ANTIOXIDANTS IN PATIENTS WITH DENGUE VIRAL INFECTION

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Abstract. An alteration in the oxidation/reduction (redox) status of humans infected with virus infections may contribute to the pathogenesis and clinical manifestations of the disease. Alterations in redox markers begin prior to the onset of clinical symptoms, suggesting early changes in the oxidant/antioxidant balance. Early identification of redox markers may be of clinical usefulness in the management of patients with dengue virus infection. We conducted a hospital based comparative cross sectional study of 55 patients serologically confirmed to have dengue infection and 55 clinically healthy age and sex matched subjects as controls to assess oxidative stress in acute dengue virus infection. Blood samples were drawn on the fifth day after symptom onset and analyzed for Trolox equivalent antioxidant capacity (TEAC), reduced glutathione (GSH), glutathione peroxidase (GPx) and paraoxonase (PON) activity. The results showed significantly lower levels of plasma TEAC, serum PON and erythrocyte GSH and GPx activity among dengue patients than in controls. Of the antioxidants investigated, PON appeared to be the most sensitive marker of oxidative stress in dengue virus infection. Serum PON may be a potentially useful marker of oxidative stress in patients with dengue virus infection.

Keywords: dengue, Trolox equivalent antioxidant capacity, reduced glutathione, glutathione peroxidase, paraoxonase

INTRODUCTION

Dengue virus is a major cause of mosquito borne diseases worldwide, with a thirty fold increase in incidence during the past 50 years (WHO, 2013). Dengue infections can be caused by any of four closely related dengue viral serotypes (Callaway, 2007; Weave and Vasilakis, 2009). The initial, or primary, infection may cause symptoms from mild to severe. Subsequent infections with other serotypes, called secondary dengue infection, may lead to more severe disease (Malavige *et al*, 2012), such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). However, the pathogenesis of dengue virus infection and the factors that

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lead to severe clinical disease are poorly understood.

The severity of dengue infection varies widely, suggesting immunopathological, genetic and virus type factors may play a role in disease severity (Gubler, 1998; Malavige *et al*, 2004 ; Dong *et al*, 2007; Mathew and Rothman, 2008). Cross reactive memory T cells and antibodies may also contribute to pathology by altering the cytokine profile during a secondary dengue infection (Strephen *et al*, 2002; Mathew and Rothman, 2008; Weave and Vasilakis, 2009). Several human HLA-class I and class II alleles, polymorphisms in the tumor necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β) genes have been associated with development of DHF and DSS (Chen *et al*, 2009). Host genetic factors, such as glucose 6-phosphate dehydrogenase (G6PD) deficiency, may also contribute to increased replication of dengue virus in monocytes, causing abnormal cellular oxidation/reduction (redox) equilibrium, suggesting cellular redox status plays a role in regulating virus replication and virulance (Nkhoma *et al*, 2009; Popovic-Dragonjig *et al*, 2011; Wang *et al*, 2012).

Oxidative stress may result when cellular antioxidant defense mechanisms are unable to keep pace with the detoxification of reactive oxygen species (ROS). Overproduction of ROS is neutralized by various mechanisms; glutathione peroxidase (GPx) has been reported to be a more sensitive antioxidant enzyme in dengue infection (Lizette *et al*, 2004). Alteration in redox markers probably commences before the onset of clinical symptoms, suggesting early compromise of oxidant/antioxidant balance (Raymond *et al*, 2009). Therefore, early identification of redox markers may be useful in managing patients with dengue infection. Oxidative stress markers, such as malonaldehyde (a lipoperoxide), reduced glutathione (GSH) and GPx have been studied to evaluate oxidative stress in dengue infections (Lizette *et al*, 2004). Most of these markers have been studied in the extra-cellular fluid of dengue patients. Extra cellular fluid concentrations of antioxidants may be altered plasma leakage (intravascular hypovolemia) in dengue patients, so measurement of intracellular (erythrocyte) concentrations of antioxidants may be more useful to study oxidative stress in dengue patients.

Many studies have shown the antioxidant properties of paraoxonase (PON) (Mackness *et al*, 1991; Raymond *et al*, 2009). PON is primarily of toxicological importance, where it is known to detoxify various organophosphates, but it also plays an important role in protecting low density lipoproteins (LDL) and high density lipoproteins (HDL) from oxidation, thereby preventing vascular inflammation and atherosclerosis (Mackness *et al*, 1991; Raymond *et al*, 2009). PON may confer protective action against oxidants. However, there are no published studies of PON activity during dengue infection. Therefore, we determined to compare the antioxidant activities of GPx and GSH in erythrocytes, serum PON and Trolox equivalent antioxidant capacity (TEAC) levels in patients with dengue infection with controls.

