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Non-invasive Sampling for Assessment of Oxidative Stress and Pro-inflammatory Cytokine Levels in Beta-Thalassaemia Major Patients

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Abstract

Background: Beta-thalassaemia (β -thalassaemia) major patients are severely anaemic and require life-long blood transfusions for survival. These patients require iron-chelation therapy as a result of iron overload due to the monthly blood transfusions. The iron over load can cause oxidative damage and pro-inflammation and therefore, hasten mortality. Thus, regular monitoring of the oxidative stress and pro-inflammation status may be useful in these patients.

Methods: Measurement of biomarkers is usually performed on serum samples but the evaluation in non-invasive samples such as saliva would be more favourable in paediatric cases. In this study, the levels of pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6) as well as oxidative indices such as lipid hydro peroxide, advanced oxidation protein products (AOPP), ferric reducing antioxidant power (FRAP), uric acid (UA) and glutathione peroxidase (GPx) activity in a total of 65 β -thalassaemia major patients (all on iron chelation) and 55 healthy control subjects were assessed. All the above biochemical parameters, measured using well established assay techniques, were detectable in saliva samples.

Results: Non parametric analyses showed that lipid hydroperoxide (LH) and glutathione peroxidase (GPx) activities were significantly higher in β -thalassaemia major patients. All other parameters were not significantly different between patient and control groups implying that iron chelation therapy was successful in attenuating oxidative stress. Strong positive correlation was observed between FRAP and UA levels. There was also a notable difference in tumour necrosis factor- α (TNF- α) between the patients and healthy controls when analysed according to ethnicity and age. AOPP level in β^+ -thalassaemia homozygous patients were significantly higher than β^+/β^0 -compound heterozygous and β^0 -thalassaemia homozygous patients.

Conclusion: Saliva may serve as a reliable, non-invasive sample which can be used to assess oxidative indices and pro-inflammatory cytokines in β -thalassaemia major patients.

Keywords: β -thalassaemia major; saliva; oxidative stress; biochemical analysis

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Introduction

Beta-thalassaemia is a common genetic disorder in the Mediterranean region and South East Asian countries including Malaysia. It is estimated that 4.5% of Malaysians carry β -globin gene mutations (1). More than 3,000 patients registered with the Malaysia Thalassaemia Registry were identified with HbE/ β -thalassaemia and β -thalassaemia major (2). Beta thalassaemia is caused by mutations in the β -globin gene complex (3). The β -globin gene mutations cause a reduction or zero synthesis of β -globin chains. The reduction of β -globin chain production results in increase of free α -globin chains and α -globin chain toxicity. These will lead to erythrocyte membrane fragility and destruction of erythrocytes (4). β -thalassaemia carriers can be slightly anaemic or asymptomatic while β -thalassaemia major children are severely anaemic. Beta-thalassaemia major patients require life-long blood transfusion to maintain their haemoglobin levels higher than 9.5g/dl (5). However, rapid destruction of erythrocytes and hypertransfusion lead to iron overload. Even though iron chelation therapy is carried out, iron overload which leads to oxidative stress still poses a problem in thalassaemia major patients (6, 7). Several studies have reported the presence of oxidative stress in β -thalassaemia major patients (6-8). However, these reports used blood samples for analysis which involved invasive sampling procedures. To date, there is a lack of reports on the assessment of oxidative stress indices in β -thalassaemia major using non-invasive samples. Non-invasive sampling has gained attention due to its simplicity and convenience. Non-invasive sampling can be readily performed with minimal supervision and potential risks of adverse effects such as haematoma and nerve injury due to phlebotomy can also be avoided (9).

Non-invasive samples such as saliva have been previously used for biochemical assays (10-12). A published report showed significant cor-

relation between analytes present in saliva and serum samples (13). Subsequent reports have successfully compared the antioxidant indices and cytokine levels between healthy individuals and patients of various disorders using non-invasive samples (12-15). Saliva samples may serve as an alternative sample for biochemical assays especially when plasma samples cannot be obtained from subjects. It is expected that the use of non-invasive samples will be well accepted, especially by paediatric patients and parents. The success of using saliva samples in monitoring oxidative stress and pro-inflammatory status in β -thalassaemia major patients may provide a good alternative platform to determine effective iron chelation therapy in these patients.

