PHYTOTHERAPY RESEARCH
Phytother. Res. (2010)
Published online in Wiley InterScience
(www.interscience.wiley.com) **DOI**: 10.1002/ptr.3116

FULL PAPER

Amelioration of Oxidative Stress in Red Blood Cells from Patients with β-thalassemia Major and Intermedia and E-β-thalassemia Following Administration of a Fermented Papaya Preparation

Eitan Fibach[1]*, Ee-Shien Tan[2], Saumya Jamuar[2], Ivy Ng[2], Johnny Amer[1] and Eliezer A. Rachmilewitz[3]

[1]Department of Hematology, Hadassah – Hebrew University Medical Center, Jerusalem, Israel
[2]Department of Paediatric Medicine and Genetics Service, KK Women's and Children's Hospital, Singapore
[3]Department of Hematology, The E. Wolfson Medical Center, Holon, Israel

In β-hemoglobinopathies, such as β-thalassemia (thal) and sickle cell anemia, the primary defects are mutations in the β-globin gene. However, many aspects of the pathophysiology are mediated by oxidative stress. Fermented papaya preparation (FPP), a natural health food product obtained by biofermentation of carica papaya, has been shown to limit oxidative stress both *in vitro* and *in vivo*. We studied the effect of FPP on two groups of β-thal patients: β-thal, major and intermedia, (in Israel) and E-β-thal (in Singapore). The results indicated that in both groups FPP treatment increased the content of reduced glutathione (GSH) in red blood cells (RBC), and decreased their reactive oxygen species (ROS) generation, membrane lipid peroxidation, and externalization of phosphatidylserine (PS), indicating amelioration of their oxidative status, without a significant change in the hematological parameters. Since the turnover of the erythron is relatively slow, it is possible that longer duration of treatment, probably with the addition of an iron chelator, is required in order to achieve the latter goals. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: hemoglobinopathies; thalassemia; erythrocyte; free radicals; antioxidants; flow cytometry.

INTRODUCTION

Fermented papaya preparation (FPP), a natural health food product obtained by biofermentation of carica papaya, has been shown to limit oxidative stress both *in vitro* and *in vivo* (Santiago *et al.*, 1991; Osato *et al.*, 1995; Marcocci *et al.*, 1996; Marotta *et al.*, 1997). In β-hemoglobinopathies, such as β-thal and sickle cell anemia, the primary defects are mutations in the β-globin gene. However, many aspects of the pathophysiology are mediated by oxidative stress (Hebbel *et al.*, 1982; Rachmilewitz and Schrier, 2001; Rund and Rachmilewitz, 2005). RBC (Amer *et al.*, 2003; 2006) as well as platelets (Amer and Fibach, 2004) and polymorphonuclear leukocytes (Amer and Fibach, 2005; Amer *et al.*, 2006) of these patients have increased levels of ROS, membrane lipid peroxidants and exposed PS concomitant with lower levels of GSH compared with their counterparts derived from normal donors.

In the present study, we tested the effect of treatment of two groups of thalassemic patients, β-thal major and intermedia (in Israel) and β-thal E (in Singapore) with FPP given orally on the oxidative status in their blood cells.

* Correspondence to: Eitan Fibach, Department of Hematology, Hadassah – Hebrew University Medical Center, Ein-Kerem, Jerusalem 91120, Israel.
E-mail: Fibach@yahoo.com

MATERIALS AND METHODS

FPP. A product of yeast fermentation of *Carica papaya Linn.* It was supplied as sachets, each containing 3g powder, by Osato Research Institute, Gifu, Japan. The compositions of its principal components are listed in Table 1.

Patients. The β-thal group included 8 patients with β-thal intermedia and 3 with β-thal major (Table 2). 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 for 10 h, 5–7 days a week. In polytransfused patients, blood samples were obtained at least three weeks following the previous transfusion. The E-β-thal group included seven patients. These patients were not on regular blood transfusions and had not received any transfusion 10 weeks prior to

Received 30 August 2009
Revised 10 December 2009
Accepted 17 December 2009

Copyright © 2010 John Wiley & Sons, Ltd.

