ORIGINAL ARTICLE

The Clinical Effects of Fermented Papaya Preparation® (FPP®) on Oxidative Stress in Patients with HbE/ β -Thalassaemia

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ABSTRACT

Background: Red blood cells (RBC) of patients with thalassaemia are under continuous oxidative stress. Fermented papaya preparation® (FPP®) has been shown to have an antioxidative effect and is postulated to reduce the oxidative stress on RBC.

Objective: To study the clinical effects of FPP® treatment in patients with HbE/β-thalassaemia on RBC indices, oxidative stress and quality of life scores.

Method: Patients with HbE/β-thalassaemia who do not receive regular blood transfusion were included in the study and were given FPP® daily (3gm 2 times a day) for 12 weeks. Peripheral blood samples were obtained at the initiation of the study and at 4-weekly intervals thereafter for a period of 12 weeks. The following parameters were measured:

1. Haemoglobin (Hb), mean corpuscular volume (MCV), reticulocyte count;
2. Oxidation studies: production of reactive oxygen species (ROS) and intracellular glutathione content (GSH), spontaneously and in response to oxidative stress;
3. Quality of life (QoL) at the start and at the end of 12 weeks using health survey questionnaires.

Results: Seven patients (5 females and 2 males) were recruited to the study from January 2006 to April 2006. Median age of the study population was 19 years (range 4 to 27yrs). In vitro analyses showed production of significantly less ROS and more GSH following treatment. There was no significant difference in the Hb, MCV, reticulocyte count, clinical parameters or QoL scores. FPP® was well tolerated by all the patients.

Conclusion: Although oxidative stress parameters were decreased, FPP® did not have any significant effect on the Hb levels or QoL. Longer studies on larger sample size are required to study the long-term clinical effect of FPP® on clinical parameters in patients with Hb E/β-thalassaemia.

Keywords: antioxidants, erythrocytes, haemoglobin, thalassaemia

BACKGROUND

Thalassaemia is a congenital haemolytic anaemia resulting from partial or complete absence of one of the major α or β globin chains of HbA ($\alpha_2\beta_2$). It is the commonest genetic disease in South-East Asia. The incidence of α and β thalassaemia trait is 2.92% and 0.93%, respectively. The incidence of HbE, a haemoglobin variant due to mutation in the β globin gene which results in both quantitative and qualitative defect of the β globin chain, is 0.64%[1]. A child who inherits both HbE and β globin chain mutation may present with thalassaemia intermedia. Thalassaemia intermedia encompasses a broad clinical spectrum from mild anaemia to transfusion dependent anaemia. While iron loading is less accelerated than transfusional iron accumulation in patients with thalassaemia major, patients with thalassaemia intermedia

often develop cardiac disease, hepatic fibrosis, endocrine abnormalities and other complications of iron overload due to increased gastrointestinal iron absorption[2].

Red blood cells of patients with thalassaemia are under continuous oxidative stress[3] due to generation of high levels of reactive oxygen species (ROS) and reduced content, due to increased consumption, of anti-oxidants such as glutathione (GSH)[3,4]. The causes of this stress are both intracellular (example, the presence of abnormal Hb and its degradation products, such as free globin chains, heme and free iron) as well as extracellular (example, iron overload). The oxidative stress has major deleterious effects mainly on the cell membrane. This results in the externalisation of phophatidylserine (PS) moieties-negatively charged phospholipids that have been shown to mediate erythrophagocytosis by macrophages in the reticuloendothelial system[5,6]. It has been shown that thalassaemic RBC have abundant exposure of PS, which has been speculated to play a role in ineffective erythropoiesis (due to increased apoptosis of erythroid precursors) and short life span of mature RBC due to increased extravascular haemolysis[7].

Fermented papaya preparation (FPP®) is a product of yeast fermentation of *Carica papaya Linn*. The composition of its principle components has been previously described[8,9]. It has been shown to limit oxidative stress both in vitro and in vivo[10–13]. Being an antioxidant, FPP® is expected to decrease the oxidative damage to thalassaemic mature RBC and erythroid precursors in the bone marrow and hence improve haemoglobin (Hb) levels in these patients. We have previously reported on in-vitro amelioration of oxidative stress in a heterogenous population of patients with β thalassemia major and intermedia, including HbE/ β thalassemia[9].

We performed a pilot study to assess the clinical effects of FPP® treatment in patients with HbE/ β-thalassaemia on (1) RBC indices such as Hb, mean corpuscular volume (MCV), and reticulocyte count; (2) on oxidative stress parameters such as generation of ROS, GSH, lipid peroxidation within the RBC, and (3) quality of life scores.

