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ORIGINAL ARTICLE

Oxidative stress contributes to hemolysis in patients with hereditary spherocytosis and can be ameliorated by fermented papaya preparation

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Abstract In the present study, we questioned the role of oxidative stress in hereditary spherocytosis (HS), where red blood cells (RBC) have a shortened survival due to primary deficiency in membrane proteins. Using flow cytometry techniques, we showed that RBC derived from 17 HS patients of seven families generate more reactive oxygen species, membrane lipid peroxides, and less reduced glutathione than normal RBC. Following in vitro incubation of HS-RBC from seven patients with a fermentation bioproduct of *Carica papaya* (fermented papaya preparation (FPP)) with known antioxidative properties, oxidative stress markers were significantly reduced. Similar results were obtained following treatment with FPP for 3 months of 10 adult HS patients, as well as decreased tendency to undergo hemolysis. The hemoglobin levels increased by >1 g/dl, mean corpuscular hemoglobin concentration decreased by >1 g/dl, and the reticulocyte count decreased by 0.93%. Concomitantly, lactic dehydrogenase decreased by 17% and indirect bilirubin by 50%. A significant decrease in malonyldialdehyde was also detected. These data indicate that oxidative stress plays an important role in the pathophysiology of HS which can be ameliorated by an antioxidant such as FPP. Additional clinical trials with FPP and other antioxidants are warranted.

Keywords Hereditary spherocytosis · Oxidative stress · Hemolysis · Fermented papaya preparation (FPP)

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Introduction

The oxidative status of cells, which is determined by the balance between pro-oxidants such as the reactive oxygen species (ROS), and antioxidants, is a major regulator of cellular functions. Impaired balance between pro- and antioxidants causes oxidative stress which may result in oxidation of proteins, lipids, and DNA with the final outcome of premature cell aging and apoptosis [1, 2]. Oxidatively stressed red blood cells (RBC) have been observed in various congenital and acquired hemolytic anemias, including thalassemia, sickle cell anemia, congenital dyserythropoietic anemia, G6PD deficiency, and paroxysmal nocturnal hemoglobinuria (PNH) as well as in

myelodysplastic syndrome. Although the primary etiology is different in these anemias, oxidative stress mediates several of their pathologies, mainly hemolysis [3].

Hereditary spherocytosis (HS) is a genetic disorder of the RBC skeleton with primary deficiency in spectrin, ankyrin-1, band 3, or protein 4.2 associated with chronic hemolytic anemia [4]. Secondary protein deficiencies resulting from oxidative stress are often observed and may be involved in the clinical manifestations of the disease [5].

In the present study, we explored the oxidative status of HS-RBC and its contribution to hemolysis. In addition, we evaluated the effects of an antioxidant, fermented papaya preparation (FPP), a biofermentation product of *Carica papaya*, on HS-RBC, both in vitro and in vivo. The antioxidant effects of FPP have been previously demonstrated in patients with beta thalassemia and PNH [6, 7].

Patients and methods

Patients

For the in vitro studies, blood samples were collected from 17 patients (mean age, 14.6±9.5 years) from seven different families of Palestinian origin with documented family history of HS. All patients had clinical and laboratory findings consistent with mild to severe HS, diagnosed on the basis of spherocyte morphology, elevated mean corpuscular hemoglobin concentration (MCHC; (33–38 g/dl), with a mean value of 35.47 g/dl, increased osmotic fragility (>75%), splenomegaly, and non-immune-mediated hemolysis. Seven patients were splenectomized. All patients received folic acid at a dose of 5 mg per day since early childhood. Twelve patients received 5 to58 U of blood (mean, 21 U) since childhood.

Blood samples were collected simultaneously from age-matched healthy donors, who did not receive any medication during the 4-week period prior to the study.

Participants who were included in the in vivo study were 10 (eight males and two females) HS patients (mean age 25± 11 years), from three different families of Palestinian origin (Table 1). The patients had a mean hemoglobin (Hb) level of 11.3±1.5 g/dl and mean MCHC of 34.5±0.9 g/dl and were sporadically transfused since childhood, (8±5 of packed cells units) with a mean ferritin level of 111 ng/ml. Six patients were splenectomized, and all of them received folic acid, 5 mg a day since early childhood.

Patients were under regular follow-up at the European Hospital and the Naser Pediatric Hospital in Gaza. Laboratory examinations were carried out at Wolfson Hospital, Holon and Hadassah-Hebrew University Medical Center, Jerusalem. The study was approved by local Helsinki committees in the European and Naser hospital in Gaza, and written informed consent was received from all the studied patients.

