

·基础研究·

长期血液透析患者红细胞膜 F₂-isoprostanes 及细胞膜流动性的研究

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摘要:【目的】探讨建立血浆和红细胞膜的 iPF_{2α}-III 评估血液透析 (HD) 患者氧化应激状态, 并研究细胞膜流动性与氧化应激的关系。【方法】对 49 名长期血液透析患者和 31 例健康人检测其血浆和红细胞细胞膜 F₂-isoprostanes (一个特定的同分异构体 iPF_{2α}-III) 作为体内氧化应激评价的指标, 同时利用 DPH 和 TMA DPH 评估细胞膜流动性作为一种间接的细胞功能标志。【结果】血液透析患者和正常对照组相比, 氧化应激增高, 血浆 iPF_{2α}-III (0.450 ± 0.173 与 0.236 ± 0.107 nmol/L, $P < 0.01$) 和红细胞膜 iPF_{2α}-III (32.5 ± 7.9 与 25.7 ± 8.7 pmol/g, $P < 0.01$)。与正常对照组比较血液透析病人红细胞膜流动性降低, DPH (0.230 2 ± 0.004 0 与 0.224 6 ± 0.004 6, $P < 0.01$) 和 TMA DPH (0.271 0 ± 0.004 5 与 0.264 7 ± 0.007 1, $P < 0.01$), 血浆的 iPF_{2α}-III 及红细胞膜流动性无相关; 血液透析患者红细胞膜的 iPF_{2α}-III 与核心区域细胞膜流动性呈负相关 ($r = 0.630$, $P < 0.01$)。在血液透析病人与健康对照组相比较中发现, 血液透析病人中的不同时段的红细胞膜上的 iPF_{2α}-III 的含量较健康对照组增高, 相对应红细胞的流动性减低。线性回归分析显示血液透析病人不同时段的红细胞膜 DPH 流动性与红细胞的膜 iPF_{2α}-III 含量相关 (幼红细胞 $r = 0.491$, $P = 0.015$; 成熟红细胞 $r = 0.563$, $P = 0.004$; 衰老红细胞 $r = 0.460$, $P = 0.024$)。【结论】血液透析患者由于长期处于氧化应激增高的状态, 此状态造成不同时段的红细胞膜的表面及核心区域的均受到损害。通过检测红细胞膜的 iPF_{2α}-III 可以评估体内红细胞氧化应激状态的指标。

关键词: 氧化应激; iPF_{2α}-III; F₂-isoprostanes; 细胞膜的流动性; 血液透析

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Erythrocyte Membrane F₂-isoprostanes Relate to Membrane Fluidity in Patients on Long-term Hemodialysis

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Abstract: 【Objective】 To determine whether plasma and erythrocyte membrane iPF_{2α}-III can be established as a marker for assess the increased oxidative stress in hemodialysis (HD) patients and to verify the potential relationships with membrane fluidity related to oxidative stress in HD patients. 【Methods】 Forty nine patients on long-term hemodialysis and 31 healthy subjects were recruited. Plasma and erythrocyte membrane F₂-isoprostanes (one specific isomer iPF_{2α}-III) was investigated as a marker for in vivo oxidative stress assessment. Membrane fluidity in both core with 1,6-diphenylhexatriene (DPH) and surface regions with trimethylammonium-DPH (TMA-DPH) were assessed and used as an indirect marker of cellular function. 【Results】 Both plasma iPF_{2α}-III (0.450 ± 0.173 vs. 0.236 ± 0.107 nmol/L, $P < 0.01$) and membrane iPF_{2α}-III (32.5 ± 7.9 vs. 25.7 ± 8.7 pmol/g protein, $P < 0.01$) showed that hemodialysis patients had increased oxidative stress than control subjects. Erythrocyte membrane fluidity was decreased in HD patients compared with normal controls measured by DPH (0.2302 ± 0.0040 vs 0.2246 ± 0.0046, $P < 0.01$) and TMA-DPH (0.2710 ± 0.0045 vs 0.2647 ± 0.0071, $P < 0.01$) anisotropy. There was no correlation between plasma total iPF_{2α}-III and membrane fluidity; and erythrocyte membrane iPF_{2α}-III was inversely correlated with the core region membrane fluidity in HD patients ($r = 0.630$, $P < 0.00$). Furthermore, membrane iPF_{2α}-III content and membrane anisotropy values from different fractions according

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to their ages as old, middle and young erythrocytes in HD patients were significantly higher than those in the corresponding fractions of healthy controls. Comparing with erythrocytes in the young fraction, membrane $iPF_{2\alpha}-III$, DPH anisotropy and TMA-DPH anisotropy in the older cells were all significantly higher. Linear regression analysis, the effects of membrane $iPF_{2\alpha}-III$ on DPH anisotropy in different aged cells were similar for the HD patients (Young $r = 0.491$, $P = 0.015$; Middle age $r = 0.563$, $P = 0.004$; Old $r = 0.460$, $P = 0.024$). 【Conclusions】 HD patients were under increased oxidative stress and oxidative damages on both core and surface regions of erythrocyte membranes, and all circulating erythrocytes of different ages were affected. Membrane $iPF_{2\alpha}-III$ content can be considered as a tissue marker for in vivo erythrocyte oxidative stress assessment.

Key words: oxidative stress; erythrocytes; F_2 -isoprostanes; membrane fluidity; hemodialysis

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Accumulating evidence has suggested that an increased oxidative stress may be a significant contributor to the accelerated pathologies associated with hemodialysis(HD)^[1]. HD patients were associated with shortened erythrocytes survival and increased hemolysis. Increased lipid peroxidation has implications for stability and integrity of the erythrocyte membrane. This may disrupt cell membrane architecture, thereby affecting membrane fluidity and cell function^[2-3].