METHODS

Recruitment of Patients

Inclusion Criteria

Patients with HbE/β thalassaemia who are on regular outpatient follow-up with KK Women's and Children's Hospital (KKH) with:

1. Baseline Hb <10g/dL, and

2. Patients who are not regularly transfused and did not receive transfusion at least 10 weeks prior to initiation of the study.

The study was approved by the institutional review board (IRB) of KKH. All patients or parents (for children less than 18 years) gave their written informed consent to participate in the study.

Patients were given 2 sachets (3gm per sachet = 6gm per day) of FPP® daily for 12 weeks. Peripheral blood samples (3ml blood, collected in ethylenediaminetetraacetic acid [EDTA] tube) were obtained at the initiation of the study and at 4 weekly intervals thereafter for a period of 12 weeks for complete blood count and cellular oxidative parameters.

Laboratory Methods

Complete Blood Count

Complete blood count, including Hb, MCV and reticulocyte count, was measured on the blood sample using Cell-dyne 3500 (Abbott, USA).

Cellular Parameters

For reactive oxygen species (ROS) assay, RBC were incubated with 2',7'-dichlorofluorescin diacetate (DCF) (Molecular Probes, Invitrogen, Carlsbad, California, USA), dissolved in methanol, at a final concentration of 0.4mM. Upon crossing the cellular membrane, DCF undergoes deacetylation by intracellular esterases producing a non-fluorescent compound that becomes highly green fluorescent following oxidation by ROS[14]. For glutathione (GSH) assay, RBC were incubated with 40μM of mercury orange. Mercury orange reacts with the SH group of GSH to produce red-orange fluorescence[14]. For lipid peroxidation assay, RBC suspensions ($5x10^6$ cells/ml) in PBS were labelled with 50μM N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (fluor-DHPE) (Sigma) dissolved in ethanol.

Table 1. Patient characteristics.

Serial No	Age (years)	Gender	Mutation 1	Mutation 2
1	12	Female	HbE	IVS 1-nt1 (G/T)
2	27	Male	HbE	45kb Fil deletion
3	22	Female	HbE	45kb Fil deletion
4	19	Female	HbE	IVS 1-nt5 (G/C)
5	7	Male	HbE	Cds 41 / 42 (-TTCT)
6	20	Female	HbE	Cds 71 / 72 (+A)
7	4	Female	HbE	Hb Khon Kaen

The oxidative status of RBC was modulated by incubating RBC with hydrogen peroxide (H_2O_2). For ROS and GSH assays, RBC were first incubated for 1 hour either with or without 2mM H_2O_2, then stained with DCF for determination of ROS or with mercury orange for determination of GSH. To assay lipid peroxidation, RBC were labelled first with fluor-DHPE and then stimulated without or with H_2O_2.

RBC treated as above for the measurement of different oxidative parameters were analysed by a fluorescence-activated cell sorter, FACSCalibur™ (Becton Dickinson, San Jose, USA) using flow cytometric methods described previously by Amer *et al*[14]. The mean fluorescence channel (MFC) of the entire RBC population was calculated for ROS, GSH and lipid peroxidation by the FACSCalibur™ and CellQuest® software (Becton-Dickinson). The MFC of DCF- and mercury orange-stained cells was proportional to the ROS and GSH content, respectively, while that of fluor-DHPE- stained cells was reciprocal to their lipid peroxide content.

Quality of Life

Patients were given RAND 36-item Short Form Health Survey (SF-36) at the initiation and at the end of the study to assess for any change in their quality of life with FPP® treatment. SF-36 is a set of generic, coherent and easily administered quality-of-life measures. These measures rely upon self-reporting and are widely used by managed care organisations. The SF-36 includes questions on general health, physical functioning, role limitations due to physical health or emotional problems, energy level, emotional being, social functioning and pain. Each question is pre-assigned a score from 0 to 100 and a final score is calculated by adding up the individual scores, with a maximum score of 3,600. The questionnaires were administered in a standardised manner and were completed during the first and the last visits.

Statistical Analysis

SPSS v14.0 (for Microsoft Windows) was used for statistical analysis. Wilcoxon sum rank test was used to compare the pre and post treatment parameters. Values are reported as mean (SD). The confidence interval was set at 95% and $p<0.05$ was considered as significant.

RESULTS

Patient Characteristics

Seven patients were recruited in the study as illustrated in Table 1. There were 5 females (71%) and the median age of the study cohort was 19 years (range 4 to 27 years). All patients had HbE/β-thalassaemia and all had known β-thalassaemia mutations as shown in Table 1. The mean baseline Hb was 7.7g/dL (1). None of the patients had received blood transfusion within 10 weeks of the enrollment period.

Laboratory Parameters

Results of the parameters pre- and post-FPP® treatment are summarised in Table 2 (overleaf). There was no significant change in the Hb levels of these patients. The mean Hb was 7.7g/dL (1), at baseline, and 7.6g/dL (0.9), ($p=0.348$), after 12 weeks of FPP® treatment.