Materials and Methods

Sixty-five β -thalassaemia major patients with ages ranging from 5 to 25-years old were recruited during their monthly blood transfusions in University Malaya Medical Centre (UMMC). These patients were categorized according to gender (25 males and 40 females), age groups [below 10-years ($n = 20$), 11 – 20-years ($n = 34$) and above 20-years ($n = 11$)], ethnicity [Malay ($n = 37$), Chinese ($n = 20$) and Others (consist of Indian and patients with interracial marriage in their ancestry, $n = 8$)], and genetic classification of β -thalassaemia major (β^+/β^+ , $n = 9$), β^+/β^0 -compound heterozygotes ($n = 39$) and β^0/β^0 -thalassaemia homozygotes (β^0/β^0 , $n = 17$) (16). Age-matched healthy controls ($n=55$) were also recruited from the public. Smokers and individuals with severe anaemia due to other causes, serious and chronic diseases and under drug treatment were excluded. Body mass index (BMI), defined as weight in kilograms divided by the square of height in meters was determined for each participant. BMI values of ≥ 28.0 for men and ≥ 28.8 for women were considered as obese as per the published criteria of the World Health Organization (WHO)

(17). The Medical Ethics Committee (MEC) of University Malaya Medical Centre (UMMC), (MEC Ref. No: 727.1) approved this study, and conducted as per the Guidelines and Declaration of Helsinki. Written and oral consents were obtained from all patients and healthy controls or their legal guardians. Participation in this study was on a voluntary basis.

Subjects were asked to rinse their mouth with water prior to sample collection. Subsequently, the subjects expectorated at least 5 mL of their saliva into wide mouth 50 mL centrifuge tubes. The samples were subjected to centrifugation at $2,500 \times g$ for 10 minutes at 4°C . Aliquots of the resulting supernatant was stored at -80°C immediately. Prior to performing the assays, the sample aliquot was thawed once and centrifuged at $10,000 \times g$ for 10 minutes at 4°C to remove mucin. Five oxidative indices and two cytokines were measured using the saliva samples. All samples were assayed in triplicates.

The total non-enzymic antioxidant level was measured using Ferric Reducing Antioxidant Power (FRAP) assay. The measurement was based on the reduction of ferric-2,4,6-tripyridyl-triazine complex (Fe^{3+} -TPTZ) to the ferrous form (Fe^{2+} -TPTZ). The standard curve obtained using ferrous sulphate heptahydrate was used to calculate the FRAP value (18).

The advanced oxidation protein product level (AOPP) was estimated by measuring the absorbance of the samples mixed with potassium iodide in acidic condition. Chloramine-T was used as standard to calculate the concentration (19).

The level of lipid peroxidation was estimated by measuring lipid hydroperoxide concentration (LH). Samples (containing malonaldehyde) were incubated at 45°C with methyl-phenyl indole and hydrochloric acid and the resulting coloured complex was measured spectrophotometrically at 586nm. The malonaldehyde present in the samples was quantitated using 1,1,3,3-tetraethoxypropane as the standard (20).

Estimation of glutathione peroxidase activity (GPx) was performed using Glutathione Peroxidase Assay Kit (Cayman Chemical Company, MI, Cat: 703102). Measurement of uric acid concentration was performed using Uric Acid Fluorometric Assay Kit (BioVision Inc, CA, Cat: K608-100). The cytokines tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6) concentration was measured using ELISA MAX Deluxe kits (Bio Legend, CA, Cat: 43204 and 43504, respectively).