F_2 -isoprostanes are formed by free radical peroxidation of arachidonic acid (AA) in situ on phospholipids and subsequently released in free form by the action of phospholipase^[4-5]. Measurement of F_2 -isoprostanes or one specific biologically active isomer $iPF_{2\alpha}-III$ in plasma is considered to be a reliable marker for studies of lipid peroxidation in human diseases and for assessment of the in vivo effects of antioxidants^[6]. Plasma isoprostanes, a sensitive and reliable index of lipid peroxidation in vivo, had been used to show increased oxidative stress in HD patients^[7-8]. However, measurement of plasma and urine F_2 -isoprostanes is a "whole-body" oxidative stress measurement. Circulating F_2 -isoprostanes levels had not been examined whether it can accurately reflect membrane peroxidation and disease does not necessarily imply a causative link, none of these markers was used to demonstrate a direct pathophysiological link between oxidative damage and increased oxidative stress in HD patients.

Assessment of esterified F_2 -isoprostanes in tissues of interest would allow a direct assay of lipid peroxidation at an oxidant damage site^[9]. The major store of AA in mammalian cells is esterified at the sn-

2 position of glycerophospholipids on cellular membranes. Peroxidation of lipid-esterified AA rather than free AA is the most likely source for F_2 -isoprostanes formation in vivo^[10]. Circulating erythrocytes, a tissue with limited biosynthetic capacity and poor repair mechanism, are exposed to unusual oxidative stress and would offer a number of advantages for assessment of in vivo oxidative damages. Erythrocyte membrane F_2 -isoprostanes content could be used as a marker for in vivo oxidative stress. Abnormal susceptibility of membrane lipids to peroxidation is believed to reflect a similar abnormality in other organs and tissues as well as an impaired antioxidant mechanism^[11]. Membrane fluidity is attributed to the dynamic of lipid constituents, which express the behavior of membrane lipid bilayers and regulate the structural and dynamic properties. Membrane fluidity is thought to play an important role in various cellular functions, changes in membrane fluidity have been related to various pathologic states^[12-13].

In this study, plasma and membrane $iPF_{2\alpha}-III$ were measured by immunoaffinity chromatography following gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS). Membrane fluidity was measured by a spectrofluorometer using 1, 6-diphenylhexatriene (DPH) and trimethylammonium-DPH (TMA-DPH) as probes in the human erythrocytes ghost membrane preparations. DPH and TMA-DPH are cylindrically shaped molecules with absorption and fluorescence emission transition dipoles aligned approximately parallel to their long molecular axis. DPH is a relatively hydrophobic molecule and position itself in the deep hydrophobic regions of the lipid bilayer. The derivative

TMA-DPH contains a cationic trimethylammonium substituent and acts as a surface anchor. The fluidity of the regions in which the dyes are situated affects their rate of rotation and hence the degree of depolarization of fluorescent light. The anisotropy value is a quantitative expression of this effect and may be regarded as having an inverse relationship with the fluidity of the lipid bilayer^[13].

The aim of this study is to determine whether erythrocyte membrane $iPF_{2\alpha}$ -III can be established as a new marker for assess the increased oxidative stress in HD patients and to verify the potential relationships between erythrocyte membrane $iPF_{2\alpha}$ -III content and membrane fluidity related to oxidative stress in vivo.

1 Materials and methods

1.1 Materials

$iPF_{2\alpha}$ -III standard, 3, 3', 4, 4'-tetradeuterated $iPF_{2\alpha}$ -III ($iPF_{2\alpha}$ -III-d4) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The polyclonal rabbit antibody-coated resin for affinity purification of $iPF_{2\alpha}$ -III was obtained from Assays Design Inc. (Ann Arbor, MI, USA). 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1, 3, 5-hexatriene (TMA-DPH) were purchased from Molecular Probes (Eugene, OR, USA). Pentafluorobenzyl bromide (PFB-Br) and N, O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) were supplied by Supelco Incorporation (Bellefonte, PA, USA). N, N-diisopropylethylamine (DIPEA), indomethacin, butyrate hydroxytoluene (BHT), Dulbecco's phosphate buffered saline (DPBS) and human albumin standard, were from Sigma-Aldrich Inc. (St. Louis, MO, USA). Dimethylsulfoxid (DMSO) was supplied by Merck & Co., Inc. (Whitehouse Station, NJ, USA). Organic solvents of HPLC grade: chloroform, 2, 2, 4-trimethylpentane (iso-octane), n-hexane, methanol, ethanol, acetone and acetonitrile were purchased from Merck KGaA (Darmstadt, Germany). Phosphate buffered saline (PBS, 10 mmol/L, pH 7.4) was supplied by Amersco Inc. (Solon, OH, USA).

Deionized water was from a Milli-Q purification system from Millipore (Molsheim, France).

1.2 Subjects

Patients on long-term HD were recruited from the Division of Nephrology, Department of Medicine & Therapeutics, Prince of Wales Hospital (Hong Kong SAR, China). The study population included 49 adult Chinese patients (26 male and 23 female, aged 56.2 ± 10.6 , 35-74 years) who were on HD for 57.8 ± 45.3 months. All patients were routinely dialyzed for two to three times weekly. Blood samples were collected from the HD patients at the start of the dialysis treatment (before the anticoagulant injection). For control subjects, 31 healthy Chinese subjects (17 male and 14 female, aged 52.4 ± 3.5 , 46-61 years) were recruited. For healthy controls, venous blood samples were collected by venipuncture after an overnight fast. The HD patients were clinically stable without any overt active inflammatory disease. Their predialysis serum creatinine and urea concentrations were 1096 ± 257 mol/L and 33.3 ± 7.5 mmol/L, respectively. To exclude common diseases, routine laboratory blood (fasting glucose, liver function test, renal function test and complete blood picture) and urine tests (protein) were performed. In accordance with the Helsinki II Declaration (1989), the design and execution of the project were explained thoroughly to the subjects, and their informed consents were obtained.

