

Fermented Papaya Preparation as Redox Regulator in Blood Cells of β -Thalassemic Mice and Patients

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Many aspects of the pathology in β -hemoglobinopathies (β -thalassemia and sickle cell anemia) are mediated by oxidative stress. Fermented papaya preparation (FPP) was tested for its antioxidant effects: the scavenging effect was determined spectrofluorometrically in a cell-free system using 2'-7'-dichlorofluorescein-diacetate (DCF). Both spontaneous and H₂O₂-induced DCF oxidations were decreased by FPP in a dose-dependent fashion. Using flow cytometry, it was shown that *in vitro* treatment of blood cells from β -thalassemic patients with FPP increased the glutathione content of red blood cells (RBC), platelets and polymorphonuclear (PMN) leukocytes, and reduced their reactive oxygen species, membrane lipid peroxidation and externalization of phosphatidylserine. These effects result in (a) reduced thalassemic RBC sensitivity to hemolysis and phagocytosis by macrophages; (b) improved PMN ability to generate oxidative burst – an intracellular mechanism of bacteriolysis, and (c) reduced platelet tendency to undergo activation, as reflected by fewer platelets carrying external phosphatidylserine. Oral administration of FPP to β -thalassemic mice (50 mg/mouse/day for 3 months) and to patients (3 g \times 3 times/day for 3 months), reduced all the above mentioned parameters of oxidative stress ($p < 0.001$ in mice and $p < 0.005$ in patients). These results suggest that FPP, as a potent antioxidant, might alleviate symptoms associated with oxidative stress in severe forms of thalassemia. Copyright © 2008 John Wiley & Sons, Ltd.

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INTRODUCTION

A growing body of experimental and clinical evidence points to the important role played by oxidative stress in β -hemoglobinopathies, β -thalassemia and sickle cell anemia (Chan *et al.*, 1999; Comporti *et al.*, 2002). Although the primary defects in these diseases are mutations in the β -globin gene, many aspects of the pathology are mediated by oxidative stress (Hebbel *et al.*, 1982; Rachmilewitz and Schrier, 2001; Rund and Rachmilewitz, 2005). Using flow cytometry, it was previously shown that RBC (Amer *et al.*, 2003, 2006) as well as platelets (Amer and Fibach, 2004) and polymorphonuclear leukocytes (PMN) (Amer and Fibach, 2005; Amer *et al.*, 2006) derived from patients with β -thalassemia and sickle cell anemia are under oxidative stress. They contain lower levels of reduced glutathione (GSH), concomitant with increased levels of reactive oxygen species (ROS), membrane lipid peroxidation and exposed phosphatidylserine (PS) compared with their counterparts derived from normal donors.

It has been shown that fermented papaya preparation (FPP), a natural health food product obtained by biofermentation of *Carica papaya*, is able to limit

the oxidative stress both *in vitro* and *in vivo* (Marotta *et al.*, 1997; Santiago *et al.*, 1991). In the present study FPP was tested for its antioxidant effects *in vitro* and *in vivo* on RBC, platelets and PMN of β -thalassemic mice and patients.

MATERIALS AND METHODS

Fermented papaya preparation (FPP). FPP is a product of yeast fermentation of *Carica papaya* Linn. It was supplied as sachets, each containing 3 g powder by Osato Research Institute, Gifu, Japan. The composition of its principle components has been described previously (Aruoma *et al.*, 2006). For *in vitro* experiments, the powder was dissolved in double distilled water, and a stock solution of 10 mg/mL was made and stored at 4 °C.

Patients. Eight patients with β -thalassemia intermedia and three with β -thalassemia major were studied (Table 1). They had different mutations in their β -globin genes. Those with thalassemia major were frequently transfused, while those with thalassemia intermedia were not. Five patients received iron chelation with deferioxamine administered subcutaneously by a pump 10 h 5–7 days a week. In polytransfused patients, blood samples were obtained prior to transfusion, i.e. at least 3 weeks following the previous transfusion. Informed consent was obtained in all cases.

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Table 1. The patient population studied

Pat. No.	Sex	β -Thal.	Mutation	Blood transf.	Iron chelation ^a
1	M	Inter. ^b	IVS1,6/IVS1,6	+/- ^c	-
2	F	Inter.	IVS1,6/IVS1,1	+ ^d	+
3	F	Inter.	IVS1,6/IVS1,6	+/-	-
4	M	Inter.	IVS1,6/IVS1,1	+	+
5	M	Inter.	TATA/TATA	+/-	-
6	M	Inter.	TATA/TATA	+/-	-
7	F	Inter.	IVS1,6/IVS1,6	-	-
8	M	Inter.	TATA/TATA	-	-
9	M	Major	IVS1,110/IVS1,110	+	+
10	M	Major	FS44/Poly A	+	+
11	F	Major	FS44/Poly A	+	+

^a Deferioxamine; ^b intermedia; ^c occasionally; ^d at least once a month.

Mice. The founders of a thalassemic mouse colony were obtained from Dr S. Rivella, Weill Medical College of Cornell University, NY, NY. Heterozygotes (Hbb^{th3/+}) mice, exhibit severe anemia (7–9 g/dL of hemoglobin [Hb]), abnormal RBC morphology, splenomegaly and hepatic iron deposition (Yang *et al.*, 1995). Animals were bred at the animal facility of the Sharett Institute, Hadassah Hospital, Jerusalem, Israel. FPP treatment started when the mice were 1 month old.

Blood collection and cell isolation. Peripheral blood (0.5 mL) samples of both normal donors and thalassemia patients were obtained. The blood was diluted with equal volume of Ca⁺⁺- and Mg⁺⁺-free Dulbecco's phosphate buffered saline (PBS) (Biological Industries, Kibbutz Beit-HaEmek, Israel), and mixed with a double volume of 3% gelatin (Sigma, St Louis, MO) in PBS and left to stand for 30 min at room temperature. The supernatant, containing RBC, but enriched for leukocytes and platelets, was collected, washed and used within 2 h of blood withdrawal. In mice, blood samples (20 μ L) were drawn from their tail vein.

Assays for RBC hemolysis and phagocytosis. To measure the protective effect of FPP on RBC hemolysis, RBC (5×10^6 /mL) were suspended in a HEPES (10 mM) buffer containing 2.5 mM CaCl₂ and 170 mM NaCl, pH 7.4, and incubated overnight with various amounts of FPP. RBC were then centrifuged, resuspended in PBS and counted. Hemolysis was calculated as the percentage of lysed RBC compared with the RBC input. The results were confirmed by spectrophotometric measurement (Fibach, 1993) of the hemoglobin content in the hemolysate.