Table 1. Composition of FPP

Component	(g/100 g FFP)	Component	(mg/100 g FFP)
Moisture	8.2	Amino acids	
Protein	0.3	Arginine	12
Fat	>0.1	Lysine	4
Ash	>0.1	Histidine	3
		Phenylalanine	10
Carbohydrates	91.50	Tyrosine	7
Glucose	87.58	Leucine	14
Maltose	0.19	Isoleucine	8
Isomaltose	0.11	Methionine	4
Maltotriose	0.01	Valine	11
Panose	0.05	Alanine	11
		Glycine	9
		Proline	9
		Glutamic acid	32
		Serine	9
		Threonine	7
		Aspartic acid	20
		Tryptophan	2
		Cystine	-

The composition analysis was carried out and authenticated by the Japan Food Research Laboratories, Fukuoka, Japan.

Table 2. Patient population

Pat. No.	Sex	β-Thal.	Mutation	Blood transf.	Desf.[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 Aq	+	+
11	F	Major	FS44 / Poly A	+	+
12	M	Major	FS44 / FS44	+	+
Sg					
13	F	Inter	HbE/ IVS 1–nt1 (G/T)	–	–
14	M	Inter	HbE/45kb Fil deletion	–	–
15	F	Inter	HbE/45kb Fil deletion	–	–
16	F	Inter	HbE/IVS 1–nt5 (G/C)	+/–[c]	–
17	M	Inter	HbE/Cds 71/72 (+A)	+/–[c]	–
18	F	Inter	HbE/Cds 41/42 (–TTCT)	–	–
19	F	Inter	HbE/Hb Khon Kaen	–	–

[a]= deferioxamine treatment; [b]= intermedia; [c]= irregularly; [d]= every 4 weeks.

commencement of the study. Informed consent was obtained in the two groups of patients.

The β-thal patients were treated with FPP (3 g three times a day after meals) for three months. The E-β -thal group was treated with FPP (3 g two times a day after meals).

Peripheral blood (0.5 ml) samples were obtained prior and during treatment. The blood was diluted with equal volume of Ca^{++}– and Mg^{++}–free Dulbecco's phosphate buffered saline (PBS).

Flow cytometry measurements of oxidative stress markers. Cells were analyzed either before or after 15 min stimulation with 0.5 mM H_2O_2. For ROS measurement, RBC were incubated with 100 μM (final concentration) 2′-7′-dichlorofluorescin diacetate (DCF) (Sigma, St Louis, MO, USA) for 15 min at 37°C in a humidified atmosphere of 5% CO_2 in air. For measuring GSH content, cell concentrates were incubated for 3 min at room temperature with [1-(4-chloromercury-phenyl-azo-2-naphthol)] (mercury orange) (Sigma, St

Copyright © 2010 John Wiley & Sons, Ltd.

Louis, MO, USA) at final concentration of 40 μM, following washing in PBS. For lipid peroxidation, RBC suspensions were labeled with 40 μM *N*- (fluorescein-5-thiocarbamoyl) 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Molecular Probes Inc., Eugene, OR,USA) dissolved in ethanol. The cells were incubated for 1 h at 37°C in a humidified atmosphere of 5% CO_2 in air, with continuous agitation, centrifuged once to remove unbound label, and resuspended in PBS. Externalization of PS was determined following washing of the cells in Ca^{++}-binding buffer and staining with isothiocyanate-conjugated Annexin-V (IQ products, Groningen, the Netherlands) (Eldor and Rachmilewitz, 2002).

Following staining, cells were analyzed by flow cytometry as previously described (Amer *et al.*, 2004). For each parameter the Mean Fluorescence Intensity (MFI) of at least 30,000 RBC was calculated using the FACS-equipped CellQuest^R software (FACS-calibur, Becton-Dickinson, Immunofluorometry Systems, Mountain View, CA, USA). In each assay, unstained cells, both treated and untreated, served as control. The MFI 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; the MFI of fluor-DHPE-stained cells is reversely proportional to their extent of lipid peroxidation.

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

RESULTS AND DISCUSSION

Blood samples were drawn and analyzed for ROS and GSH prior and at different times during treatment. Figure 1 shows the average values for all treated patients before and during the treatment. In the two groups of patients, a marked decrease in ROS and an increase in GSH were observed in RBC, as well as a decrease in lipid peroxidation and in externalization of PS residues. However, despite the significant changes in all the oxidation parameters tested, there were no significant changes in the hematological parameters, including complete blood count, RBC indices, reticulocytes and hemoglobin (Hb) levels.