1.3 Measurement of $iPF_{2\alpha}$ -III

For the measurement of plasma $iPF_{2\alpha}$ -III, venous blood (4 mL) was collected into a lithium-heparin tube containing indomethacin (in 5% sodium bicarbonate solution) at a final concentration of 15 mol/L as cyclooxygenase inhibitor. The blood tube was centrifuged at 2 400 g for 10 min at 4 °C to separate plasma from blood cells. Aliquots (1 mL) of plasma were transferred to Eppendorf tubes containing BHT as free radical scavenger at a final concentration of 20 μ mol/L. The samples were stored at -70 °C until analysis.

For the preparation of erythrocyte membranes to measure membrane $iPF_{2\alpha}$ -III and membrane fluidity,

the buffy coat was removed by aspiration with a plastic pipette after removal of plasma. The packed erythrocytes (1 mL) were haemolyzed by 12 mL distilled water, followed by centrifugation at $8\,000 \times g$ for 15 min. The supernatant was aspirated carefully without disturbing the pellet which contained ghost membranes. The procedure was repeated at least 3 times until the supernatant became clear. The resulting membrane pellet was liquefied by vortexing with DPBS to a volume of 1 mL. BHT was added at a final concentration of 20 $\mu\text{mol/L}$ and stored at -70°C .

To study circulating erythrocytes of different ages, 24 HD patient and 10 healthy control samples were randomly selected. Erythrocytes in each blood sample were fractionated into sub-populations according to ages by the protocol described by Garby et al^[14]. The erythrocytes were washed three times with five volumes of DPBS and the packed erythrocytes were transferred to a 10 mL OptiSeal™ polyallomer centrifuge tube fitted to a swinging bucket rotor, type 70.1 Ti, of the Beckman Model XL-70 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA, USA). The sample was spun at $35\,000 \times g$ at 4°C for 2 h. After centrifugation, the tube was immediately frozen to -70°C . While the sample was still frozen inside the centrifuge tube, a cutter was used to cut the tube into three equal parts. The age of the cell fractions was defined by their positions in the tube, as young cells at the top layer, middle age cells at the center and old cells at the bottom of the tube. Each fraction was collected into a test tube and the contents were thawed. Erythrocyte ghost membranes in each fraction were then prepared as described above.

Plasma or membrane (0.5 mL) and 500 pg $\text{iPF}_{2\alpha}\text{-III-d4}$ was hydrolyzed with 0.5 mL of 1 mol/L NaOH. BHT, final concentration of 200 $\mu\text{mol/L}$, was added to avoid in vitro lipid peroxidation during the following processes. The sample was incubated at 40°C for 60 min in a shaking air bath, neutralized with 0.5 mL of 1 mol/L HCl, diluted to 10 mL with phosphate buffer (0.05 mol/L, pH 7.4) containing 0.01% thimerosal, and then centrifuged at $2400 \times g$ for 10 min to remove any precipitate. An immunoaffinity

column was prepared by settling 150 μL antibody-coated resins into the 1-mL reservoir fitted with 20 μm polyethylene frits (Varian Inc., Palo Alto, CA, USA). The resulting column was then filled with 0.05 mol/L phosphate buffer solution with 0.01% thimerosal, and stored at 4°C until use. The subsequent immunoaffinity extraction was automated using a Gilson ASPEC XL Sample Processor (Gilson, Villiers-le-bel, France). The sample was applied to a preconditioned immunoaffinity column (IAC) containing the anti- $\text{iPF}_{2\alpha}\text{-III}$ antibodies immobilized on the CNBr-activated aepharose 4B gel. The IAC was washed with 20 mL of distilled water, and $\text{iPF}_{2\alpha}\text{-III}$ and $\text{iPF}_{2\alpha}\text{-III-d4}$ were then eluted with 5.5 mL of acetone-distilled water (95:5, volume ration). The eluant was evaporated to dryness with nitrogen. The immunoaffinity column was regenerated after thoroughly washings sequentially with 20 mL of distilled water, 10 mL of acetone-distilled water (95:5, volume ration), 10 mL of ethanol-distilled water (90:10, volume ration), and 20 mL of distilled water. Quantitation of $\text{iPF}_{2\alpha}\text{-III}$ by GC-NICI-MS on Trace MS Plus system

$\text{iPF}_{2\alpha}\text{-III}$ standard solutions and purified $\text{iPF}_{2\alpha}\text{-III}$ from plasma/membrane samples were treated with a mixture of PFB-Br (25 μL , 10% in acetone) and DIPEA (25 μL , 20% in acetone) at 60°C for 10 min. Excess reagents were evaporated under nitrogen. The $\text{iPF}_{2\alpha}\text{-III}$ esters were treated with 50 μL of BSTFA and 5 μL of DIPEA (20% in acetone) at 60°C for 5 min. Excess reagents were removed under nitrogen. The PFB ester TMS ether derivatives were then reconstituted in 40 μL of iso-octane with 30 s of sonication and vortexing. After centrifugation at 13 000 r/min for 2 min, the supernatant was analyzed by GC-MS.