To measure the effect of FPP on phagocytosis of RBC by macrophages, mononuclear blood cells obtained from normal donors were cultured according to the two-phase liquid culture procedures as previously described (Fibach, 1998). Adherent macrophages from the first and second phases of the culture were collected by trypsinization, washed, resuspended in fresh alpha medium containing 10% fetal calf serum and recultured in multi-well dishes. Normal and thalassemic RBC were diluted to 5×10^6 /mL with either PBS or ABO-compatible normal or thalassemic plasma and incubated for 30 min at 37 °C with or without 0.5 mg/mL FPP. The PBS-diluted RBC were then washed and treated for additional 30 min with different con-

centrations of t-butylhydroperoxide (BHP). All RBC samples were washed and placed in macrophages-containing plates. After 24 h incubation, the non-phagocytosed RBC were harvested and counted microscopically using a hemocytometer. The extent of phagocytosis was calculated as the percent of phagocytosis per the number of RBC input.

Flow cytometry measurements of oxidative stress markers. For ROS measurement, mouse and human cells were incubated with 100 μ M (final concentration) 2'-7'-dichlorofluorescein diacetate (DCF) (Sigma) for 15 min at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cells were analysed either before or after 15 min stimulation with 0.5 mM H₂O₂. For measuring GSH content, cell concentrates were incubated for 3 min at room temperature with [1-(4-chloromercuriphenylazo-2-naphthol)] (mercury orange) (Sigma) at a final concentration of 40 μ M for human cells and 20 μ M for mouse cells, following washing of the cells in PBS. Externalization of phosphatidylserine (PS) was determined following washing of the cells in Ca⁺⁺-binding buffer and staining with isothiocyanate-conjugated Annexin-V. Platelets were also stained for the membrane P-selectin with phycoerythrin-conjugated anti-CD62-P antibodies as previously described (Ataga and Orringer, 2003; Eldor and Rachmilewitz, 2002).

Generation of oxidative burst by PMN was measured as follows: Blood cells enriched by gelatin separation were treated overnight with FPP, then washed with PBS and stained with DCF as mentioned above. They were then washed again and stimulated for 20 min at 37 °C with 40 ng/mL (final concentration) phorbol 12-myristate 13-acetate (PMA, Sigma). PMA was dissolved in DMSO (Sigma); the final concentration of DMSO was less than 0.025%.

Following staining, the cells were analysed with a flow cytometer (FACS-Calibur, Becton-Dickinson, Immunofluorometry systems, Mountain View, CA). CaliBRITE™-3 beads (Becton-Dickinson) were used to calibrate the FACS. Cells were passed at a rate of ~1000/s, using saline as the sheath fluid. A 488 nm argon laser beam was used for excitation. Each cell population was gated based on forward light scatter and side light scatter (Amer *et al.*, 2006). In a previous study, the identities of the gated populations thus obtained were determined by staining with fluorochrome-conjugated antibodies to CD41 (platelets), glycophorin A (RBC),

CD15 (PMN) and CD3 and CD19 (T- and B-lymphocytes, respectively) (Amer and Fibach, 2005). Cell fluorescence was measured using logarithmic amplification. For each parameter the mean fluorescence channel (MFC) of at least 10 000 cells of each population was calculated using the FACS-equipped CellQuest[®] software (Becton-Dickinson). In each assay, unstained cells, both treated and untreated, served as control. The MFC of cells stained with DCF, mercury orange and annexin-V is proportional to generation of ROS, the content of GSH and extent of external PS, respectively; while that of fluor-DHPE-stained cells is reversely proportional to their extent of lipid peroxidation (Amer *et al.*, 2004).

Spectrofluorometric analysis of ROS scavenging activity. DCF (0.1 mM) in PBS was incubated with or without different amounts of FPP and then stimulated or not stimulated with 0.4 mM H₂O₂. DCF oxidation was measured by a spectrofluorometer (excitation 488 nm; emission 530 nm). Results are expressed in arbitrary fluorescence units (AFU) (Chignell and Sik, 2003; Komarov *et al.*, 2004).

Statistical analysis. The results are expressed as the average \pm standard deviation (SD) MFC and compared using the two-sample Student's *t*-test for differences in means.

RESULTS

Effects of FPP *in vitro*

The scavenging effect of FPP was determined in a cell-free system using DCF. Two parallel experiments were performed: FPP was added to a solution of DCF for 20 min in the presence of H₂O₂ (induced DCF oxidation) or for 16 h in the absence of H₂O₂ (spontaneous DCF oxidation). Spectrofluorometric analysis showed that in both cases FPP decreased DCF oxidation in a dose-dependent fashion (Fig. 1A).

The effect of FPP on the oxidative status of blood cells derived from thalassemic patients was analysed. Following incubation with FPP, various blood cell types were assayed for oxidative state parameters by flow cytometry. The dose and time effects of FPP on ROS generation by thalassemic RBC are depicted in Fig. 1B and C, respectively. In Fig. 1B, thalassemic RBC were incubated with different concentrations of FPP for 30 min, then DCF was added and RBC were either stimulated or un-stimulated with 0.4 mM H₂O₂. The results show a dose-dependent decrease of ROS by FPP in both stimulated and unstimulated RBC. In Fig. 1C, FPP (0.4 mg/mL) was added to thalassemic RBC suspensions containing DCF, and ROS were measured at different intervals. The difference in the kinetics of ROS