Sample collection

Venous blood was drawn in tubes containing 15% (K_3) EDTA (Becton-Dickinson, Plymouth, UK). The blood was diluted with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS; Biological Industries, Kibbutz Beit-HaEmek, Israel) and used within 2 h of collection. In transfused patients, blood was collected prior to blood transfusion or at least 3 weeks following previous transfusion.

Assays

ROS, reduced glutathione (GSH), and lipid peroxides were measured in RBC following incubation with 100 μM 2′-7′-dichlorofluorescin diacetate, 40 μM [1-(4-chloromercury-phenyl-azo-2-naphthol)] (mercury orange) and 50 mM *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethyl ammonium salt, respectively [8, 9]. After being washed twice, the cells were resuspended in PBS and analyzed by flow cytometry (FACS-Calibur; Becton-Dickinson, Immunofluorometry Systems, Mountain View, CA, USA). The mean fluorescence channel (MFC) was calculated by the Cellquest® software (Becton-Dickinson), with unstained cells serving as controls (MFC in the range of 3–7). The MFC values for RBC obtained from healthy donors were as follows: ROS= 180±30, GSH=251±27, and lipid peroxidation=155±24.

In addition, blood samples were analyzed for complete blood count, and the plasma was analyzed for hemolytic parameters (lactate dehydrogenase (LDH) and indirect bilirubin) and for lipid peroxidation by measuring the levels of thiobarbituric acid-reactive substances [10], which determine the levels of malonyldialdehyde (MDA).

Hemolysis was assayed by suspending 20 μl of packed RBC in PBS or in the autologous plasma and overnight incubation in the presence of various concentrations of FPP at 37°C in humidified atmosphere of 5% CO_2 in air. Following 5-min centrifugation at 800 rpm, the supernatants were collected for Hb determination by measuring the absorbance at 540 nm.

Statistical analysis

Data were analyzed on SPSS 11.0 statistical analysis software (SPSS Inc., Chicago, IL, USA). Continuous variables such as MCHC and LDH were described using mean±standard deviation. Distribution of continuous variables were compared to normal using the Kolmogorov–Smirnov test (cut-off at $p=0.01$). All these variables were found to have approximately normal distributions. Repeat-

Table 1 Age, gender, and initial hematological parameters of 10 HS patients who received FPP for 3 months

Patients'initials/age/ gender (F/M)	No. of P.C[a] units	Hb >13 g/dl[c]	MCHC 31–34 g/dl[b]	Reticulocytes <1%[b]	Indirect bilirubin 0.1–1 mg/dl[b]	LDH 105–333 U/L[b]	MDA <0.7 nm/ml[b]
FMN/20/M	12	10.9	34.1	9	3	552	1.3
FN/28/M[c]	9	11	35	7	2	489	1.8
FS/24/M[c]	6	12.2	33	3	1.8	666	1.9
JM/34/M[c]	5	13	35	6	2	497	1.7
KN/42/M	6	10	34	12	1.7	622	1.8
MM/23/F[c]	4	12.3	34	5	1	458	1.4
MN/14/F	5	11.2	34.1	6	4	792	1.7
SKN/16/M	18	8	34	9	1.4	550	1.8
SN/42/M[c]	13	12.5	35	5	1.3	425	1.7
PN/20/M[c]	2	12	36.3	4	1.2	457	1.2
Mean values±SD Mean age, 26.3±10	8±5	11.3±1.5	34.5±0.9	6.6±2.7	1.94±0.9	550±114	1.6±0.24

[a] Packed cells

[b] Normal range

[c] Splenectomized

ed measures analysis was used to examine change in each of the variables over four time points, followed post-hoc by paired *t* test. Comparisons between cases and controls were made using the *t* test for independent samples. All tests are two sided and considered significant at $p<0.05$.

Results

In vitro study

Flow cytometry analysis demonstrated that RBC from seven HS patients generated increased ROS (1.65-fold) and membrane lipid peroxides (3.04-fold) compared with RBC obtained from age- and sex-matched control donors. Conversely, control donors had GSH levels 2.05-fold higher than in HS patients.

We studied the effects of FPP, a fermentation bioproduct of *C. papaya* with known antioxidative properties [6, 7, 11] on HS-RBC. Incubation of HS-RBC for 2 h with 0.1 mg/ml FPP resulted in a 41% decrease in ROS and 322% increase in GSH (Fig. 1a). The effect of FPP on hemolysis was determined following incubation of HS-RBC with FPP (Fig. 1b,c). The results showed less hemolysis in RBC incubated with FPP.