Samples were analyzed by NICI mode on a Trace GC2000 series gas chromatograph coupled to a Trace MS Plus mass spectrometer (ThermoFinnigan, San Jose, CA, USA) using methane (1.3 mL/min) as the reagent gas. Chromatography was carried out with SPB-1701 column (30 m $\times$ 0.25 mm, 0.25 μm film thicknesses, Supelco Incorporation, Bellefonte, PA,

USA) using helium as the carrier gas (1 mL/min). The injection was performed by an AS2000 auto-sampler (ThermoFinnigan, San Jose, CA, USA), and the temperature of the injector was kept at 250 °C. Sample injection volume was 2 μ L. The oven temperature was programmed from 150 °C to 235 °C at a rate of 15 °C/min, increased from 235 °C to 280 °C at 10.3 °C/min, and then maintained at 280 °C for 10 min. The Trace MS Plus was programmed for optimal detection with electron energy set at 70 eV, emission current set at 150 μ A, ion energy set at 1.2 eV, and ion energy ramp set at 0.5 mV/amu. Selected ion monitoring (SIM) was performed for the carboxylate anion ($[M-181]^-$, loss of $CH_2C_6F_5$) at ions m/z 569 and 573 for $iPF_{2\alpha}$ -III and $iPF_{2\alpha}$ -III-d4, respectively. Peak identification was based on comparison of retention times with standards. Sample $iPF_{2\alpha}$ -III concentrations samples were calculated by using the ratios of the peak heights of m/z 569 to that of m/z 573 against a calibration curve. The recovery was $(96.4 \pm 2.3)\%$ ($n=8$), within-batch and between-batch precision CV were 4.8 % ($n=12$) and 7.0 % ($n=8$), respectively.

1.4 Measurement of erythrocyte membrane protein content

The protein content of membrane suspensions was determined by the fluorescence emission intensity of tryptophan using human albumin as a standard on a spectrofluorometer (PerkinElmer LS-50B)^[13]. A frozen erythrocyte membrane sample was thawed in the dark at room temperature. An aliquot of 30 μ L membrane suspension was added to 3 mL DPBS and mixed thoroughly. The mixture was transferred to a quartz cuvette. The fluorescence intensity of tryptophan in the membrane preparation was recorded by a LS 50B spectrofluorometer. Concentration of the membrane suspension was calculated by the measured fluorescence intensity against a calibration curve. The within-batch precision CV and between-batch precision CV were 4.3% and 5.2%, respectively.

Fluorescence intensities and anisotropy values were measured by a computer controlled spectrofluorometer (PerkinElmer LS-50B) equipped with the

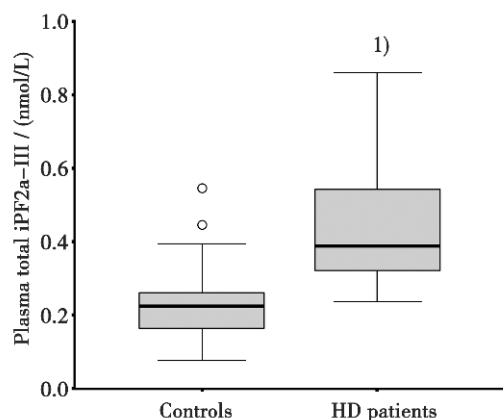
Fast Filter Wheel Accessory. All measurements were made at 37 ± 0.5 °C in a thermostatic controlled cuvette holder. For measurement of the fluorescence anisotropy, the sample was excited with vertically polarized light. The emission intensity was measured parallel (I_v) and perpendicular (I_{vh}) to the vertical plane of polarization of the excitation light. Anisotropy was calculated from the following equation: $Anisotropy = (I_v - G \cdot I_{vh}) / (I_v + 2G \cdot I_{vh})$, where G was an instrument correction factor, determined before each measurement. The instrument parameters for the spectrofluorometer were as follow: Excitation slit width: 5 nm for DPH and 4 nm for TMA-DPH, Excitation wavelength: 360 nm, Emission slit width: 5 nm for DPH and 4 nm for TMA-DPH, Emission wavelength: 430 nm, Stirrer setting: high, Integration time: 5 seconds, Cut-off filter: 390 nm.

For the measurement of DPH anisotropy, 1.5 mL of 2 μ mol/L DPH (in DPBS) was added to an equal volume of membrane suspension with a final protein concentration of 0.25 g/L. The mixture was incubated at 37 °C for 45 min in the dark. For the measurement of TMA-DPH anisotropy, 3 mL of membrane suspension (protein content of 0.25 g/L) was equilibrated at 37 °C. 3 μ L of 1 mmol/L TMA-DPH (in DMSO) was added. The mixture anisotropy was taken after 5 min incubation at 37 °C in the dark. Calculation of the fluorescence anisotropy was performed using PerkinElmer computer software FL WinLab 3.00. For DPH and TMA-DPH, the within-batch precision CV ($n=10$) were 1.89%, 1.61%, respectively, the between-batch precision CV ($n=10$) were 2.11%, 1.69%, respectively.

1.5 Statistics

Data distribution was evaluated using the Shapiro-Wilks test. Distributions of plasma/membrane $iPF_{2\alpha}$ -III, DPH and TMA-DPH anisotropy values were Gaussian distributed. Data were presented as mean $\pm$ SD. Parametric Student t -test and ANOVA followed by post-hoc LSD test were used. Associations between the different variables were examined by Pearson's correlation analyses. The causal relationships between them were studied by linear regression

analysis. Statistical significance was set at $P < 0.05$. All analyses were carried out with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).



o Individual data points for the extremes of the data. 1) denotes $P < 0.01$ compare to controls.