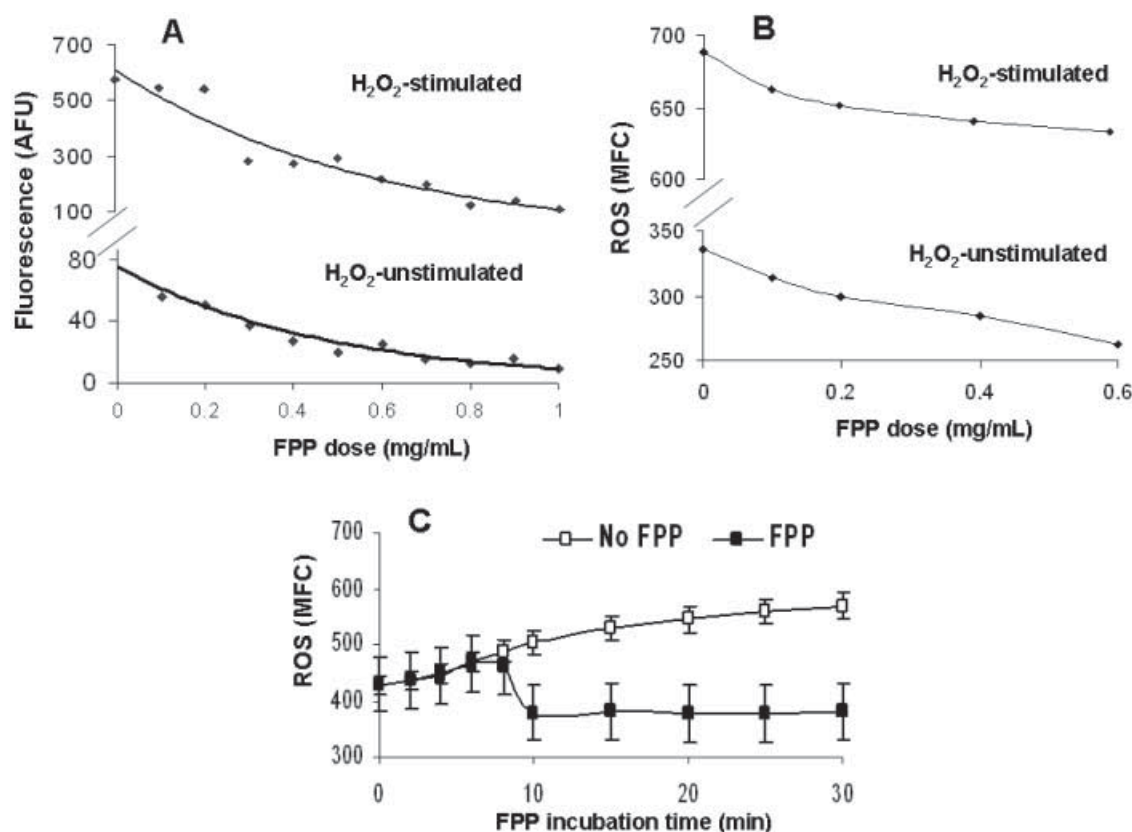


Figure 1. The effect of FPP on extra- and intra-cellular ROS. (A) To determine the scavenging effect of FPP, a solution of DCF (0.1 mM) in PBS was incubated with different concentrations of FPP, either with or without 0.4 mM H₂O₂. DCF oxidation was determined by spectrofluorometric measurement of fluorescence (excitation 488 nm; emission 530 nm). Incubation duration was 16 h with H₂O₂, and 20 min without H₂O₂. Results are presented in arbitrary fluorescence units (AFU). (B) FPP dose-effect on ROS was measured by incubating thalassemic RBC ($1-2 \times 10^6$ /mL) at 37 °C with different concentrations of FPP for 30 min. The cells were washed, stained with DCF and stimulated or un-stimulated with 0.4 mM H₂O₂ followed by ROS measurement by flow cytometry. Results are presented as mean fluorescence channels (MFC) (mean \pm SD of four experiments). (C) FPP time effect was measured by incubating thalassemic RBC ($1-2 \times 10^6$ /mL) containing DCF at 37 °C with FPP (0.4 mg/mL). ROS of FPP-treated and untreated RBC were measured at different intervals. FPP-untreated RBC served as control. Results are presented as MFC (mean \pm SD of four experiments).

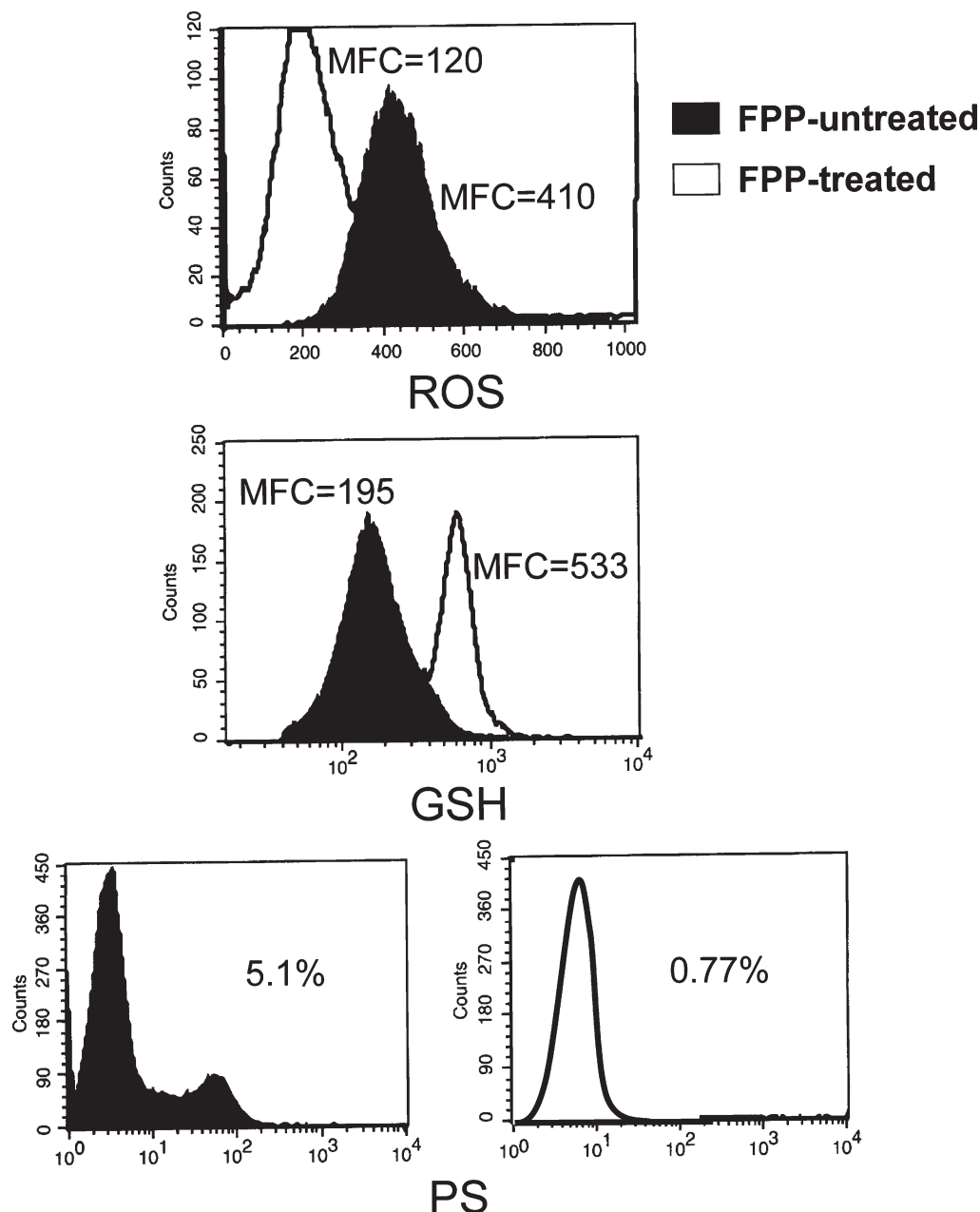


Figure 2. Flow cytometry analysis of oxidative state parameters in thalassemic RBC. Peripheral blood cells from a thalassemia patient were subjected to gelatin separation followed by overnight incubation with or without 0.1 mg/mL FPP. The cells were then washed and stained for reactive oxygen species (ROS) (after H_2O_2 stimulation), reduced glutathione (GSH) and phosphatidylserine (PS) by flow cytometry, following gating of the RBC on the basis of forward and size light scattering, as described in Materials and Methods. Fluorescence distribution histograms of RBC incubated with (white) or without FPP (black) are shown. The mean fluorescence channels (MFC) for ROS and GSH, and the percentages of positive cells – for PS, are also indicated.

generation between RBC with and without FPP appeared after 10 min and increased following further incubation.

Figure 2 illustrates flow cytometry results of FPP effects on oxidative parameters. FPP was added, at 0.1 mg/mL, to a suspension of thalassemic RBC cells; following overnight incubation, the cells were washed, labeled with either DCF, mercury orange or annexin-V and their ROS and GSH content as well as externalization of PS, respectively, were determined. The results show that treatment with FPP increases the GSH content of thalassemic RBC and reduces their ROS, as well as their PS exposure.