Oxidative stress in thalassemia is caused primarily by the RBC abnormalities – degradation of unstable Hb which ends up in free globin chains and heme with eventual iron release. Another contributing factor to iron overload is increased intestinal absorption and regular blood transfusions. Consequently, free iron species – non-transferrin-bound iron (NTBI) and labile plasma iron catalyzing the formation of ROS, have been identified (Esposito *et al.*, 2002).

Chronic oxidative stress in blood cells, such as the case in thalassemia, affects their function: We have previously shown that RBC become sensitive to hemolysis and to phagocytosis by macrophages (Fibach and Rachmilewitz, 2008), causing their shorter survival in the circulation, and consequently become sensitive to chronic anemia; neutrophils have reduced capacity to generate oxidative burst – an intracellular mechanism of bacteriolysis (Wiener, 2003), a possible cause for recurrent infections; and platelets tend to become activated (Iuliano *et al.*, 1997), leading to frequent thromboembolic complications (Eldor and Rachmilewitz, 2002). Various mechanisms have been suggested to be involved in these defected cellular functions: increased susceptibility of RBC 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). Neutrophils' 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 (Hampton *et al.*, 1998). In platelets, oxidants, by increasing ROS, have been shown to induce their activation [15].

We have previously shown that all these functional defects in thal cells could be ameliorated by antioxidants [13], including FPP (Amer *et al.*, 2008). The effect of FPP could be due to its high content of glutamic acid, glycine and methionine which are substrates for glutathione synthesis (Santiago *et al.*, 1991; Osato *et al.*, 1995; Marcocci *et al.*, 1996). In addition, GSH sparing effect could also be an option, since decreased generated ROS requires less GSH for their neutralization.

The concept of using phytochemicals such as indicaxanthin (Tesoriere *et al.*, 2006) and curcumin (Thephinlap *et al.*, 2009) as antioxidants in β thalassemic RBC has shown promising results. FPP was previously demonstrated to have an effect *in vivo*: oral administration into rats showed a significant inhibition of the formation of thiobarbituric acid reactive substances, which is an index of lipid peroxidation in 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). In the present study of oral treatment with FPP of two groups of β-thalassemic patients with β-thal major and intermedia, and with E-β-thal from different countries showed a significant reduction in all the tested parameters of oxidative stress in their blood cells, without a significant improvement in the hematological parameters. Since the turnover of the erythron is relatively slow, it is possible that longer duration of treatment is required in order to achieve the latter goals. In addition, a combination treatment with an antioxidant, like FPP, with an iron chelator might yield better results.

Acknowledgements

This work was partially funded by a grant from Osato Research Institute, Japan. EF and ER are consultants to the Osato Research Institute.

Copyright © 2010 John Wiley & Sons, Ltd.