Statistical analysis

Statistical analysis was performed using SPSS version 15 (SPSS Inc., Chicago, USA). Data shown are expressed in mean \pm SEM unless indicated otherwise. Box and whisker plot was used to show distribution of salivary GPx activity and pro-inflammatory cytokine levels in β -thalassaemia patients. The significance of differences between means at a confidence limit of 95%, set at $P < 0.05$, was assessed using one-way analysis of variance (ANOVA), followed by Tukey-Kramer test for multiple variable comparison. On the other hand, the significance of differences between median was set at $P < 0.05$ and assessed using Mann-Whitney U test. Comparison of parameters according to demographic distribution of β -thalassaemia major patients was carried out using Kruskal-Wallis H test. A Mann-Whitney U test with a significance level adjusted by a Bonferroni correction (formula = $\alpha/\text{number of test}$) was conducted as a post-hoc test.

Results

Comparison of salivary oxidative indices and cytokine levels, between β -thalassaemia major patients and healthy controls.

In the present study, parametric analysis showed that the mean salivary LH level and GPx activity were significantly higher in the β -thalassaemia patients compared to healthy control subjects

(Table 1). The mean salivary LH level was elevated approximately by 3-fold ($p < 0.05$) while GPx activity was increased by 60% compared with healthy controls ($p < 0.01$). However, non parametric analysis showed that the enzyme activity was increased by 35% ($U = 781.50$, $p < 0.01$). (Figure 1)

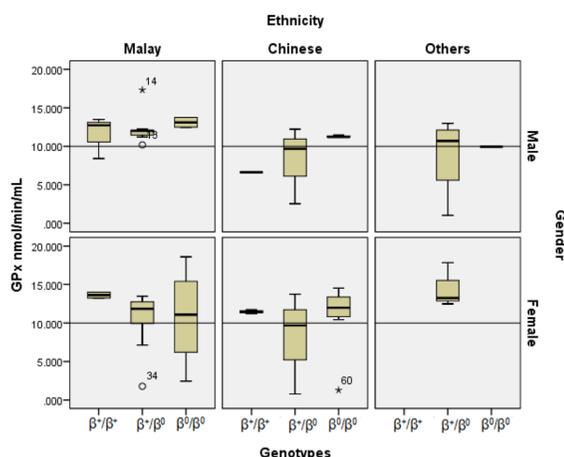


Figure 1. Whisker plot analysis of salivary GPx activity in β -thalassaemia patients compared to healthy control. Salivary GPx was measured using Glutathione Peroxidase Assay Kit. Data represent triplicate assays for samples from patients ($n=65$) and healthy control ($n=55$). Level of significance was set at $p < 0.05$.

By assuming the data (Table 2) distribution was normal, there was a highly significant difference in salivary TNF- α level between the ethnic groups ($F(2,62) = 6.17$, $p < 0.01$) and age groups ($F(2,62) = 3.56$, $p < 0.05$). From the Tukey's post-hoc test, the level of salivary TNF- α was higher in patients from other ethnicity (19.42 ± 5.54 pg/mL) compared with Malay ethnicity (7.81 ± 1.03 pg/mL). The level of salivary TNF- α was also significantly higher in the patients aged between 11 – 20-years (12.86 ± 1.92 pg/mL) compared with patients aged above 20-years (4.32 ± 1.01 pg/mL). Using non-parametric statistical analysis, significant difference was observed in AOPP level depending on the

genetic classification of β -thalassaemia, ($\chi^2(2) = 6.38$, $p < 0.05$). The Mann-Whitney U test with Bonferroni correction was conducted on this parameter with significance level set to $p < 0.017$ as a post-hoc test. No significance of AOPP level was observed between β^+ -thalassaemia homozygotes patients and β^+/β^0 -compound heterozygotes patients ($U = 90.00$, $p = 0.02$), β^+ -thalassaemia homozygous patients and β^0 -thalassaemia homozygous patients ($U = 39.00$, $p < 0.04$) and between β^+/β^0 -compound heterozygous patients and β^0 -thalassaemia homozygous patients ($U = 269.00$, $p < 0.27$).

TNF- α and IL-6 level were significantly different between the age groups ($\chi^2(2) = 7.74$, $p < 0.05$) and ethnicity ($\chi^2(2) = 6.27$, $p < 0.05$). Box and whisker plot of salivary TNF- α & IL-6 distribution showed that, TNF- α (Figure 2A) and IL-6 (Figure 2B) levels were significantly lower in patients aged above 20-years (sub-group B) compared with patients aged below 10-years (sub-group A) ($U = 52.00$, $p < 0.05$) and patients aged between 11 – 20-years (sub-group C) ($U = 87.00$, $p < 0.01$). The pro-inflammatory cytokine levels were also higher in β -thalassaemia major patients from Malay ethnicity compared with Chinese and other ethnicity ($U = 81.50$, $p < 0.05$).