Figure 3 summarizes the ROS and GSH results obtained with RBC, platelets and PMN derived from 11 β -thalassemia patients. The differences between the

average MFC of cells incubated with or without FPP were highly significant ($p < 0.0001$), indicating reduced oxidative stress following FPP treatment.

Oxidative stress in RBC induces both intra- and extra-vascular hemolysis resulting in their short survival. To study the effect of *in vitro* treatment with FPP on hemolysis and phagocytosis the following experiments were carried out:

RBC were diluted in Ca^{++} -containing-buffer and incubated overnight with various amounts of FPP. The results indicated severe hemolysis of thalassemic RBC compared with normal RBC (data not shown) which was alleviated, in a dose-dependent manner, by FPP, (Fig. 4); at a concentration above 0.2 mg/mL it inhibited hemolysis by >90%.

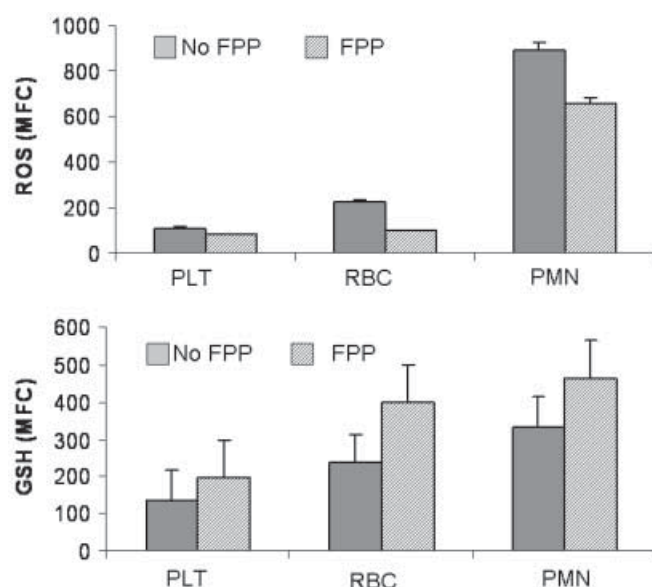


Figure 3. The *in vitro* effect of FPP-treatment on the oxidative status of cells from thalassemic patients. Peripheral blood cells derived from 11 patients with β -thalassemia were stained for reactive oxygen species (ROS) and reduced glutathione (GSH) as described in legends to Fig. 1. Platelets (PLT), red blood cells (RBC) and polymorphonuclear leukocytes (PMN) were analysed by flow cytometry following gating on the basis of forward and side light scattering and the mean fluorescence channel (MFC) determined. The results show the average MFC of the patients \pm SD. The differences between the cells with or without FPP were highly significant ($p < 0.0001$).

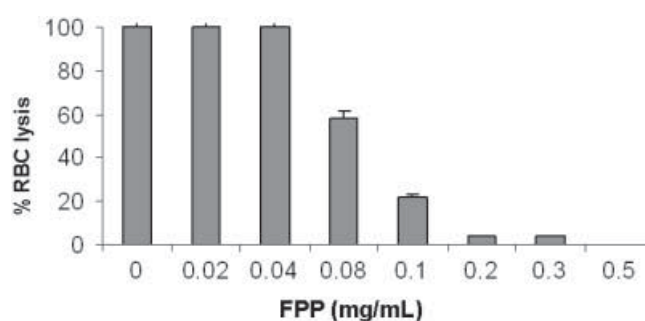


Figure 4. The *in vitro* effect of FPP-treatment on RBC lysis. Thalassemic RBC were diluted with phosphate buffered saline (PBS) to 5×10^6 /mL and incubated overnight at 37°C with the indicated amounts of FPP. The cells were centrifuged, the RBC in the pellet were resuspended in PBS and counted, and the percentage (%) of RBC lysis was calculated. The results show a dose-dependent decrease in the lysis of RBC (mean \pm SD derived from four patients).

Extravascular hemolysis was simulated by determining the ability of RBC to undergo phagocytosis by macrophages *in vitro*. Normal and thalassemic RBC were diluted with PBS and incubated overnight with or without FPP, followed by exposure for 30 min to various concentrations of the potent oxidant BHP, washed and placed in macrophage-containing plates for overnight incubation, as described in Materials and Methods. The results (Fig. 5A, C) show that phagocytosis was dose-dependently induced by BHP in both normal

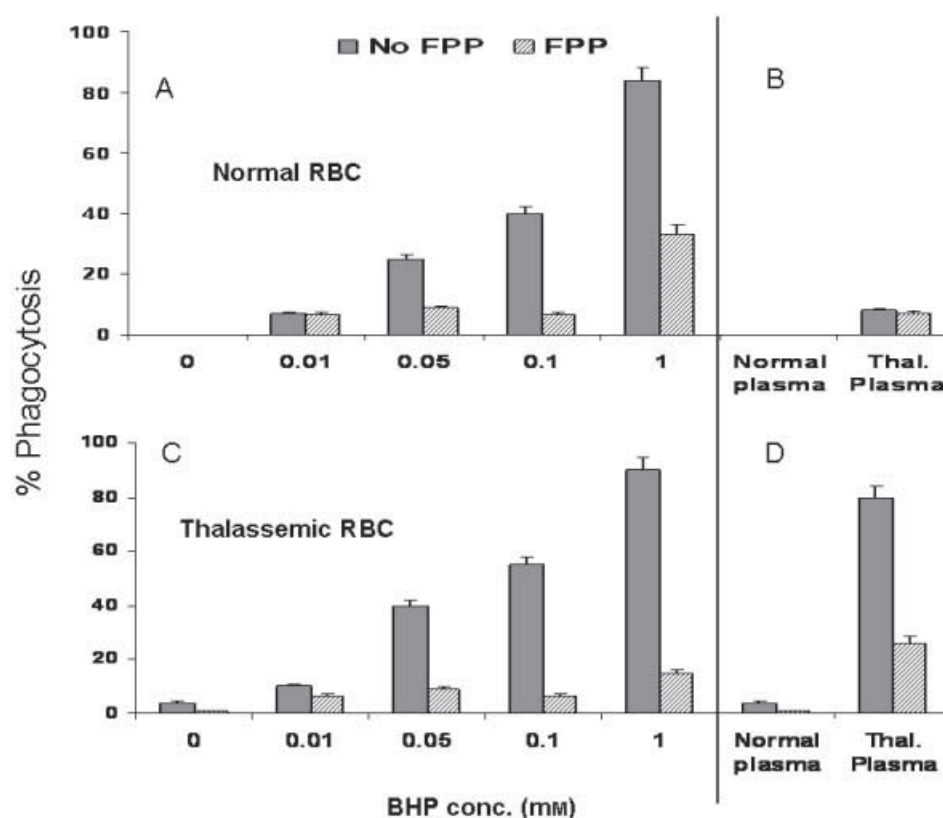


Figure 5. Effect of FPP on phagocytosis of RBC. Normal (A, B) and thalassemic (C, D) RBC were diluted to 5×10^6 /mL with either phosphate buffered saline (A, C) and with ABO-compatible normal or thalassemic plasma (B, D) and incubated for 30 min at 37°C with or without 0.5 mg/mL FPP. The buffer-diluted RBC were then treated for additional 30 min with the indicated concentrations of *t*-butylhydroperoxide (BHP). All samples were washed and placed in macrophage-containing plates, as described in Materials and Methods. After 24 h, non-phagocytosed RBC were counted microscopically. The percent (%) of phagocytosis was calculated per the RBC input. The results show the mean \pm SD values of RBC derived from four patients or four normal donors.

and thalassemic RBC. Pre-incubation with FPP reduced this effect of BHP. These results imply that oxidative stress is involved in the susceptibility of RBC to undergo phagocytosis and that FPP inhibits phagocytosis, probably by its antioxidative effect on RBC.