MATERIALS AND METHODS

Subjects and sampling

A hospital based comparative cross sectional study was carried out at the Medical Unit of the Colombo South Teaching Hospital, Sri Lanka during a dengue epidemic period in late 2012. The study sample consisted of 55 (25 males and 30

females) patients serologically confirmed (IgM against dengue antigen) to have dengue infection and 55 (25 males and 30 females) clinically healthy age and sex matched volunteers from the same community as controls. Blood samples were obtained from patients and controls during the same period. Blood samples from patients were obtained on day 5 after the onset of fever (Lizette *et al*, 2004).

Patients with renal disorders, diabetes mellitus or those taking antioxidant vitamin supplements were excluded from the study. The study protocol was approved by the Ethics Review Committee, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka. Written informed consent was obtained from all participants prior to collection of blood samples.

Collection of blood samples

Approximately 4.0 ml of venous blood sample was obtained from each participant who was not fasting. Two milliliters of blood was transferred into a heparin coated vial to examine for GPx, GSH and TEAC. The remaining aliquot was transferred into a serum separating vial to determine serum PON activity. Samples for the PON assay were stored at -20°C until analyzed. The GPx, GSH and total TEAC assays were performed within 3 hours of blood collection.

Laboratory analysis of samples

The TEAC assay was performed according to the method of Huang *et al* (2005). This assay was based on the reduction of 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid color intensity by antioxidants.

PON levels in the serum were measured using a arylesterase/paraoxonase commercial assay kit (ZMC Cat. No. 0801199 Zepto Metrix Corporation, Buffalo, NY). Arylesterase/paraoxonase catalyzes the cleavage of phenyl acetate resulting in phenol formation and the rate of formation of phenol was measured by monitoring the increase in absorbance at 270 nm.

Isotonic saline prewashed red blood cell GSH and GPx concentrations were measured. GSH levels were measured using the method of Lewis *et al* (2006) with colorimetry while GPx activity was measured using a Randox commercial assay kit (Ransel test kit, Randox Laboratoris, Crumlin, UK). Hemoglobin (Hb) concentration was measured to convert the units to GPx per gram of Hb following the method of Lewis *et al* (2006).

Serological diagnosis of acute dengue infection was made using the dengue IgM rapid solid phase immunochromatographic assay method (Bio Line Standard Diagnostics, Suwon, Korea). Only dengue IgM positive patients were included as study patients.

Statistical analysis

Analysis of data was performed using SPSS for windows version 17.0 (IBM; Armonk, NY). Since the probability-probability (PP) plot revealed the variables involved were not normally distributed, the Mann-Whitney *U* test was used to compare the means between the patient and control groups. Results were reported as median (IQR) and differences. A *p*-value <0.05 was considered significant. A Box-and-Whisker plot was used for comparing antioxidant activity between patients and controls.

RESULTS

There were no significant differences in variables between males and females. The antioxidant results for patients and controls are shown in Table 1. The medi-

Table 1
Median GSH, TEAC, PON and GPx levels in dengue patients and controls.

Antioxident	Dengue IgM positive patients (*n*=55) Median (Range)	Control Subjects (*n*=55) Median (Range)	*U*[a]	*z*	*p*-value
GSH (mg/l packed RBC)	32.5 (10.9-46.3)	37.9 (28.8-44.1)	1,021.5	-2.94	<0.05
TEAC (μg/ml)	29.1 (28.1-36.5)	36.3 (30.7-41.2)	54.0	-8.72	<0.001
PON (kU/l)	30.3 (16.0-37.6)	57.3 (45.6-61.3)	0.00	-9.04	<0.001
GPx (U/gHb)	33.6 (22.2-37.5)	35.9 (29.5-48.2)	686.0	-4.94	<0.05

[a]Results of the Mann-Whitney *U* test; GSH, reduced glutathione; TEAC, Trolox equivalent antioxidant capacity; PON, paraoxonase; GPx, glutathione peroxidase.

ans of all the parameters were significantly higher among controls than patients. However, the difference of the median antioxidant levels between patients and controls were higher for PON and this was followed by the medians for the TEAC, GPx and GSH.