There was no significant improvement in the MCV after treatment with FPP®. The mean MCV values were 67.5fL (12.6) at baseline, and 68.4 fL (13.6),

Table 2. Changes in red blood cells parameters before and after treatment with fermented papaya preparation (FPP®) for 12 weeks.

	Pre FPP®	Post FPP®	*p* value
Mean ROS[†]-unstimulated (MFC[#])	119 (41.1)	65 (12.7)	0.018
Mean ROS[†]-stimulated (MFC[#])	369 (299.8)	141 (50.2)	0.028
Mean GSH[‡]-unstimulated (MFC[#])	114 (22.1)	218 (109.1)	0.028
Mean GSH[‡]- stimulated (MFC[#])	57 (15.8)	157 (56.5)	0.018
Mean lipid peroxide-unstimulated (MFC[#])	99 (16.3)	140 (19.6)	0.018
Mean lipid peroxide-stimulated (MFC[#])	57 (12.6)	69 (22.4)	0.237
Mean haemoglobin (g/dL)	7.7 (1)	7.6 (0.9)	0.348
Mean MCV[§] (fL)	67.5 (12.6)	68.4 (13.6)	0.398
Mean reticulocyte count (%)	6.3 (3.3)	5.8 (4.1)	0.237
Median QoL[¶]	2955 (2100-3265)	2950 (2200-3170)	0.866

[†]ROS: Reactive oxygen species
[#]Mean fluorescence channel
[‡]GSH: Glutathione
[§]MCV: mean corpuscular volume
[¶]QoL: Quality of life scores
Values are reported as mean (SD) or median (range)

(p=0.398) after 12 weeks. Reticulocyte counts did not decrease significantly as well. The mean reticulocyte count at baseline was 6.3 % (3.3), and after 12 weeks was 5.8 % (4.1), (p=0.237).

Cellular Parameters

ROS Assay

Following staining with DCF, the mean baseline MFC of the H_2O_2-unstimulated RBC was 119 (41.1). Stimulation with H_2O_2 increased the mean MFC to 369 (299.8). These results indicate a higher ROS generation in response to an oxidative stress (H_2O_2). After treatment with FPP® for three months, the mean MFC of the H_2O_2-unstimulated RBC decreased significantly to 65 (12.7), (p=0.018) and the mean MFC of H_2O_2-stimulated RBC decreased significantly to 141 (50.2), (p=0.028) (Fig. 1).

GSH Assay

The mean MFC of the unstimulated and H_2O_2-stimulated RBC were 114 (22.1) and 57 (15.8), respectively, at baseline, and 218 (109.1), (p=0.028) and 157 (56.5), (p=0.018), respectively, after treatment with FPP® which indicate a significantly higher production of intracellular GSH (Fig. 2).

Lipid Peroxidation

The mean MFC of the unstimulated and H2O2-stimulated RBC were 99 (16.3) and 57 (12.6), respectively, at baseline, and 140 (19.6), (p=0.018) and 69 (22.4), (p=0.237), respectively, indicating a decrease in lipid peroxides after treatment with FPP®.

Quality of Life

Quality of life (QoL) assessment by the SF-36 health survey did not show any significant change with FPP® treatment as shown in Table 2. Analysis of QoL scores by sub-headings also did not show any significant difference.

DISCUSSION

Oxidative damage has been associated with multiple health problems including metabolic, immunological and cardiovascular disorders as well as cancer[15]. There is evidence that antioxidants can ameliorate parameters of oxidative stress with consequent improvement in several clinical and laboratory parameters[16]. In thalassaemia, the initial abnormality is due to the impaired globin chain synthesis. However, the damage to various cellular components is at least partially mediated by oxidative stress[17]. GSH is considered an important scavenger of ROS and is a key component of the intracellular anti-oxidant system[14]. Initiation of lipid peroxidation by the oxygen-derived free radicals leads to oxidation of polyunsaturated fatty acids into lipid hydroperoxides. This is considered as a significant stage in the pathogenic process related to oxidative stress. FPP® ameliorated these