In another set of experiments, normal and thalassemic RBC, diluted in normal or thalassemic plasma, were incubated overnight with or without FPP, and then added to macrophage-containing plates. The results (Fig. 5B) show that thalassemic plasma induced susceptibility to phagocytosis in both normal and thalassemic RBC; the presence of FPP reduced this effect of thalassemic plasma.

Oxidative stress in PMN results in a deficient ability to generate oxidative burst (Amer and Fibach, 2005; Wiener, 2003). This may account for their reduced bactericidal activity and recurrent bacterial infections in thalassemic patients. To determine the *in vitro* effect of FPP on the ability of PMN to generate respiratory burst, blood cells derived from thalassemic patients were incubated overnight with or without FPP. They were then stained with DCF, washed and challenged with phorbol myristate acetate (PMA). ROS in PMN were measured by flow cytometry following gating. The results indicate (Fig. 6A) that FPP reduced (by 34%)

their basal level of ROS (prior to challenge with PMA) and improved their response to PMA, as shown by the increase in the ratio of ROS generated by PMA-treated vs untreated PMN; it was 1.12 and 2.53 in FPP-untreated and FPP-treated PMN, respectively.

Platelets in thalassemia are also affected by oxidative stress (Amer and Fibach, 2004; Iuliano *et al.*, 1997; Pratico *et al.*, 1992). We studied the *in vitro* effect of FPP on platelet activation markers: Externalization of PS – by staining with annexin-V and expression of P-selectin by staining with antibodies to CD62P. The results (Fig. 6B) show high expression of these markers on thalassemic platelets. FPP significantly decreased (40%) the proportion of platelets carrying PS, but had only a modest (20%) effect on the expression of P-selectin.

Effects of FPP *in vivo*

The effect of FPP was first determined in thalassemic mice. Ten mg/mL of FPP was added to their drinking water; this was calculated to provide on the average 50 mg/mouse/day. Prior to treatment and on various intervals after initiating the treatment up to 3 months, blood samples were drawn and RBC were analysed for ROS, GSH, lipid peroxidation and exposed PS compared with mice without FPP in their drinking water. The results (Fig. 7) demonstrated that treatment with FPP significantly reduced all the tested parameters of oxidative stress.

Based on these results, a group of 11 patients with homozygous β -thalassemia were treated with FPP (3 g three times a day after meals). Blood samples were drawn and analysed for ROS and GSH prior and at different times during treatment. Figure 8 shows the average values for all treated patients before and 3 months after beginning of treatment. In all patients a marked decrease in ROS and an increase in GSH were observed in RBC, platelets and PMN. Despite the significant changes in all the oxidation parameters tested, there were no significant changes in the hematological parameters both in mice and men, including complete blood count, RBC indices, reticulocytes and Hb levels.

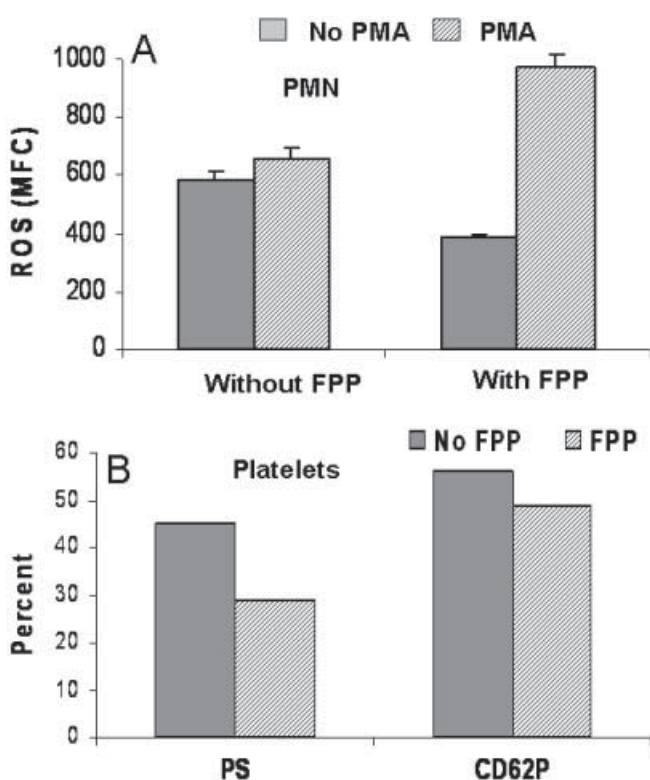


Figure 6. The *in vitro* effect of FPP on the functionality of thalassemic PMN and platelets. PMN (A) or platelets (B) from thalassemic patients were incubated overnight with or without 0.1 mg/mL FPP. PMN were analyzed for their reactive oxygen species (ROS) generation, either without (basal) or with phorbol myristate acetate (PMA, 40 ng/mL for 20 min at 37 °C). ROS were determined by flow cytometry and the mean fluorescence channel (MFC) determined. The ratio of ROS of PMA-treated and untreated PMN indicates their ability to respond by respiratory burst. Platelets were analysed for the activation markers external phosphatidylserine (PS) by staining with annexin-V, and P-selectin by staining with antibodies to CD62P. The percentages of platelets carrying PS or CD62P are shown. The results show the mean \pm SD values of PMN and platelets derived from four patients.