Correlation analysis between oxidative stress indices and cytokine levels in the β -thalassaemia major patients

Significant correlations were observed between FRAP/UA ($r = 0.70$, $p < 0.01$), AOPP/IL-6 ($r = 0.31$, $p < 0.05$), LH/UA ($r = 0.26$, $p < 0.05$) and UA/TNF- α ($r = 0.29$, $p < 0.05$). Using non-parametric statistical analysis, significant correlations were observed between FRAP/UA ($r_s(63) = 0.72$, $p < 0.01$), FRAP/AOPP ($r_s(63) = 0.43$, $p < 0.01$), AOPP/UA ($r_s(63) = 0.25$, $p < 0.05$), LOOH/IL-6 ($r_s(63) = -0.25$, $p < 0.05$) and UA/TNF- α ($r_s(63) = -0.25$, $p < 0.05$).

Table 1. Comparison of salivary oxidative stress indices and cytokine levels between β -thalassaemia major patients and healthy controls

Parameters (unit)	β -thalassaemia major patients						Healthy controls					
	Mean ^a	SD	SEM	Percentile		Mean ^a	SD	SEM	Percentile			
				25%	50% ^b	75%			25%	50% ^b	75%	
FRAP (μ M)	208.29	137.94	17.11	107.92	184.76	271.30	99.24	13.38	141.79	197.50	246.55	
AOPP (μ M)	120.98	158.08	19.61	16.77	71.51	154.32	117.31	15.82	43.64	76.67	121.51	
LH (μ M)	0.68*	1.58	0.20	0.12	0.26	0.41	0.19	0.03	0.12	0.19	0.37	
GPx (nmol/min/mL)	10.93**	3.67	0.46	10.08	11.72**	12.81	4.84	0.65	0.76	8.66	10.95	
UA (nmol/mL)	101.76	65.59	8.14	47.73	97.26	132.84	42.23	5.69	57.90	82.72	120.32	
TNF- α (pg/mL)	10.65	9.60	1.19	4.49	7.13	14.69	6.62	0.89	5.00	7.98	14.52	
IL-6 (pg/mL)	28.47	32.86	4.08	8.09	18.24	32.54	35.06	4.73	9.58	13.89	23.16	

^a Unpaired t-test

^b Mann-Whitney U test

* $p < 0.05$ indicates significant difference between groups

** $p < 0.01$ indicates significant difference between groups

Table 2. Correlation analysis between salivary oxidative stress indices and cytokines in β -thalassaemia patients^{a, b}
Correlation analysis between parameters using Pearson's product-moment correlation

<i>r</i>	FRAP	AOPP	LOOH	GPx	UA	TNF- α
AOPP	0.24					
LOOH	0.06	-0.09				
GPx	0.06	0.10	0.06			
UA	0.70**	0.02	0.26*	0.09		
TNF- α	-0.15	0.08	-0.17	-0.16	-0.29*	
IL-6	0.10	0.31*	-0.06	0.08	0.02	0.14

(ii) Correlation analysis between parameters using Spearman's rank order correlation

<i>r</i> _s	FRAP	AOPP	LOOH	GPx	UA	TNF- α
AOPP	0.43**					
LOOH	0.17	-0.05				
GPx	0.14	0.19	0.13			
UA	0.72**	0.25*	0.19	0.14		
TNF- α	-0.19	0.09	-0.21	-0.17	-0.25*	
IL-6	0.06	0.22	-0.25*	0.01	0.10	0.13

