

ORIGINAL ARTICLE

# Erythrocyte Oxidative Damage in Chronic Fatigue Syndrome

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**Background.** It has been hypothesized that a link exists between erythrocyte metabolism (particularly redox metabolism) and erythrocyte shape and that both are related to erythrocyte deformability. The aim of this research is to confirm the results of earlier studies and to investigate a correlation between erythrocyte morphology and erythrocyte oxidative damage in chronic fatigue syndrome (CFS).

**Methods.** Reduced glutathione (GSH), malondialdehyde (MDA), methemoglobin (metHb) and 2,3-diphosphoglyceric acid (2,3-DPG) were measured in 31 patients suffering from CFS and 41 healthy control subjects. Scanning electron microscopic studies of the erythrocytes from both groups were also carried out.

**Results.** There was evidence of oxidative damage in CFS with statistically significant increases in 2,3-DPG ( $p < 0.05$ ), metHb ( $p < 0.005$ ) and MDA ( $p < 0.01$ ). The CFS patients in this study also had significantly more stomatocytes in their blood than the normal subjects ( $p < 0.005$ ).

**Conclusions.** There is a strong likelihood that the increase in erythrocyte antioxidant activity is associated with the presence of stomatocytes. The results of this study provide further evidence for the role of free radicals in the pathogenesis of CFS and a link between erythrocyte metabolism and erythrocyte shape. © 2007 IMSS. Published by Elsevier Inc.

**Key Words:** Erythrocyte, Morphology, Biochemistry, Free radicals, Oxidative damage.

## Introduction

A role of free radical scavenging for erythrocytes has previously been demonstrated, which is additional to their established role of gas exchange (1–7). It has also been shown that patients suffering from chronic fatigue syndrome (CFS) had significantly different erythrocyte profiles compared to controls with erythrocyte distribution width (RDW) being the primary regression factor differentiating these groups (8). Erythrocyte shape changes have been described in rheumatoid arthritis, multiple sclerosis and particularly CFS (9–14). It is proposed that in carrying out their role of free radical scavenging, erythrocytes become damaged by oxidation, which consumes endogenous reducing substances (15). This damage then leads to shape changes and increased rigidity by alteration in the erythrocyte lipid

bilayer (peroxidation) and oxidation of labile groups in the proteins of the cytoskeleton. We investigated changes in erythrocyte antioxidant levels and morphological changes associated with CSF. Statistical significance was determined using Student's *t*-test with significance set at  $p < 0.05$ . To investigate erythrocyte oxidative damage, reduced glutathione (GSH), malondialdehyde (MDA), methemoglobin (metHb) and 2,3-diphosphoglyceric acid (2,3-DPG) were measured in patients suffering from CFS and healthy control subjects. Scanning electron microscopic (SEM) studies were also carried out on the erythrocytes from both groups. Full blood counts were also performed.

## Materials and Methods

### Ethics

This study was approved by the Ethics in Human Research Committee of Charles Sturt University. All subjects received an information sheet and consented to the procedure.

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### Participants

Patients with chronic fatigue syndrome were recruited through the Chronic Fatigue Syndrome Society, and their diagnosis confirmed by a clinician according to established criteria (16). Controls were, as far as possible, age- and gender-matched persons not from the patient's immediate family and not apparently suffering from any of the defined symptoms. Stipulating a large difference between groups (effect size = 0.8) and  $p < 0.05$  provided a power of 0.97 that we will obtain a significant result and reject our null hypothesis that no difference is seen for antioxidants between the CSF and control groups (17). All subjects were provided with a questionnaire addressing onset events and symptom incidence.

### Blood Sampling

Samples were taken from an antecubital vein by an experienced phlebotomist without trauma using a plastic 20 ml syringe and 19 g needle. Specimens were placed in appropriate anticoagulant commencing with the SEM fixative, immediately upon withdrawal of the needle.

### Methods

**Scanning Electron Microscopy (SEM).** Three drops of whole blood were added directly from the syringe to 5 ml of 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, within seconds of being withdrawn. Fixation was allowed to proceed for at least 24 h before processing. The cells were washed twice in cacodylate buffer, dehydrated with two washes in 70% ethanol, two washes in 95% ethanol, two washes in absolute ethanol and two washes in acetone. One drop of the cell suspension was applied to an acetone-washed coverslip and allowed to dry. These preparations were stored in a dessicator until microscopy could be performed. The coverslip was fixed to an aluminum stub using a colloidal silver adhesive and gold coated using a sputter coater (SPI). Electron microscopy was carried out using a Jeol Model JSM-840 scanning electron microscope at 15 keV. Images were digitally captured using the Oxford Isis EDS system (autobeam program) and saved to a CD-ROM. The cells in the digital images were examined macroscopically and classified according to their

shapes. The morphological classifications are represented in Figure 1.

Erythrocyte malondialdehyde (MDA), erythrocyte reduced glutathione (GSH), 2,3-diphosphoglycerate (2,3-DPG) and methemoglobin (MetHb) were measured as originally described (18–21).