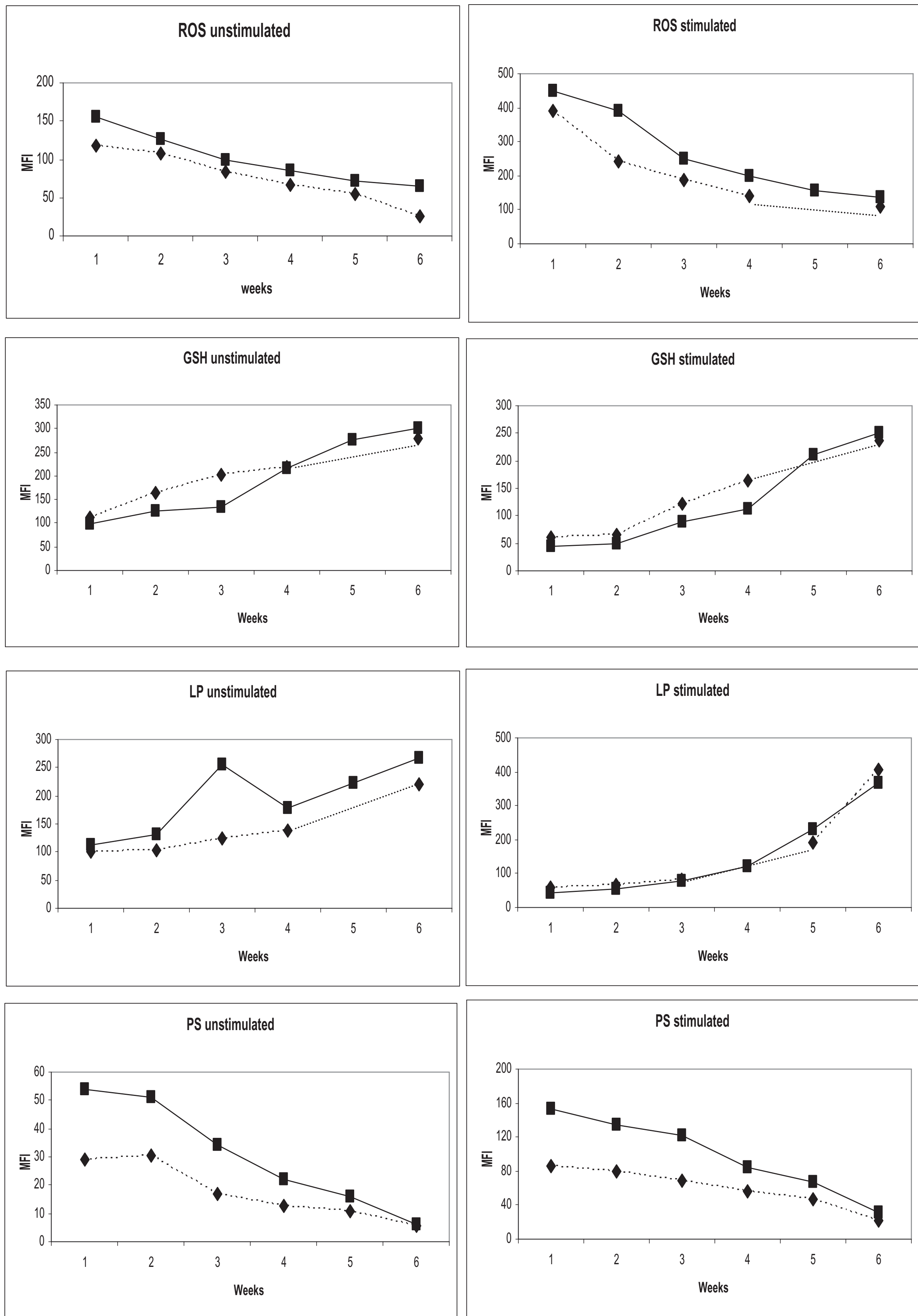

Figure 1. Changes in parameters of oxidative stress following administration of FPP to patients with β-thalassemia (–■) and E-β thalassemia (---▲). The data present the Mean Fluorescence Index (MFI) of cells stained for reactive oxygen species (ROS), reduced glutathione (GSH), lipid peroxidation (LP) and external phosphatidylserine (PS) in H_2O_2-stimulated (right) and unstimulated (left) samples. Note that ROS, GSH and PS for cells stained for LP, the MFI is reversely proportional to their LP. The results show a decrease in ROS, LP and externalization PS concomitant with an increase in GSH.

Copyright © 2010 John Wiley & Sons, Ltd.

REFERENCES

Amer J, Atlas D, Fibach E. 2008. N-acetylcysteine amide (AD4) attenuates oxidative stress in beta-thalassemia blood cells. *Biochim Biophys Acta* **1780**: 249–255.

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.

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.

Eldor A, Rachmilewitz EA. 2002. The hypercoagulable state in thalassemia. *Blood* **99**: 36–43.

Esposito BP, Breuer W, Slotki I, Cabantchik ZI. 2002. Labile iron in parenteral iron formulations and its potential for generating plasma non-transferrin-bound iron in dialysis patients. *Eur J Clin Invest* **32**(Suppl 1): 42–49.

Fibach E, Rachmilewitz E. 2008 The role of oxidative stress in hemolytic anemia. *Curr Mol Med* **8**: 609–619.

Hampton MB, Kettle AJ, Winterbourn CC. 1998. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92**: 3007–3017.

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.

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.

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.

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-normalyzer) and its by-product. *Free Radic Biol Med* **11**: 379–383.

Shinar E, Rachmilewitz EA. 1990. Oxidative denaturation of red blood cells in thalassemia. *Semin Hematol* **27**: 70–82.

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.

Thephinlap C, Phisalaphong C, Fucharoen S, Porter JB, Srichairatanakool S. 2009. Efficacy of Curcuminoids in Alleviation of Iron overload and Lipid Peroxidation in Thalassemic Mice. *Med Chem* **5**: 474–482.

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.

Copyright © 2010 John Wiley & Sons, Ltd.