Fig 1 shows the Box-and-Whisker plot of the antioxidant concentrations in patients and controls. The Box-and-Whisker plot showed a greater difference in TEAC and PON activity between patients and controls (patients had lower activity than controls) than in GSH and GPx levels, suggesting that of the biomarkers tested, PON was a more sensitive marker of oxidative stress in dengue infection.

DISCUSSION

Oxygen derived free radicals are abundant in all human cells, being produced via many enzymatic reactions. Low levels of ROS serve as signaling molecules for metabolic regulation (Messer *et al*, 2002). Under normal physiological conditions there is a balance between ROS generated by the body and the antioxidant defense system. When this balance is shifted in favor of ROS, the free radicals react with polyunsaturated fatty acids resulting in lipid peroxidation (De Silva *et al*; 1998). This ROS mediated peroxidation of membrane lipids can lead to cellular damage, endothelial injury and microvascular dysfunction (Udupi and Rice-Evans, 1992; Valero *et al*, 2002).

Virus infections may stimulate the generation of ROS in polymorphonuclear leukocytes through a direct interaction between the virus antigen and the plasma membrane of the phagocytes (Malavige *et al*, 2012) resulting in a disturbance of redox equilibrium that can lead to pathology in a variety of wide range of virus infections (Peterhans *et al*, 1987; Reis *et al*, 2008; Seet, 2009) including dengue infection (Raymond *et al*, 2009). Oxidative stress is higher during febrile stage (days 3-5) with dengue infection compared to the convalescent stage, suggesting alteration in redox markers may begin before the onset of clinical symptoms (Reis *et al*, 2008). The host inflammatory response appears to be an important contributor to the pathology in dengue infection. This concept motivated us to study changes in antioxidant activity during acute dengue infection.

Fluctuations in antioxidant enzymes levels in the blood and body fluids have been reported for many diseases (Canakci