Full blood count (FBC) was performed using a Sysmex SE 9500 hematology analyzer (Roche Diagnostics, Nuna-wading, Victoria, Australia) on an EDTA anticoagulated, whole blood specimen. Reticulocytes were stained with new methylene blue and counted microscopically. Results were expressed as a percentage of the 1000 cells counted.

### Statistics

Data were expressed as means  $\pm$  SD. Raw data expressed as percentages were arcsine transformed prior to analysis. Data were tested as to whether it was normally distributed using the Levene's test for homogeneity of variance. Student's *t*-test was used to assess categorical data between groups. ANOVA was used to analyze other parameters in the control and CFS groups;  $p < 0.05$  was considered statistically significant.

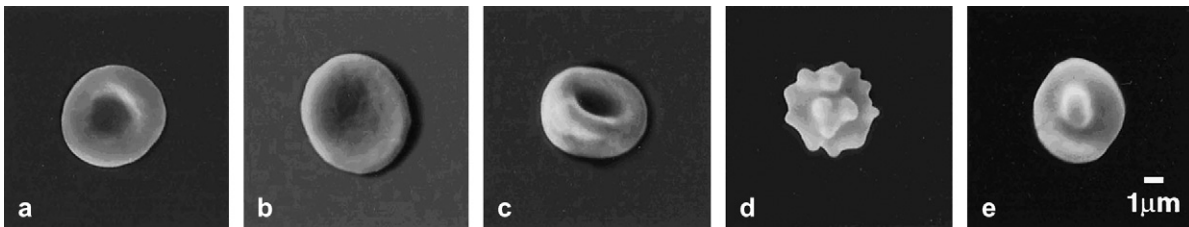
### Results

Thirty three patients (10 males and 23 females) were recruited together with 41 age- and gender-matched control subjects (18 males and 23 females). Mean age of the CFS group was  $41 \pm 29$  years and of the control group,  $40 \pm 25$  years.

There was no difference in any of the FBC parameters and reticulocyte count between the control group and the CFS group (Table 1).

Erythrocyte 2,3-DPG, MDA and methHb were significantly increased in the CFS group compared with the controls ( $p < 0.05$ , 0.01, and 0.005, respectively) (Table 2). Erythrocyte reduced glutathione showed no significant difference.

Stomatocytes were significantly increased in the CFS group compared with the controls ( $p < 0.005$ ). There was no significant difference between groups with respect to the other morphological classifications (Table 3).



**Figure 1.** Erythrocyte morphological subclasses. (a) biconcave disc, (b) leptocyte, (c) stomatocyte, (d) acanthocyte, and (e) burr cell.

**Table 1.** Means  $\pm$  SD of FBC parameters\*

		Hb (g/L)	Hct	MCV (fL)	RDW (%)	WCC ( $\times 10^9/L$ )	Reticulocytes (%)
Controls	Males ( $n = 18$ )	148.9 $\pm$ 11.9	0.43 $\pm$ 0.04	89.2 $\pm$ 4.3	12.9 $\pm$ 0.9	6.5 $\pm$ 1.4	1.35 $\pm$ 0.5
	Females ( $n = 23$ )	136.2 $\pm$ 8.8	0.41 $\pm$ 0.03				
CFS	Males ( $n = 10$ )	150.9 $\pm$ 9.8	0.44 $\pm$ 0.03	89.7 $\pm$ 3.2	12.6 $\pm$ 0.8	7.0 $\pm$ 2.0	1.25 $\pm$ 0.4
	Females ( $n = 23$ )	137.9 $\pm$ 6.4	0.41 $\pm$ 0.02				

FBC, full blood count; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; RDW, erythrocyte distribution width; WCC, white cell count.

\*No statistically significant difference for these parameters was noted between groups.

## Discussion

The causes of CFS are as yet undetermined, but studies have shown that certain infectious diseases, multiple nutrient deficiencies, food intolerance, or extreme physical or mental stress may trigger chronic fatigue. Oxidative stress is a term used to describe the body's prolonged exposure to oxidative factors that cause more free radicals than the body can neutralize. Free radicals are produced as a by-product of normal metabolic functions. Thus, antioxidants function as modulators of cellular homeostasis including detoxification of oxyradicals and metals as well as potent free radical scavenger. Loss of these mechanisms lead to muscular fatigue and myalgia associated with CFS (22,23).

There was no significant difference between the CFS group and the normal group as far as the FBC tests and reticulocytes were concerned. It has been reported previously that CFS patients had significantly different RDW than controls (8), but this could not be repeated here or in the earlier study (9). The reticulocyte results suggest that there is no reduction in erythrocyte lifespan in CFS.