^a Pearson's product-moment correlation

^b Spearman's rank order correlation

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

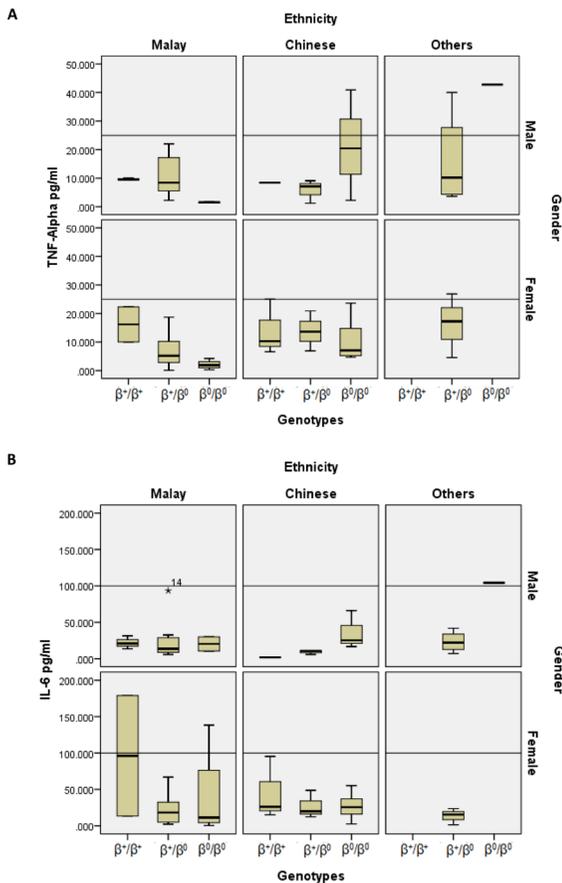


Figure 2. Whisker plot analysis of salivary pro-inflammatory cytokines (A) TNF- α and (B) IL-6 levels in β -thalassaemia patients compared to healthy control. Salivary pro-inflammatory cytokines (A) TNF- α (B) IL-6 levels were measured using commercial double-antibody sandwich ELISA. Data represent triplicate assays for samples from patients (n=65) and healthy control (n=55). Level of significance was set at $p < 0.05$. Pro-inflammatory cytokines (A) TNF- α (B) IL-6 levels were higher in β -thalassaemia major patients from Malay ethnicity compared with Chinese and Indian ethnicity.

Discussion

The present study demonstrated that oxidative stress indices and pro-inflammatory cytokines were successfully measured using saliva samples. The enzyme GPx is one of the numerous

enzymatic antioxidants preventing erythrocyte breakdown by hydroxyl radicals (21). The radicals are able to oxidize polyunsaturated lipids such as those found in the cell membrane, forming LH (22). The lipid hydroperoxides serve as substrates for selenoperoxidases which include GPx and therefore, the concurrent increase of these two parameters are expected. On the other hand, non-parametric statistical analysis showed a lack of significant difference in LH (indicator of lipid damage) and AOPP (indicator of protein damage) but an increase in GPx activity was evident when compared between patient and healthy control groups. This suggests enzymatic antioxidant was active in defending against free radical induced oxidative damage in these patients.

Although UA was usually associated with oxidative stress, it also possesses antioxidant ability (23, 24). This could be the factor affecting the relationship between UA and AOPP, LH and TNF- α .

Increase in AOPP level was reported to be associated with monocyte activation (25). The increase in monocyte activation may have led to cellular damage and increased amount of purine and UA. UA, in turn may also have help in attenuating protein oxidation by preventing protein nitration as reported by an earlier publication (26). This could be the possible explanation of positive moderate correlation between UA/AOPP using non-parametric analysis. In aspect of UA/LH relationship, the increase in LH suggests the presence of cellular damage or lysis, which increases the amount of DNA and UA due to purine metabolism released in the plasma and saliva, indirectly (27). As UA is also able to prevent further lipid peroxidation, this may help in attenuating LH production (28).

