenesis.
- Cochlear vascular occlusion has been proposed as a potential mechanism of hearing damage. Some researchers have focused their attention on the haemorheological profile, but this mechanism has not yet been elucidated.
- An association of SSNHL with oxidative stress has been demonstrated; however, its contribution to haemorheological profile in SSNHL patients is still unknown.

What does this paper add?

- We aimed to find out if haemorheological profile is altered by oxidative stress in SSNHL patients.
- Erythrocyte-derived reactive oxygen species (ROS) and erythrocyte lipid peroxidation positively correlated with whole blood viscosity and erythrocyte deformability in SSNHL patients.
- ROS alter erythrocyte membrane rigidity, playing a key role in SSNHL pathogenesis.

Acknowledgment

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References