In vivo study

FPP was supplied as sachets containing 3 g each. The patients received one sachet three times a day after meals, for 3 months. Blood samples were drawn before and during the treatment (Fig. 2). After 3 months, Hb levels increased from 11.2 to 12.4 g/dl ($p<0.0001$), and the MCHC decreased from 34.5 to 33.4 g/dl ($p=0.009$) and the reticulocyte count from 6.5% to 5.57% ($p=0.011$). Concomitantly, LDH decreased from 550 to 458 U/L ($p=0.033$) and the indirect bilirubin from 1.940 to 0.97 mg/dl ($p=0.015$). A significant decrease in MDA, from 1.6 to 1.486 nmol/ml ($p=0.019$), was also detected (Fig. 2).

Discussion

The RBC is a preferential target for oxidative stress due to its unique function in transporting oxygen. Hb is prone to auto-oxidation processes in which ROS and oxidized Hb (methemoglobin) are produced [12, 13]. In normal mature RBC, ROS are balanced and detoxified by antioxidant defense systems such as GSH [2]. However, in various pathologies, including both congenital and acquired hemolytic anemias, this balance is impaired—resulting in oxidative stress [3, 8].

In the present study, using flow cytometry, we documented the presence of oxidative stress in 17 HS patients from seven Palestinian Arab families. This was demonstrated by increased generation of ROS and lipid peroxides and lower levels of GSH in their RBC, compared with healthy control donors. Similar results were obtained in four Ashkenazi Jewish patients with HS (data not shown). These results are consistent with previous reports on the presence of oxidative stress in HS [13, 14].

The finding of oxidative stress in RBC from patients with HS can be explained in part by the higher rate of Hb auto-oxidation and methemoglobin formation [12, 13],

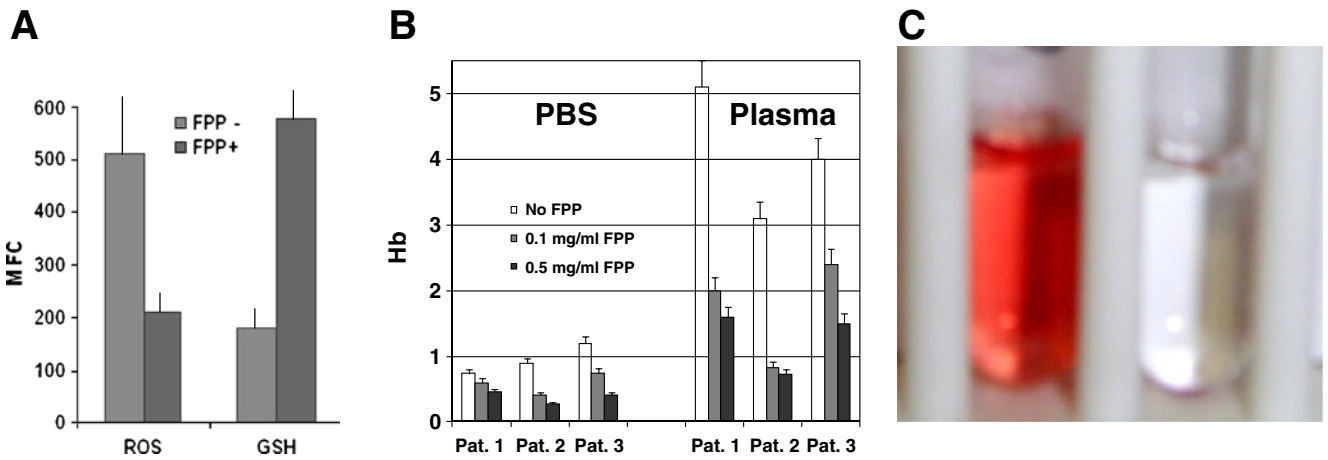

Fig. 1 **a** The in vitro effect of FPP on oxidative stress and hemolysis of HS-RBC. RBC from HS patients were diluted in PBS and incubated for 2 h with or without 0.1 mg/ml FPP. The cells were then assayed for ROS and GSH. The results are expressed as the average MFC of 17 patients. **b** RBC from three HS patients were diluted in PBS or in their autologous plasma and incubated overnight at 37°C with the indicated concentrations of FPP. The cells were then centrifuged, and the Hb concentration in the supenatant was measured. **c** RBC from one HS patient were diluted in PBS and incubated overnight with or without 0.1 mg/ml FPP. Hemolysis is present in the absence of FPP (*left* tube) but not following incubation with FPP (*right* tube)

which may be caused by the increased intracellular Hb concentration (MCHC) due to RBC dehydration. Accumulation of oxidized Hb close to the RBC membrane promotes localized oxidant damage to skeletal proteins and phospholipids, ultimately disrupting the membrane structure and function [12, 13]. It has also been reported that spectrin in HS-RBC is highly sensitive to oxidative stress, which may contribute to membrane damage [13–15].