Recent experimental evidence suggests that excess plasma UA is able to induce inflammatory cytokines including TNF- α in medical con-

ditions (29). The salivary UA provides minimal contribution in reducing salivary TNF- α production. The attenuation of protein oxidation and lipid peroxidation may be attributed to UA (antioxidant) and this could prevent more cellular damage and reduce pro-inflammatory cytokine production. The non-parametric statistical analysis in the present study showed that reduction of LH provided a weak induction to increase the IL-6 secretion (30). IL-6 has been reported to exhibit anti-inflammatory properties as well, by preventing further destruction of epithelial cells (31). This is possibly due to the IL-6 ability to induce cells to enter mitosis (32) and therefore, one should not rule out the possibility that the decreased LOOH level could favour IL-6 to induce cell proliferation.

The level of oxidative indices may differ depending on the ethnicity as observed in previous investigations (33, 34). Some minor differences in the food intake preferences may exist among the different ethnic groups in Malaysia and this may indirectly affect the level of antioxidants especially in the current patient cohort.

In the present study, the TNF- α level compared between ethnicities showed that the low sample size (8 of 'other' vs 37 of Malays) might have played a major role in the observation. Both parametric and non-parametric statistical analyses are sensitive to a huge difference in sample size. The duration of treatment and compliance towards chelation therapy could be the factor affecting the cytokine level between age groups. Poor compliance towards chelation treatment will not remove the accumulated iron adequately and thus, cause complications in patients (35). Due to inadequate removal of excess iron, redox imbalance still occurs and patients may still be under oxidative stress.

External factors which are not assessed such as co-inheritance of α -globin gene mutations and polymorphisms such as *XmnI* which are able to

reduce the amount of free α -globin chains may have helped in reducing the oxidative stress severity (36). Salivary fluid component comprised of gingival crevicular fluid also contains serum exudates. This fluid was further mixed with other fluids from salivary glands and bronchial and nasal secretions (37) which could result in analyte dilution. Therefore, it is possible that the concentration or activity of the salivary oxidative indices and cytokine were lower compared with findings reported with plasma levels (38).

A previous study on β -thalassaemia major patients attending monthly blood transfusion in UMMC showed that iron overload and oxidative stress were still observed in the patients although chelation therapy was prescribed (6). However, the patients recruited in the previous study were on single iron chelation therapy using deferoxamine (DFO). In the present study, 23% of the patients had undergone combination iron chelation treatment involving combination of DFO and deferiprone (DFP), DFO and deferasirox (DFX) or DFP and DFX.

Published data has reported better compliance with combination chelation therapy (39) and the patients also showed improvement in endocrine glands' and cardiac functions. In addition, clinical observation also reported that DFX alone may reduce oxidative stress in transfusion dependent patients (40). These factors could be the contributors to the improvement of the oxidative stress status in β -thalassaemia major patients. Compared with diets of the healthy controls, 48% of the β -thalassaemia major patients were prescribed with supplementary vitamins such as Vitamins C, D, B12 and folic acid. Vitamins such as ascorbic acid may contribute to the estimation of total non-enzymic antioxidant status/level by FRAP assay (41, 42). The supplement may have helped increase the antioxidant ability in β -thalassaemia major patients to the level almost similar to healthy controls.

Conclusion

In this study, the measurement of oxidative stress indices and cytokine levels of β -thalassaemia major patients and healthy controls could be successfully carried out using non-invasive saliva samples. The analytes of interest were within detectable range and could be measured using in-house methods and commercial assay kits. The oxidative indices among patients were mostly comparable to healthy controls probably due to better compliance towards chelation therapy, combination chelation therapy and good patient management.

Disclosures: None

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Authors Contribution

MRAR performed the biochemical assays and prepared the draft of the article. URK and JAMAT designed this study and revised the article draft for correction. URK and MRSR revised the statistical analysis for this article. All authors have read and approved the final version of this manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

Abbreviations

(AOPP) advanced oxidation protein product; (β -thalassaemia) beta-thalassaemia; (β -globin) beta – globin; (BMI) Body mass index; (DFO) deferoxamine; (DFX) deferasirox; (FRAP) Fer-

ric Reducing Antioxidant Power; (GPx) glutathione peroxidase; (IL) Interleukins; (LH) lipid hydro peroxide; (TNF- α) tumor necrosis factor-alpha; (UA) uric acid; (UMMC - HEC) University of Malaya Medical Centre – Human Ethical Committee; (WHO) World Health Organisation.

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