Fig.1 Plasma total $iPF_{2\alpha}$ -III concentration in hemodialysis patients ($n = 49$) compared to in healthy controls ($n = 31$)

2 Results

2.1 Plasma total $iPF_{2\alpha}$ -III, erythrocyte membrane $iPF_{2\alpha}$ -III and membrane fluidity in HD patients and control subjects

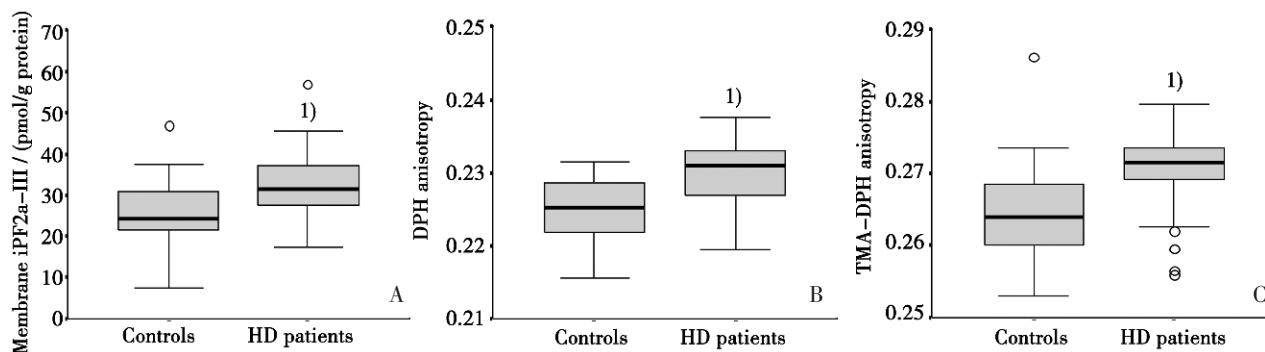
Plasma total $iPF_{2\alpha}$ -III was significantly higher in HD patients (0.450 ± 0.173 nmol/L) than that in control subjects (0.236 ± 0.107 nmol/L, $P < 0.01$) (Fig.1). Erythrocyte membrane $iPF_{2\alpha}$ -III was significantly higher in HD patients than that in controls (32.5 ± 7.9 vs. 25.7 ± 8.7 pmol/g protein, $P < 0.01$) (Fig.2A). Both DPH and TMA-DPH anisotropy values in HD patients were significantly higher than those in controls (DPH anisotropy: 0.2302 ± 0.0040

vs. 0.2246 ± 0.0046 , $P < 0.01$; TMA-DPH anisotropy: 0.2710 ± 0.0045 vs. 0.2647 ± 0.0071 , $P < 0.01$) (Fig.2B,C). The anisotropy results suggested that both core and surface regions of the erythrocyte membranes were more rigid in HD patients than in healthy subjects.

2.2 Relationship between plasma/membrane $iPF_{2\alpha}$ -III and membrane fluidity in HD patients

Pearson's correlation analysis was used to study the relationships between the plasma $iPF_{2\alpha}$ -III, membrane $iPF_{2\alpha}$ -III and membrane fluidity. There was no significant relationship between plasma total $iPF_{2\alpha}$ -III and erythrocyte membrane $iPF_{2\alpha}$ -III ($r = 0.208$, $P = 0.152$). There was no significant correlation between plasma $iPF_{2\alpha}$ -III with neither DPH anisotropy ($r = -0.007$, $P = 0.960$) nor TMA-DPH anisotropy ($r = 0.147$, $P = 0.313$) suggesting that measurement of plasma $iPF_{2\alpha}$ -III is a "whole-body" oxidative stress measurement in HD patients and did not correlate with erythrocyte membrane fluidity, and thus cannot be considered as a marker for in vivo erythrocyte oxidative stress assessment.

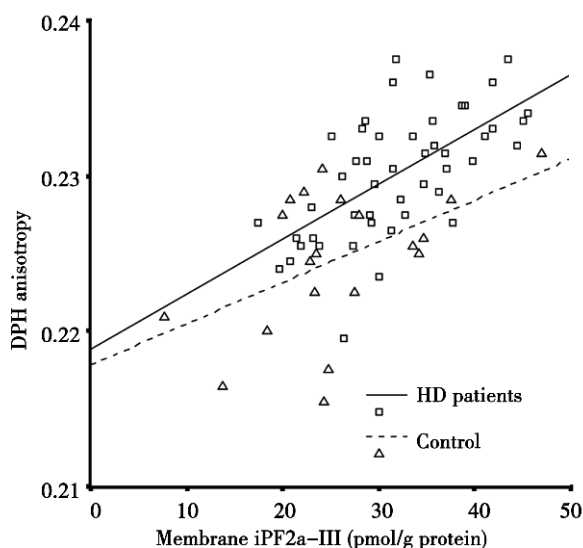
Fig.3 shows that there was a significant positive correlation between erythrocyte membrane $iPF_{2\alpha}$ -III and DPH anisotropy ($r = 0.630$, $P < 0.001$) in 49 HD patients and ($r = 0.496$, $P < 0.05$) in 20 health controls. But there was no correlation between erythrocyte membrane $iPF_{2\alpha}$ -III and TMA-DPH anisotropy ($r = -0.006$, $P = 0.966$). This indicated that an increase in membrane $iPF_{2\alpha}$ -III was associated with a decrease in erythrocyte membrane fluidity in the core region, but not with the surface region. Slope



o Individual data points for the extremes of the data. 1) denotes $P < 0.01$ compare to controls.

Fig.2 Erythrocyte membrane $iPF_{2\alpha}$ -III (A), DPH anisotropy (B), and TMA-DPH anisotropy (C) in haemodialysis patients ($n = 49$) and controls ($n = 20$)

values of the linear regression equations between DPH anisotropy values and membrane $iPF_{2\alpha}$ -III for HD patients and healthy control subjects were 3.54×10^{-4} pmol/g protein and 2.65×10^{-4} pmol/g protein, respectively (Fig.4). These slope values were of the same order of magnitude, suggesting that the contribution of membrane $iPF_{2\alpha}$ -III content to membrane fluidity in the core region was similar for both HD patients and healthy controls.