Our results demonstrated that treatment with FPP ameliorated the oxidative parameters in HS both in vitro and in vivo. The antioxidant properties of FPP [11, 16] could be attributed to its high content of glutamic acid, glycine, and methionine, which serve as a substrate for glutathione synthesis [16].

In vivo treatment resulted not only in decreased oxidative stress parameters in the RBC as well as in the

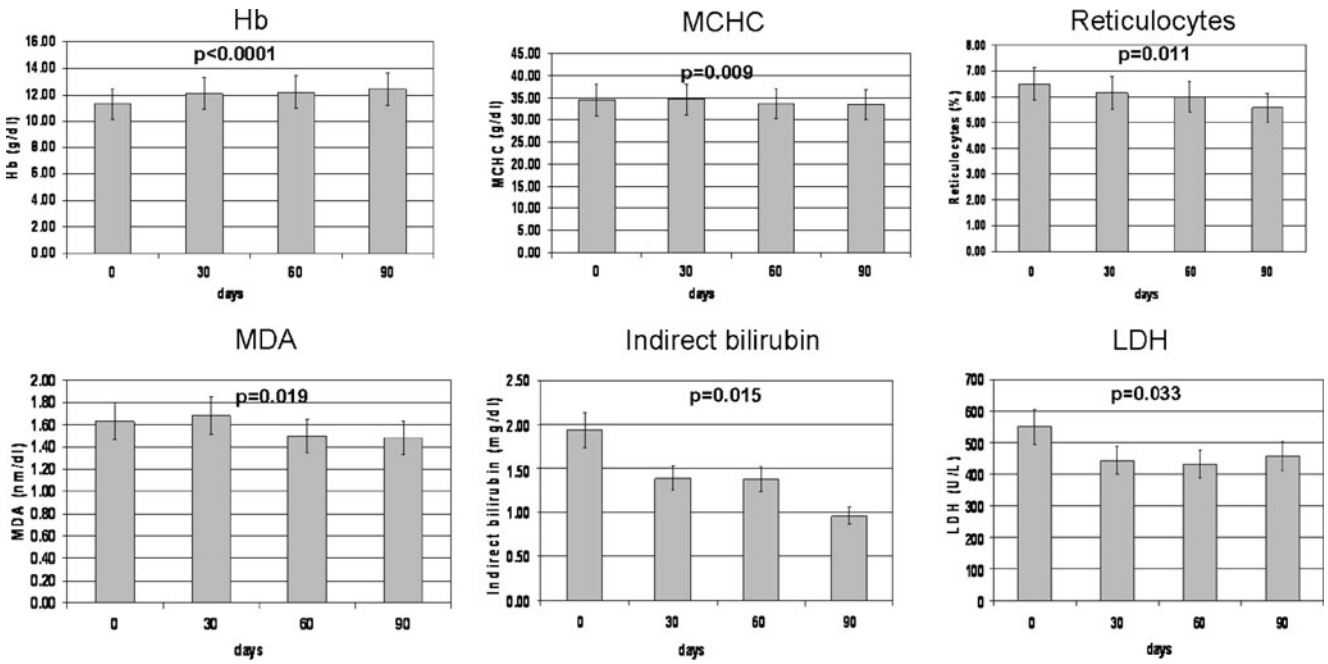

Fig. 2 The figure represents the mean dynamic changes in patients with HS treated with FPP for 3 months. Blood samples were analyzed before and during FPP treatment. Hb, MCH, and reticulocyte levels in addition to levels of hemolytic (indirect bilirubin and LDH) and oxidative stress parameters (MDA) were measured

plasma (MDA), but was also associated with decrease in hemolytic markers (indirect bilirubin and LDH) concomitant with an increase in Hb of more than 1 g/dl and decreases in the MCHC and reticulocytes.

Amelioration of oxidative stress parameters by FPP has also been reported in thalassemia but without significant changes in hematological parameters [17]. A possible explanation could be the fact that free iron species such as labile plasma iron and intra cellular labile iron are higher in thalassemia, resulting in increased generation of ROS, compared with HS or PNH, where improvement in hematological parameters has been found [18].

In conclusion, oxidative stress, which appears to play an important role in the pathophysiology of HS by contributing to hemolysis, could be ameliorated by administration of an antioxidant such as FPP. These preliminary findings warrant a well-designed randomized clinical trial with FPP in HS patients compared with a parallel placebo group to verify the treatment efficacy.

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