DISCUSSION

Fermented papaya preparation (FPP) is a natural Japanese health food product, obtained by biofermentation of *Carica papaya*. Previous studies suggested that it has antioxidative properties (Maccocci *et al.*, 1996; Osato *et al.*, 1995; Santiago *et al.*, 1991). The β -hemoglobinopathies, β -thalassemia and sickle cell anemia, are diseases in which oxidative stress mediates many aspects of the pathology (Hebbel *et al.*, 1982; Rachmilewitz and Schrier, 2001; Rund and Rachmilewitz, 2005). It was previously shown that in these diseases, RBC (Amer *et al.*, 2003, 2004), as well as platelets (Amer and Fibach, 2004) and polymorphonuclear neutrophils (PMN) (Amer and Fibach, 2005; Amer *et al.*, 2006) are under oxidative stress. The term oxidative stress is used here not strictly as the imbalance of pro-oxidants and antioxidants but in the context of 'a disruption of redox signaling and control' as recently suggested (Jones,

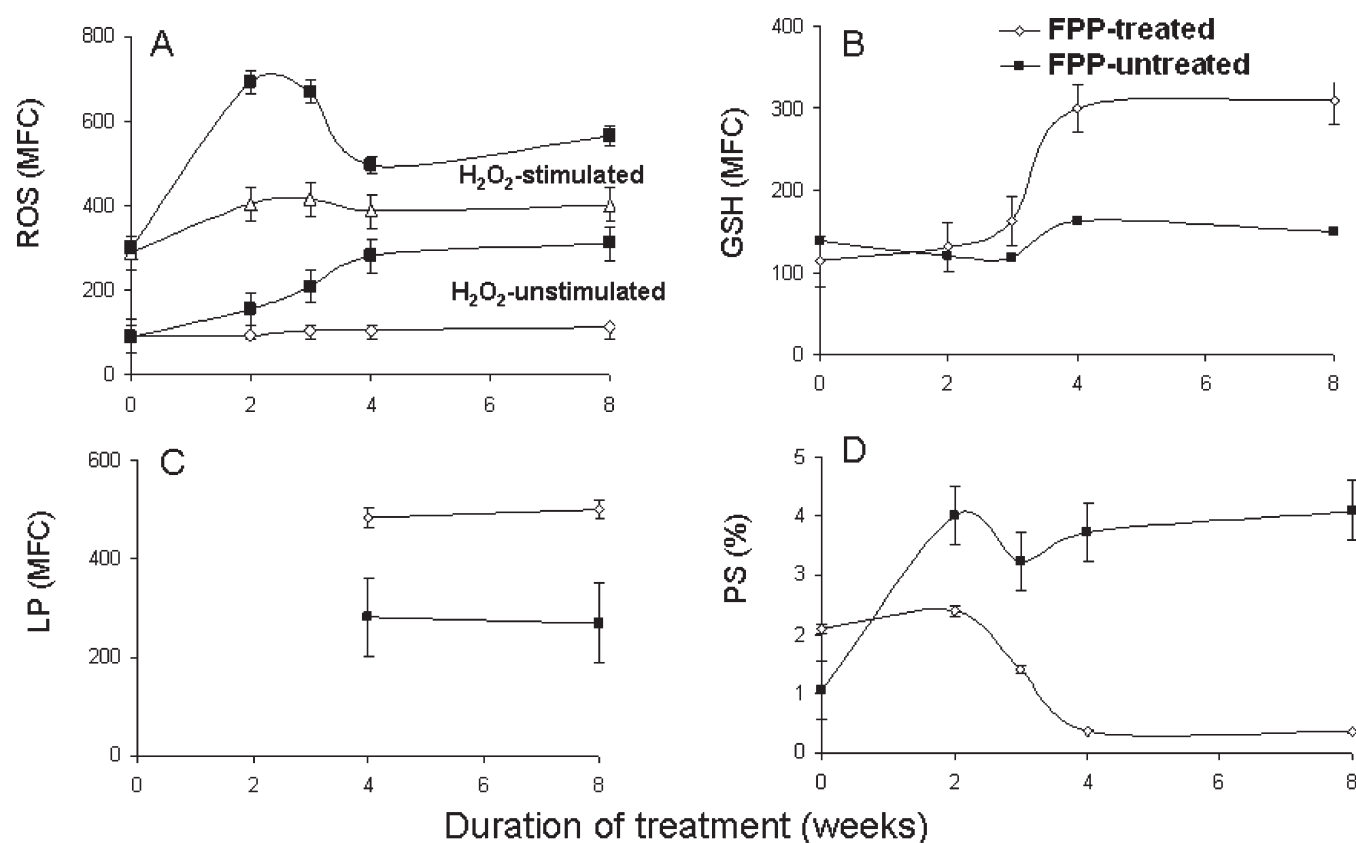


Figure 7. The *in vivo* effect of FPP in thalassemic mice. FPP, at 10 mg/mL, was added to the drinking water of heterozygous ($Hbb^{th3/+}$) thalassemic mice. Prior to treatment and on various intervals after initiating the treatment, up to 3 months, blood samples were drawn and RBC were analysed for ROS (with or without H₂O₂ stimulation) (A), GSH (B), lipid peroxidation (LP) (C) and exposed PS (D). The mean fluorescence channels (MFC) were determined for ROS, GSH and LP, while the percentage (%) of positive RBC was determined for PS. The results show the mean \pm SD values of RBC derived from four mice. The differences between the MFC and % of positive control before and after FPP administration were highly significant ($p < 0.001$).

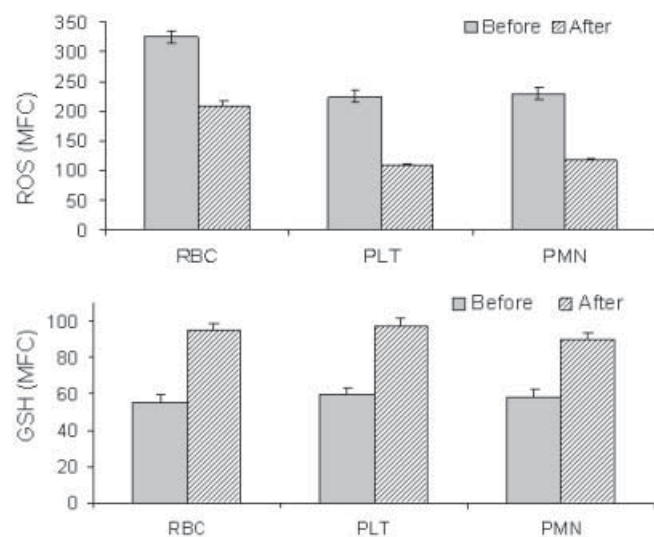


Figure 8. The effect of FPP in thalassemic patients. Nine patients with β -thalassemia were treated with PFF per os (3 g \times 3 times a day) for a 3 month period. Blood samples were drawn and their RBC, platelets (PLT) and polymorphonuclears (PMN) were analysed for ROS and GSH. The results depict the average mean fluorescence channels (MFC) \pm SD for all treated patients before and 3 months after beginning of the treatment, and indicate a significant ($p < 0.005$) decrease in ROS and an increase in GSH.

2006). When it occurs in thalassemic RBC, it may explain the anemia which is due to ineffective erythropoiesis of the erythroid precursors in the bone marrow and short survival of the mature RBC in the peripheral

blood. The oxidative stress of platelets may cause their increased tendency to undergo activation and aggregation and account for the high incidence of thromboembolic complications in these patients (Eldor and Rachmilewitz, 2002). The chronic oxidative stress of the PMN may result in ineffective bactericidal activity which may cause recurrent infections (Amer and Fibach, 2005; Klings and Farber, 2001; Okpala, 2004). The present study tested the antioxidant effects of FPP both *in vitro* and *in vivo* on blood cells of β -thalassemic mice and patients.