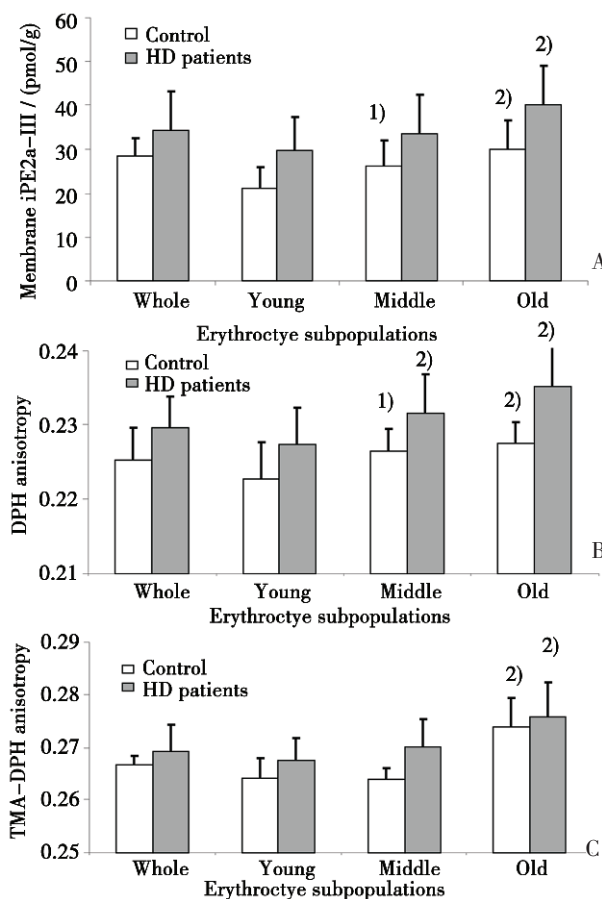
$y = 3.54 \times 10^{-4} x + 0.219, r = 0.630, P < 0.001$, and in 20 control subjects ($y = 2.65 \times 10^{-4} x + 0.218, r = 0.496, P = 0.026$)

Fig.3 Relationship between erythrocyte membrane $iPF_{2\alpha}$ -III content and DPH anisotropy in 49 HD patients

2.3 Membrane $iPF_{2\alpha}$ -III and membrane fluidity in different erythrocyte fractions

From the 49 HD patients, 24 patients were randomly selected and their erythrocytes were fractionated according to their ages as old, middle and young fractions. Table 1 summarized the membrane $iPF_{2\alpha}$ -III content and membrane anisotropy values from different fractions. Membrane $iPF_{2\alpha}$ -III content from different erythrocyte fractions in HD patients were significantly higher than those in the corresponding fractions of healthy controls. Similar results were observed for membrane anisotropy values except for TMH-DPH anisotropy of old cells ($P = 0.452$). Comparing with erythrocytes in the young fraction, ANOVA showed that membrane $iPF_{2\alpha}$ -III, DPH anisotropy and TMA-DPH anisotropy in the

older cells were all significantly higher for the HD patients (Fig.4). Thus, during the aging process of circulating erythrocytes, there was an increase in membrane $iPF_{2\alpha}$ -III content accompanied with a decrease in erythrocyte membrane fluidity in HD patients. These results supported the hypothesis that accumulation of oxidative stress products on the erythrocyte membranes was one of the phenomena contributing to the alteration in membrane functions.



Erythrocytes from haemodialysis patients were fractionated in 3 sub-populations of different mean age and cells from each sub-population were used to prepare membranes. Data are expressed as the mean $\pm$ SD. Significance was determined by ANOVA, with significance set at 1) $P < 0.05$, 2) $P < 0.01$ compared with Young subpopulations.

Fig.4 Membrane $iPF_{2\alpha}$ -III (A), DPH anisotropy (B) and TMA-DPH anisotropy (C) from 10 normal subjects and 24 haemodialysis patients

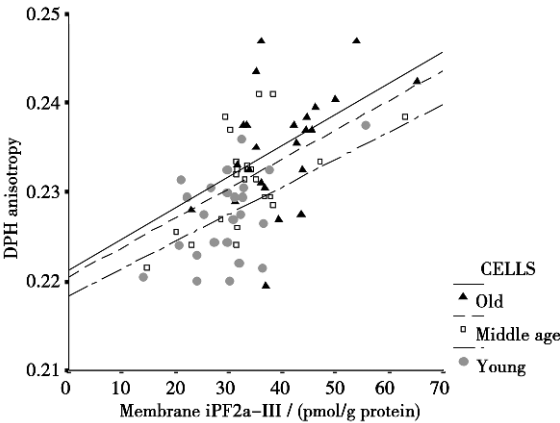
2.4 Relationship between plasma, membrane $iPF_{2\alpha}$ -III and membrane fluidity in subpopulation erythrocytes with different ages

Pearson's correlation analysis shows there was no

correlation between plasma $iPF_{2\alpha}$ -III with any of the fractionated erythrocytes DPH anisotropy values (Young: $r=0.153$, $P = 0.474$; Middle: $r = 0.087$, $P = 0.684$; Old: $r = -0.038$, $P = 0.859$) and TMA-DPH anisotropy values (Young: $r=0.041$, $P = 0.851$; Middle: $r = 0.120$, $P = 0.576$; Old: $r = 0.136$, $P = 0.528$). Furthermore there was no correlation between erythrocytes membrane $iPF_{2\alpha}$ -III and TMA-DPH anisotropy values in the corresponding fractions (Young: $r = 0.112$, $P = 0.603$; Middle: $r = 0.369$, $P = 0.075$; Old: $r = 0.343$, $P = 0.101$). There were significant positive correlations between erythrocytes membrane $iPF_{2\alpha}$ -III with DPH anisotropy in the corresponding fractions (Young: $r = 0.491$, $P = 0.015$; Middle: $r = 0.563$, $P = 0.004$; Old: $r = 0.460$, $P = 0.024$) (Fig.5). These results further demonstrated that membrane $iPF_{2\alpha}$ -III can serve as a marker for accessing in vivo oxidative stress and oxidative damage in erythrocyte membranes. Upon linear regression analysis, the effects of membrane $iPF_{2\alpha}$ -III on DPH anisotropy in different aged cells were bound to be similar (Fig.5). Slope values for the linear regression equations for the young, middle age and old fractions were 3.07×10^{-4} , 3.30×10^{-4} , and 3.49×10^{-4} , respectively.