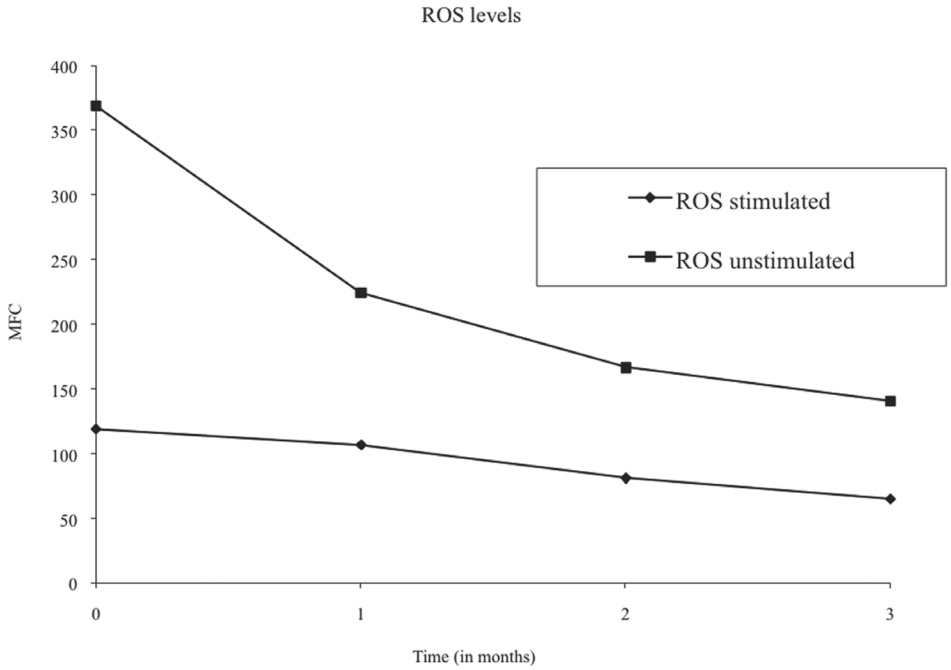

Fig. 1. Mean reactive oxygen species (ROS) levels in stimulated- and unstimulated-red blood cells after treatment with fermented papaya preparation®.

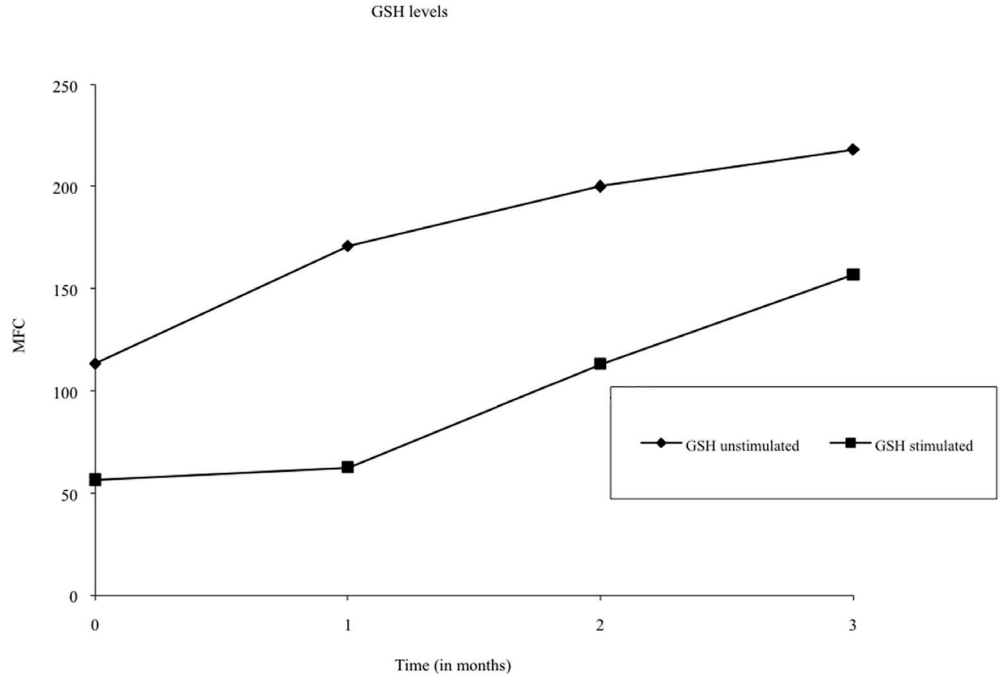

Fig. 2. Mean glutathione (GSH) levels in stimulated- and unstimulated-red blood cells after treatment with fermented papaya preparation®.

oxidative parameters in both unstimulated and hydrogen peroxide stimulated RBC[9].

However, despite the improvement in oxidative parameters, there were no significant improvements in the RBC indices (Hb, MCV or reticulocyte count), or in the quality of life.

One of the limitations of this study is the short study duration of 3 months. This period may not be sufficient to demonstrate an improvement in Hb concentrations and/or in quality of life. The dose of 6gm FPP® per day may not be enough and higher doses of FPP® may be required to show clinical improvement.

CONCLUSION

FPP® significantly reduced parameters of oxidative stress in RBC of 7 patients with HbE/β-thalassaemia and was well tolerated by the patients. However, there were no significant effects on Hb levels or in quality of life of these patients. Longer studies with larger sample size are needed to study the long-term clinical effect of FPP® before it is recommended for daily use for patients with thalassaemia intermedia.

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