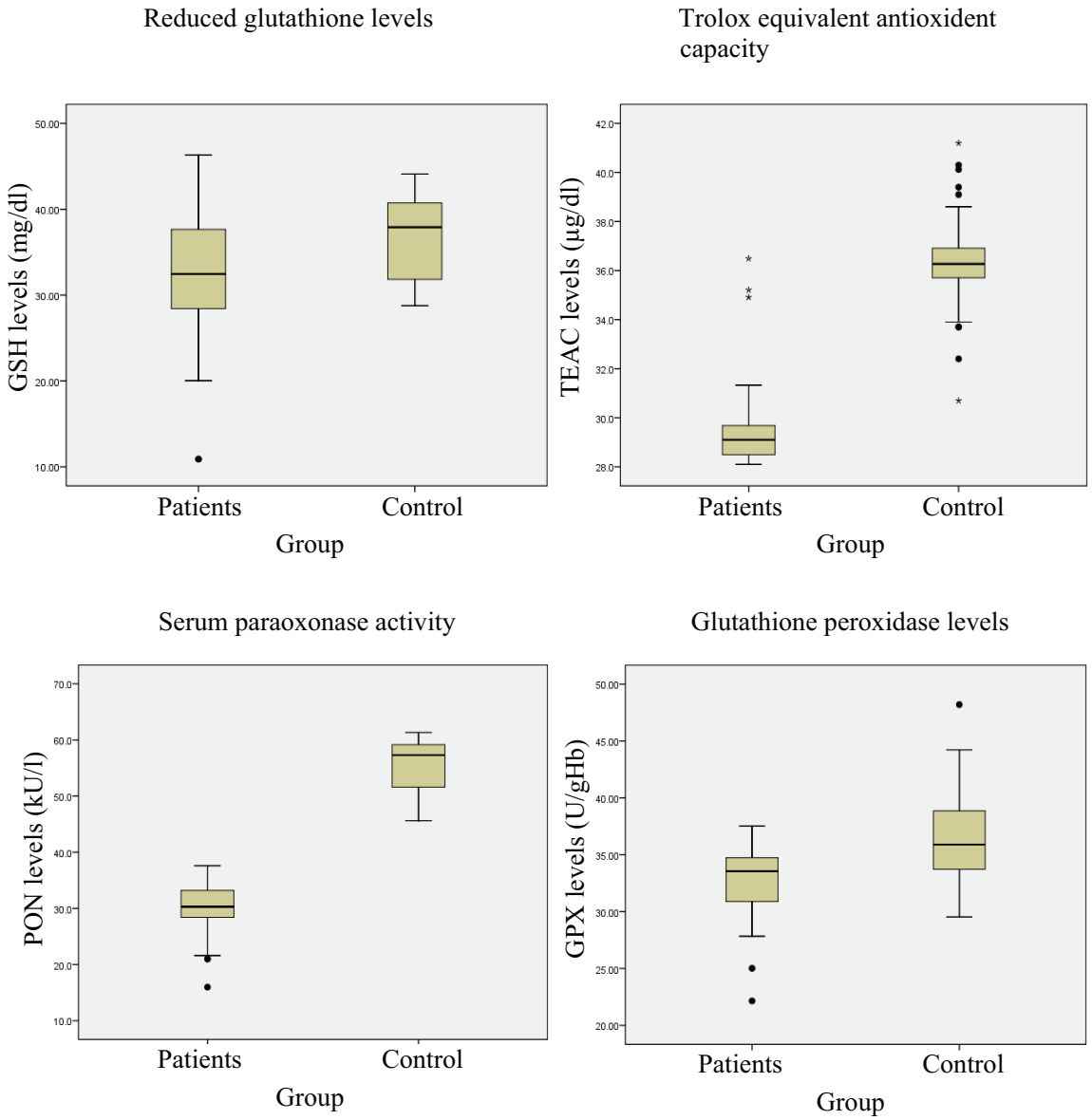

Fig 1–Comparison of antioxidant levels between dengue patients and controls.

et al, 2007). Redox disequilibrium is associated with pathology in cardiovascular (Chandrasena *et al*, 2009) and diabetes and cataracts (Chandrasena *et al*, 2006), where erythrocyte GPx activity was found to be a sensitive marker of oxidative stress. In the present study, a significantly lower level of all antioxidant (GPx , GSH, TEAC and PON) levels was observed in patients than in controls suggesting increased production of oxidants. This probably resulted in disruption of the redox balance, leading to lower levels of these antioxidants. These findings are consistent with a previous study (Lizette *et al*, 2004). However, the mechanisms linking these abnormalities

in redox equilibrium during dengue infection are yet clear. One study showed mosquito cells infected with dengue virus were capable of producing superoxide anions (Chen *et al*, 2011). Thus, the production of superoxide anions could be an explanation for the abnormalities redox balance in dengue infection.

Lower erythrocyte levels of GPx and GSH among patients in our study indicates superoxides formed by antioxidants of various thiol containing compounds (glutathione) result in decreased levels of GSH. This suggests there is increased production of hydrogen peroxide (H_2O_2). Although H_2O_2 is not an oxidative radical, it is readily converted to highly reactive hydroxyl radicals and utilization of reduced glutathione (GSH) in the presence of GPx may be a possible explanation for the lower levels of erythrocyte GSH and GPx in our patients. Replenishing intracellular GSH levels by providing N-acetylcystine, a thiol-containing compound that provides the GSH precursor cysteine, may be more effective in reducing oxidative stress in dengue virus infection (Udupi and Rice-Evans, 1992).

Our study showed significantly lower PON activity levels in dengue patients than control subjects. The difference in median antioxidant levels between patients and controls was greater for PON than the other antioxidants investigated, suggesting PON may be the most sensitive marker of oxidative stress during dengue infection. Studies have shown an inverse relationship between serum PON activity and malondialdehyde (MDA) production in patients with cardiovascular disease (Gil *et al*, 2004; Klassen *et al*, 2004; Seet *et al*, 2009) indicating PON may be involved in oxidative defense. Although the exact mechanism for PON is unclear, the reduction of PON activity in patients with dengue virus infection in our study may be due to the counteracting production of superoxide anion during the early phase of dengue infection. We believe this is the first study evaluating the association of serum PON with dengue infection.

In summary, our findings suggest a relationship between oxidative stress and the acute viral phase of dengue infection as indicated by the lower antioxidant activity of TEAC, PON, GPx and GSH. These alterations in redox markers indicate changes that may have begun early in the disease resulting in an alteration in oxidant/antioxidant balance. Of the antioxidants tested, PON appeared to be the most sensitive antioxidant marker during dengue infection. Serum PON levels may be a useful marker for assessing oxidative stress in the management of patients with dengue infection.

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