3 Discussion

In this study, plasma $iPF_{2\alpha}$ -III, membrane



Young: $y = 3.07 \times 10^{-4}x + 0.218$, $r = 0.491$, $P = 0.015$;
Middle age: $y = 3.30 \times 10^{-4}x + 0.220$, $r = 0.563$, $P = 0.004$;
Old: $y = 3.49 \times 10^{-4}x + 0.221$, $r = 0.460$, $P = 0.024$

Fig.5 Relationship between erythrocyte membrane $iPF_{2\alpha}$ -III and DPH anisotropy in different fractionated erythrocytes in 24 HD patients

$iPF_{2\alpha}$ -III and membrane fluidity were firstly studied in patients on long-term hemodialysis. It has been postulated that oxidative stress contributes to morbidity in HD patients, including cardiovascular diseases, infections, diabetes, disorders of peripheral and central nervous system, anemia and accelerated aging^[1,15].

In HD patients, the balance between pro- and anti-oxidant capacities is shifted towards an increased oxidative stress. HD may induce repetitive bouts of oxidative stress, primarily through membrane bio-incompatibility leading to blood cell activation,

Table 1 Whole and fractionated erythrocyte membrane $iPF_{2\alpha}$ -III and membrane fluidity in 24 haemodialysis patients and 10 controls (mean $\pm$ SD)

Cell population		Control (n = 10)	Hemodialysis (n = 24)	P
Membrane $iPF_{2\alpha}$ -III / (pmol/g protein)	Whole	28.6 $\pm$ 3.9	34.1 $\pm$ 9.2	0.019
	Young	21.1 $\pm$ 4.9	29.6 $\pm$ 7.8	0.003
	Middle	26.2 $\pm$ 5.7	33.4 $\pm$ 8.9	0.027
	Old	30.0 $\pm$ 6.6	40.1 $\pm$ 8.7	0.003
DPH Anisotropy	Whole	0.225 3 $\pm$ 0.004 3	0.229 6 $\pm$ 0.004 2	0.009
	Young	0.222 7 $\pm$ 0.005 0	0.227 4 $\pm$ 0.004 9	0.016
	Middle	0.226 5 $\pm$ 0.002 9	0.231 5 $\pm$ 0.005 3	0.008
	Old	0.227 6 $\pm$ 0.002 8	0.235 2 $\pm$ 0.006 6	0.000
TMA-DPH Anisotropy	Whole	0.266 7 $\pm$ 0.001 7	0.269 2 $\pm$ 0.005 1	0.039
	Young	0.264 1 $\pm$ 0.003 9	0.267 6 $\pm$ 0.004 2	0.031
	Middle	0.264 0 $\pm$ 0.002 1	0.270 1 $\pm$ 0.005 4	0.002
	Old	0.273 9 $\pm$ 0.005 5	0.275 7 $\pm$ 0.006 7	0.452

particularly polymorphonuclear leukocytes and endotoxin challenge^[16-17]. At the same time, decreased levels of anti-oxidant defense mechanisms have been demonstrated, including vitamin C, vitamin E, selenium, the GSH scavenging system and superoxide dismutase. Efforts have been undertaken to study and develop new technological and clinical solutions to decrease oxidative stress level in HD patients. However, results from these investigations were not yet conclusive^[18].

The roles of oxidative stress and usefulness of oxidative stress markers have often been evaluated in HD patients. Several lipid peroxidation markers, including MDA, were used for oxidative stress assessment in HD patients^[19]. These studies demonstrated HD patients had higher lipid peroxidation product concentrations when compared with healthy controls. Plasma isoprostanes, a sensitive and reliable index of lipid peroxidation in vivo, had been used to show increased oxidative stress in HD patients^[7-8]. However, none of these markers was used to demonstrate a direct pathophysiological link between oxidative damage and increased oxidative stress in HD patients.

For recruiting HD patients and healthy control subjects for the present study, sample size calculation was based on earlier studies in which plasma total F_2 -isoprostanes was used as a marker^[7-8,20]. Based on these studies, different sample size (12-43 subjects) was calculated for detecting the difference with 90% power and a two-sided 5% significance level. Therefore, the 49 stable HD patients and 31 healthy subjects recruited for this study were sufficient for the requirement. The clinical status, age, sex, race, and treatments of the recruited HD patients were similar to some of these studies^[20].

Pre-dialysis plasma total $iPF_{2\alpha}$ -III concentrations were significantly higher in HD patients than in control subjects (0.450 ± 0.173 nmol/L vs. 0.235 ± 0.107 nmol/L, $P < 0.001$). This result was in accordance with other studies using plasma F_2 -isoprostanes as the marker^[7-8,20-21]. The present finding was consistent with the hypothesis that HD patients

are under increased oxidative stress.

The mean erythrocyte membrane $iPF_{2\alpha}$ -III content of the HD patients was 26.5 % higher than that of the controls (32.5 ± 7.9 vs. 25.7 ± 8.7 pmol/g protein, $P < 0.01$). This result agrees with the conclusion from measuring plasma total $iPF_{2\alpha}$ -III, indicating that increased oxidative stress was present in HD patients. Furthermore, membrane $iPF_{2\alpha}$ -III contents in erythrocyte fractions of different ages from HD patients were also significantly higher than those in the corresponding fractions from healthy control subjects. Increases in young, middle and old fractions were 28.5 %, 27.4% and 33.5%, respectively. These results suggested that increased oxidative stress affected all circulating erythrocytes of different ages in HD patients.