Level of 2,3-DPG was significantly increased in the erythrocytes of the CFS group ( $p < 0.05$ ). Erythrocyte membrane function is regulated (at least in part) by 2,3-DPG, and it has been shown that the level of erythrocyte 2,3-DPG is important in determining deformability of erythrocyte membranes (24). Increased 2,3-DPG increases erythrocyte fragility and decreases deformability (25). Erythrocyte 2,3-DPG also functions as a regulator of hemoglobin oxygen affinity (26,27). Increased levels of 2,3-DPG have the effect of decreasing oxygen affinity and therefore would allow more oxygen to be delivered to the tissues in these cases. Thus, erythrocytes in CFS would be more rigid than normal and would be slow to recover from mechanical deformation. This would have the physiological effect of reducing oxygen delivery to the tissues, which may be com-

pensated for by the increased 2,3-DPG levels. Morphological studies of the CFS and control subjects demonstrated increased stomatocyte formation in CFS. This may be explained by the increased 2,3-DPG levels mentioned above and possibly by the increased oxidative markers described below.

Despite normal levels of reduced glutathione, which may have been due to the size of the sample, there were significantly increased levels of methemoglobin in CFS (mean = 1.26%) compared with controls (mean = 0.55%) ( $p < 0.005$ ), indicating oxidative damage to the hemoglobin molecule. Our power analysis provided evidence that we can expect a low probability of a type 2 error occurring, making our statistically significant results meaningful. The presence of the products of hemoglobin denaturation such as methemoglobin may cause damage to red cell membranes by oxidation of membrane sulfhydryl groups and peroxidation of lipids (28). Decreased deformability of red cell membranes can be demonstrated to be due to cross-linking of spectrin, a red cell membrane protein of major structural importance, caused by oxidative damage to sulfhydryl groups (29,30). Hemoglobin denaturation as a result of oxidation can result in the formation of Heinz bodies attached to the interior membrane surface, causing redistribution of major membrane components, viz., the anion channel, ankyrin and glycophorin (28). The anion channel and glycophorin are integral membrane proteins and ankyrin binds these to spectrin. Redistribution enhances IgG binding and reduces deformability (28).

Malondialdehyde, a product of lipid peroxidation, was significantly increased in patients with CFS ( $p < 0.01$ ). Peroxidation of erythrocyte membrane lipid in CFS is strong evidence that free radicals play a part in pathogenesis in at least some of the patients suffering from this syndrome. Peroxidation of membrane lipids with the formation

**Table 2.** Means  $\pm$  SD for erythrocyte antioxidants and oxidation products of CFS sufferers and controls

	2,3-DPG ( $\mu\text{mol/mL}$ )	GSH (mg/100 mL)	MDA (nmol/mL)	MetHb (%)
Controls ( $n = 41$ )	4.97 $\pm$ 0.99	73.10 $\pm$ 8.26	27.18 $\pm$ 14.29	0.55 $\pm$ 0.71
CFS ( $n = 33$ )	5.82* $\pm$ 0.98	74.33 $\pm$ 10.44	39.98* $\pm$ 26.64	1.26* $\pm$ 1.46

CFS, chronic fatigue syndrome; GSH, glutathione; MDA, malondialdehyde; MetHb, methemoglobin.

\*Significantly different from the control population ( $p < 0.05$ ).

**Table 3.** Erythrocyte morphology (SEM) (means  $\pm$  SD for the percentage of morphological subclasses in CFS and controls)

	Biconcave discs (%)	Leptocytes (%)	Burr cells (%)	Stomatocytes (%)	Acanthocytes (%)
Controls ( $n = 41$ )	78.5 $\pm$ 9.9	9.9 $\pm$ 4.0	4.2 $\pm$ 2.8	6.9 $\pm$ 4.2	0.3 $\pm$ 0.6
CFS ( $n = 33$ )	66.9 $\pm$ 7.4	12.5 $\pm$ 5.4	7.6 $\pm$ 3.8	12.8* $\pm$ 4.9	0.3 $\pm$ 0.4

\*Significantly different from the control population ( $p < 0.005$ ).

of malondialdehyde on the surface of red cells causes recognition by macrophages, which may lead to damage by phagocytosis (31). While our method for MDA is a manual spectrophotometric technique and high performance liquid chromatography is considered more sensitive, this did not seem to affect our results significantly.

There is evidence that erythrocytes undergo oxidative changes in conditions where free radical formation is known to be high such as rheumatoid arthritis (9) in hemodialysis (32,33) and in diabetes (34). That similar changes occur in CFS suggests that free radical formation is also a contributor to the pathology of this condition. This study confirms the presence of oxidative damage in CFS. This oxidative damage is most likely a contributor to the biochemical and morphological changes in CFS, which may result in decreased erythrocyte deformability and alter rheology and which would contribute to the clinical symptoms of chronic fatigue among others in this condition.

Further research that has recently commenced in our laboratory will determine whether there is a correlation between erythrocyte form and antioxidant levels. In addition, determination of cysteine levels and other molecules that affect erythrocyte membrane transport and antioxidant status needs to be determined.

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