FPP has been previously shown, using electron spin resonance spectrometry, to possess free radical scavenging activity (Imao *et al.*, 1998; Santiago *et al.*, 1991) and was found to suppress ROS in the Fenton reaction, the hydrogen peroxide hypochlorite, the horseradish peroxidase and the xanthine-xanthine oxidase cell-free systems (Santiago *et al.*, 1991). In the present study, scavenging activity was determined by testing inhibition of DCF oxidation in a cell-free solution. DCF becomes highly fluorescent following oxidation by ROS (Bass *et al.*, 1983). Using spectrofluorometry, the effect of FPP in reducing the basal oxidation or H₂O₂-induced oxidation was tested. The results showed, in both cases, a dose-dependent decrease in DCF fluorescence (Fig. 1), indicating the ROS scavenging potential of FPP.

Following the results *in vitro*, the effect of FPP on ROS generation was studied in blood cells from patients with thalassemia major and intermedia. The cells were incubated with 2',7'-dichlorofluorescein. Upon

crossing the cell membrane, 2',7'-dichlorofluorescein diacetate undergoes deacetylation by esterases to be trapped intracellularly as 2',7'-dichlorofluorescein, which in turn becomes fluorescent dichlorofluorescein (DCF) upon oxidation by ROS (Bass *et al.*, 1983). The cellular DCF fluorescence was measured, at a single cell level, by flow cytometry. Cells were 'gated' based on their size (forward light scattering) and granularity (side light scattering) into RBC, platelets and PMN and the fluorescence of cells in each population was measured. Although this methodology yields results in arbitrary fluorescence units rather than in molar values, it is most valuable for purpose of comparison. Figure 2A shows distribution histograms with respect to DCF fluorescence of RBC treated or untreated with FPP. The mean fluorescence intensities (MFC) showed a 3.4-fold difference. Using this method, the dose and time of incubation effect of FPP was demonstrated on thalassemic RBC (Fig. 1B, C).

Flow cytometry was also used to analyse other parameters of oxidative stress. The results showed that *in vitro* treatment with FPP, in addition to decreasing ROS, also increased the level of GSH and decreased the level of exposed PS (Fig. 2). These effects were observed in RBC as well as in platelets and PMN of thalassemic patients (Fig. 3) and are most likely related to its free radical scavenging activity. Moreover, its high content of glutamic acid, glycine and methionine are relevant substrates for glutathione synthesis (Marcocci *et al.*, 1996; Osato *et al.*, 1995; Santiago *et al.*, 1991). In addition, the GSH sparing effect could be also an option, since decreased generated ROS require less GSH for their neutralization.

Chronic oxidative stress in blood cells, such as the case in thalassemia, affects their function: RBC become sensitive to hemolysis and to phagocytosis by macrophages (Rund and Rachmilewitz, 2005; Schrier *et al.*, 2003) (Fig. 5), causing their shorter survival in the circulation, and consequently to chronic anemia; PMN have reduced ability to generate oxidative burst – an intracellular mechanism of bacteriolysis (Wiener, 2003) (Fig. 6A), a possible cause for recurrent infections; and platelets tend to undergo hyperactivation (Iuliano *et al.*, 1997), which was reflected in the present study by exposure of PS (Fig. 6B), leading to frequent thromboembolic complications. Various mechanisms have been suggested to be involved in these defective cellular functions: Increased susceptibility to hemolysis and phagocytosis is most likely the result of oxidative damage to their membrane proteins, band 4.1, band 3 and spectrin (Beneke *et al.*, 2005) and lipids (Shinar and Rachmilewitz, 1990). PMN failure to generate oxidative burst may be the result of the effect of excess cytosolic ROS on NADPH oxidase activity; possible mechanisms may include damage to the cytosolic enzyme components or to the phagosome membrane

lipids. In platelets, activation has been demonstrated to involve increased ROS generation: activators, e.g. thrombin, increase their ROS (Hanson and Harker, 1988; Eidt *et al.*, 1989) while oxidants, by increasing ROS, induce activation (Blockmans *et al.*, 1995; Iuliano *et al.*, 1997). It was previously shown that all these functional defects in thalassemic cells (RBC, PMN and platelets) could be ameliorated by antioxidants (Amer and Fibach, 2004, 2005; Amer *et al.*, 2004, 2006). In the present study, FPP showed similar protecting effects (Figs 4 and 6), in parallel to its ability to reduce ROS generation, suggesting that these effects are mediated by its antioxidative properties.

Oxidative stress in thalassemia is caused primarily by the RBC abnormalities – degradation of unstable hemoglobin which results in free globin chains and heme. Another contributing factor is iron overload due to increased intestinal absorption and regular blood transfusions. This point is reflected in the results depicted in Fig. 5 B, D; phagocytosis of thalassemic RBC was higher than that of normal RBC. However, in both cases, incubation with thalassemic plasma increased their phagocytosis to a larger extent compared with normal plasma. Several studies indicated the presence of elevated levels of free radicals released from damaged RBC (Iuliano *et al.*, 1992; Shinar and Rachmilewitz, 1993; Iuliano *et al.*, 1997) in the thalassemic plasma as well as iron-containing compounds (hemin and hemoglobin) (Shinar and Rachmilewitz, 1993) which are known to induce oxidative stress.

The concept of using phytochemicals such as indicaxanthin (Tesoriere *et al.*, 2006) as antioxidants in β -thalassemic RBC has been studied *in vitro* and showed promising results. FPP was previously demonstrated to have an effect *in vivo*: oral administration into rats showed a significant inhibition of thiobarbituric acid reactive substances formation, which is an index of lipid peroxidation in the iron-induced epileptic focus of rats (Santiago *et al.*, 1991), and an increase in superoxide dismutase activity in their cortex and hippocampus (Imao *et al.*, 1998). The present study tested the *in vivo* effects of FPP by treating β -thalassemic mice and patients and showed a significant reduction in all the tested parameters of oxidative stress in blood cells similar to the results obtained *in vitro*. However, no significant improvement was found in the hematological parameters. It is possible that more than 3 months are required in order to achieve the latter goals. Therefore, additional trials with a longer duration of treatment are currently in preparation.