Impaired erythrocyte membrane fluidity had been reported in ESRD patients undergoing maintenance HD^[3]. In this study, two probes were used to study membrane fluidity in both core and surface regions. Both DPH and TMA anisotropy values were higher for HD patients than those for control subjects (DPH: 0.2302 ± 0.0040 vs. 0.2246 ± 0.0046 , $P < 0.001$; TMA-DPH: 0.2710 ± 0.0045 vs. 0.2647 ± 0.0071 , $P < 0.001$). Both core and surface membrane fluidity of erythrocytes in HD patients were more rigid than those of the control subjects. Similar results were observed for erythrocyte fractions of different ages, suggesting that in HD patients, circulating erythrocytes of different ages suffered from oxidative damages and alterations in membrane functions.

Both membrane $iPF_{2\alpha}$ -III and anisotropy data indicated that HD patients in this study had increased in lipid peroxidation associated with altered membrane function. Increased lipid peroxidation has implications for stability and integrity of the erythrocyte membrane through disruption of membrane architecture. Molecular modeling of phosphatidylcholine with the F_2 -iPs at the sn-2 position reveals isoprostan- containing phospholipid species to be a remarkably kinked molecule^[10]. It has been suggested that increased oxidative stress and

changes in erythrocyte membrane fluidity in HD patients may contribute to the shortened erythrocyte survival. However, a direct causal relationship between oxidative stress and disease states had not been definitively demonstrated. There has been little direct evidence of a role for oxidant injury in cell membrane under physiological condition.

To study whether the conventional use of plasma $iPF_{2\alpha}$ -III provides information on membrane function, correlation analyses on plasma total $iPF_{2\alpha}$ -III and anisotropy values were performed. In HD patients, there was no correlation between plasma total $iPF_{2\alpha}$ -III and DPH ($P=0.96$)/TMA-DPH ($P=0.31$) anisotropy values of erythrocyte membranes. Furthermore, no correlation was found between plasma total $iPF_{2\alpha}$ -III and anisotropy values of fractionated erythrocytes of different aged. These results indicated that measurement of plasma total $iPF_{2\alpha}$ -III, as a "whole-body" oxidative stress measurement, does not reflect erythrocyte membrane oxidative stress in vivo.

Increased F_2 -isoprostanes levels in HD patients may derive from impaired renal clearance for isoprostanes, or as an artifact during HD. Free (non-esterified) F_2 -isoprostanes appears in the urine of subjects with normal renal function^[22], and plasma levels are expected to increase in renal failure. On the other hand, plasma free F_2 -isoprostanes concentrations were observed to decrease initially during HD^[8]. Since free isoprostanes are low molecular weight charged compounds, it suggested that free isoprostanes might be transferred to the dialysis fluid or absorbed by HD membrane during the treatment process. Plasma esterified F_2 -isoprostanes was covalently bound to plasma lipids and would not be subjected to either renal clearance or removal during HD. It would be metabolized primarily by hepatic mechanisms. Thus, plasma esterified F_2 -isoprostanes should be used as the oxidative stress index in HD patients, instead of the free F_2 -iPs fraction. Results of the present study are valid to conclude that plasma total $iPF_{2\alpha}$ -III does not reflect membrane functions in HD patients, thus could not be used as a functional

marker. Furthermore, the use of membrane $iPF_{2\alpha}$ -III content would be a better marker to overcome the above problems in studying oxidative stress in HD patients.

To evaluate whether this newly developed membrane $iPF_{2\alpha}$ -III measurement can be used as a tissue marker for in vivo erythrocyte oxidative stress assessment, correlation analyses were performed between the membrane lipid peroxidation product content and membrane fluidity. In HD patients, there was a significant positive correlation between membrane $iPF_{2\alpha}$ -III and DPH anisotropy ($r=0.612$, $P<0.001$), but not TMA-DPH anisotropy ($r=0.147$, $P=0.313$). Similar results were observed in fractionated erythrocyte subpopulations of different ages. Furthermore, there was no correlation between plasma total $iPF_{2\alpha}$ -III and erythrocyte membrane $iPF_{2\alpha}$ -III ($r=0.208$, $P=0.152$). These findings suggest that increased erythrocyte membrane $iPF_{2\alpha}$ -III led to a corresponding loss of fluidity in the core region of the erythrocyte membrane. It could be concluded that erythrocyte membrane $iPF_{2\alpha}$ -III could serve as another independent index to reflect erythrocyte oxidative stress in vivo.

To improve the present study further, other erythrocyte membrane functions could be introduced, for example, erythrocyte deformability assessment and membrane enzyme / transporter activities (such as NA,K-ATPases). Correlation of these functions could provide more supportive evidence that membrane $iPF_{2\alpha}$ -III is a tissue marker for in vivo erythrocyte oxidative stress assessment. Other factors affecting membrane fluidity, such as membrane fatty acids composition could be measured, providing more insights to the relative contribution of plasma $iPF_{2\alpha}$ -III to membrane function. Antioxidant status of the present patient group had not been studied. Such information may be useful in understanding the dynamic relationships of oxidative stress in HD patients.

In summary, an increased oxidative stress in HD patients was confirmed by measuring plasma total $iPF_{2\alpha}$ -III and erythrocyte membrane $iPF_{2\alpha}$ -III. Erythrocyte membrane fluidity, measured by DPH

and TMA-DPH anisotropy, was decreased in HD patients compared with normal controls. There was no correlation between plasma total iPF_{2α}-III and membrane fluidity; and erythrocyte membrane iPF_{2α}-III was inversely correlated with the core region membrane fluidity in HD patients. In conclusion, erythrocyte membrane iPF_{2α}-III can be considered as a functional marker for erythrocyte oxidative stress assessment.

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