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REFERENCES

- Amer J, Fibach E. 2004. Oxidative status of platelets in normal and thalassemic blood. *Thromb Haemost* **92**: 1052–1059.
- Amer J, Fibach E. 2005. Chronic oxidative stress reduces the respiratory burst response of neutrophils from beta-thalassaemia patients. *Br J Haematol* **129**: 435–441.
- Amer J, Ghoti H, Rachmilewitz E, Koren A, Levin C, Fibach E. 2006. Red blood cells, platelets and polymorphonuclear neutrophils of patients with sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants. *Br J Haematol* **132**: 108–113.
- Amer J, Goldfarb A, Fibach E. 2003. Flow cytometric measurement of reactive oxygen species production by normal

- and thalassaemic red blood cells. *Eur J Haematol* **70**: 84–90.
- Amer J, Goldfarb A, Fibach E. 2004. Flow cytometric analysis of the oxidative status of normal and thalassemic red blood cells. *Cytometry A* **60**: 73–80.
- Aruoma OI, Colognato R, Fontana I *et al.* 2006. Molecular effects of fermented papaya preparation on oxidative damage, MAP kinase activation and modulation of the benzo[a]pyrene mediated genotoxicity. *Biofactors* **26**: 147–159.
- Ataga KI, Orringer EP. 2003. Hypercoagulability in sickle cell disease: a curious paradox. *Am J Med* **115**: 721–728.
- Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. 1983. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J Immunol* **130**: 1910–1917.
- Beneke R, Bihn D, Hutler M, Leithauser RM. 2005. Haemolysis caused by alterations of alpha- and beta-spectrin after 10 to 35 min of severe exercise. *Eur J Appl Physiol* **95**: 307–312.
- Blockmans D, Deckmyn H, Vermeylen J. 1995. Platelet activation. *Blood Rev* **9**: 143–156.
- Chan AC, Chow CK, Chiu D. 1999. Interaction of antioxidants and their implication in genetic anemia. *Proc Soc Exp Biol Med* **222**: 274–282.
- Chignell CF, Sik RH. 2003. A photochemical study of cells loaded with 2',7'-dichlorofluorescein: implications for the detection of reactive oxygen species generated during UVA irradiation. *Free Radic Biol Med* **34**: 1029–1034.
- Comporti M, Signorini C, Buonocore G, Ciccoli L. 2002. Iron release, oxidative stress and erythrocyte ageing. *Free Radic Biol Med* **32**: 568–576.
- Eidt JF, Allison P, Noble S *et al.* 1989. Thrombin is an important mediator of platelet aggregation in stenosed canine coronary arteries with endothelial injury. *J Clin Invest* **84**: 18–27.
- Eldor A, Rachmilewitz EA. 2002. The hypercoagulable state in thalassemia. *Blood* **99**: 36–43.
- Fibach E. 1993. Measurement of total and fetal hemoglobin in cultured human erythroid cells by a novel micromethod. *Hemoglobin* **17**: 41–53.
- Fibach E. 1998. Techniques for studying stimulation of fetal hemoglobin production in human erythroid cultures. *Hemoglobin* **22**: 445–458.
- Hanson SR, Harker LA. 1988. Interruption of acute platelet-dependent thrombosis by the synthetic antithrombin D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone. *Proc Natl Acad Sci USA* **85**: 3184–3188.
- Hebbel RP, Eaton JW, Balasingam M, Steinberg MH. 1982. Spontaneous oxygen radical generation by sickle erythrocytes. *J Clin Invest* **70**: 1253–1259.
- Imao K, Wang H, Komatsu M, Hiramatsu M. 1998. Free radical scavenging activity of fermented papaya preparation and its effect on lipid peroxide level and superoxide dismutase activity in iron-induced epileptic foci of rats. *Biochem Mol Biol Int* **45**: 11–23.
- Iuliano L, Colavita AR, Leo R, Pratico D, Violi F. 1997. Oxygen free radicals and platelet activation. *Free Radic Biol Med* **22**: 999–1006.
- Iuliano L, Violi F, Pedersen JZ, Pratico D, Rotilio G, Balsano F. 1992. Free radical-mediated platelet activation by hemoglobin released from red blood cells. *Arch Biochem Biophys* **299**: 220–224.
- Jones DP. 2006. Redefining oxidative stress. *Antioxid Redox Signal* **8**: 1865–1879.
- Klings ES, Farber HW. 2001. Role of free radicals in the pathogenesis of acute chest syndrome in sickle cell disease. *Respir Res* **2**: 280–285.
- Komarov AM, Hall JM, Weglicki WB. 2004. Azidothymidine promotes free radical generation by activated macrophages and hydrogen peroxide-iron-mediated oxidation in a cell-free system. *Biochim Biophys Acta* **1688**: 257–264.
- Marcocci L, D'Anna R, Yan LJ, Haramaki N, Packer L. 1996. Efficacy of Bio-Catalyzer alpha.rho no.11 (Bio-Normalizer) supplementation against peroxyl radical-induced oxidative damage in rat organ homogenates. *Biochem Mol Biol Int* **38**: 535–541.
- Marotta F, Reizakovic I, Tajiri H, Safran P, Ideo G. 1997. Abstinence-induced oxidative stress in moderate drinkers is improved by bionormalizer. *Hepatogastroenterology* **44**: 1360–1366.
- Okpala I. 2004. The intriguing contribution of white blood cells to sickle cell disease – a red cell disorder. *Blood Rev* **18**: 65–73.
- Osato JA, Korkina LG, Santiago LA, Afanas'ev IB. 1995. Effects of bio-normalizer (a food supplementation) on free radical production by human blood neutrophils, erythrocytes, and rat peritoneal macrophages. *Nutrition* **11**: 568–572.
- Pratico D, Iuliano L, Pulcinelli FM, Bonavita MS, Gazzaniga PP, Violi F. 1992. Hydrogen peroxide triggers activation of human platelets selectively exposed to nonaggregating concentrations of arachidonic acid and collagen. *J Lab Clin Med* **119**: 364–370.
- Rachmilewitz EA, Schrier S. 2001. The pathophysiology of β -thalassemia. In *Disorders of Hemoglobin: Genetics, Pathophysiology and Clinical Management*, Steinberg MH, Forget BG, Higgs DR, Nagel RL (eds). Cambridge University Press: Cambridge, 233–251.
- Rund D, Rachmilewitz E. 2005. Beta-thalassemia. *N Engl J Med* **353**: 1135–1146.
- Santiago LA, Osato JA, Hiramatsu M, Edamatsu R, Mori A. 1991. Free radical scavenging action of Bio-catalyzer alpha.rho No.11 (Bio-normalizer) and its by-product. *Free Radic Biol Med* **11**: 379–383.
- Schrier SL, Centis F, Verneris M, Ma L, Angelucci E. 2003. The role of oxidant injury in the pathophysiology of human thalassemias. *Redox Rep* **8**: 241–245.
- Shinar E, Rachmilewitz EA. 1990. Oxidative denaturation of red blood cells in thalassemia. *Semin Hematol* **27**: 70–82.
- Shinar E, Rachmilewitz EA. 1993. Haemoglobinopathies and red cell membrane function. *Baillieres Clin Haematol* **6**: 357–369.
- Tesoriere L, Allegra M, Butera D, Gentile C, Livrea MA. 2006. Cytoprotective effects of the antioxidant phytochemical indicaxanthin in beta-thalassemia red blood cells. *Free Radic Res* **40**: 753–761.
- Wiener E. 2003. Impaired phagocyte antibacterial effector functions in beta-thalassemia: a likely factor in the increased susceptibility to bacterial infections. *Hematology* **8**: 35–40.
- Yang B, Kirby S, Lewis J, Detloff PJ, Maeda N, Smithies O. 1995. A mouse model for beta 0-thalassemia. *Proc Natl Acad Sci USA* **92**: 11608–11612.