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A study of oxidative stress, cytokines and glutamate in Wilson disease and their asymptomatic siblings

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ABSTRACT

Background: Free copper in Wilson disease (WD) is toxic and may reduce antioxidant, increase oxidative stress marker and thereby cytokine release and excitotoxic injury, but there is paucity of studies in humans. We report oxidative stress markers, cytokines and glutamate in neurologic WD and correlate these with their clinical severity, laboratory findings and extent of Magnetic resonance imaging (MRI) changes.
Methods: 29 patients with neurologic WD and 9 asymptomatic WD siblings were included and their clinical, treatment history, disease severity, biochemical findings and MRI changes were noted. Glutathione (GSH), total antioxidant capacity (TAC) and malonodialdehyde (MDA) were measured by spectrophotometer, cytokines by cytokine bead array and glutamate by the fluorometer.
Results: In WD patients, the glutathione (mean ± SEM, 2.20 ± 0.06 vs. 2.73 ± 0.04 mg/dl, $P < 0.001$) and TAC (1.70 ± 0.03 vs. 2.29 ± 0.02 Trolox_Eq_mmol/l, $P < 0.001$) were reduced, and MDA and glutamate (23.93 ± 0.54 vs. 19.96 ± 0.27 μmol/l; $P < 0.001$) were increased (4.7 ± 0.11 vs. 3.03 ± 0.52 nmol/ml, $P < 0.001$) compared to controls. The serum IL6 {median (IQRs), 9.42(10.92) vs. 5.2(5.34) pg/ml; $P = 0.001$}, IL8 {12.37(10.92) vs. 5.63(5.52) pg/ml; $P < 0.001$}, IL10 {8.33(8.3) vs. 2.05(1.37) pg/ml; $P = 0.001$} and TNFα {6.14(8.95) vs. 3.61(3.58) pg/ml; $P < 0.001$} were also increased in WD patients compared to controls. These changes were more marked in the neurologic WD compared to asymptomatic WD and in the untreated compared to treated patients. TAC correlated with duration of illness, serum free copper, 24 hour urinary copper and serum ceruloplasmin, and glutamate with MDA, TNFα, ceruloplasmin and 24-hour urinary copper.
Conclusions: In WD patients, antioxidants are reduced and MDA, cytokines and glutamate are increased which are more marked in symptomatic neurologic WD than asymptomatic patients.

1. Introduction

Wilson disease (WD; OMIM #277900) is an autosomal recessive disease caused by mutation in ATP7B gene spanning more than 80 kb genomic DNA on chromosome 13q4.2–q21 (Thomas et al., 1995). ATP7B gene has 21 exons, encodes 1465 amino acids and its gene products help in transporting copper (Cu) into the secretary pathway for incorporation into apoceruloplasmin and excretion into the bile (Vrabelova et al., 2005). Mutation of ATP7B gene results in impaired trafficking of Cu in and through the hepatocytes resulting in excessive accumulation of Cu in various organs such as the liver, cornea, lens and brain.

In the blood, Cu is found in the bound and in the free states. In the bound state, Cu is covalently linked to ceruloplasmin, while free Cu in the blood loosely binds to albumin and other small molecules. The level of loosely bound Cu is increased in WD, although the total serum Cu and ceruloplasmin levels usually remain low (Ogihara et al., 1995). In a normal individual, free Cu is only 10–15%, which is greatly increased in WD (Chen et al., 2012). Free Cu level is highly toxic, can cross blood–brain barrier and induces oxidative damage to the brain tissue (Choi and Zheng, 2009). Since 1993, total antioxidant capacity (TAC) of a hydrophilic antioxidant is measured in biological samples to learn how the human body reacts to oxidative and nitrosactive injury. TAC measures hydrophilic antioxidants and is highly influenced by serum uric acid level (Bruha et al., 2011). Glutathione (GSH) is a water-soluble antioxidant and is widely expressed in most of the organs. Free Cu level in WD increases and there is a reduction in the

Abbreviations: ADL, activities of daily living; BFM, Burke–Fahn–Marsden; CLD, chronic liver disease; Cu, copper; EDTA, ethylene diamine tetra-acetic acid; GSH, glutathione; KF, Kayser–Fleischer; LPO, lipid peroxidation; MRI, magnetic resonance imaging; MDA, malonodialdehyde; MMSE, Mini Mental State Examination; ROS, reactive oxygen species; TAC, total antioxidant capacity; TNFα, tumor necrosis factor-alpha; IL, interleukin; WD, Wilson disease.

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level of glutathione and other antioxidants (Schulz et al., 2000; Attri et al., 2006; Nagasaka et al., 2006) hence, there is an increase in reactive oxygen species (ROS) and lipid peroxidation (LPO) (Lovell et al., 1995; Hensley et al., 2000; Halliwell, 2001; Dalgic et al., 2005; Schlief and Gitlin, 2006). ROS are known to activate NF-Kappa B which in turn activates growth factors and anti-apoptotic molecules resulting in excessive cell proliferation (malignancy), inflammatory cytokines and adhesive molecules (Karin and Greten, 2005). In WD, Cu induces oxidative stress and thereby cytokine and glutamate release, which may be responsible for cellular injury. Insignificantly higher levels of both inflammatory and anti-inflammatory cytokines in WD patients compared to controls have been reported (Goyal et al., 2008). In another study, TAC was reduced in WD patients compared to controls and was more marked in neurologic WD which was correlated with disease severity (Bruha et al., 2011). Evaluation of antioxidant, oxidative stress marker, cytokine and glutamate together may help in understanding the mechanism of cellular injury in WD. In the available medical literature, we could not find any study correlating these markers in neurologic WD. In this communication, we therefore report the antioxidant (glutathione and TAC), oxidative stress marker [Lipid peroxidation product; malonodialdehyde (MDA)], cytokines and glutamate levels in neurologic WD and correlate these with clinical, MRI and biochemical markers of severity of WD.

2. Subjects and methods

In this study consecutive patients with neurologic WD attending to neurology service of a tertiary care teaching hospital were included prospectively. The diagnosis of neurologic WD was based on clinical, Kayser–Fleischer (KF) ring on slit lamp examination, low serum ceruloplasmin (<20 mg/dl) and high urinary Cu (>40 μg/24 h) (Roberts et al., 2008). A detailed clinical evaluation including duration of neuropsychiatric symptoms, age at onset, and history of hepatitis was noted. Pedigree chart was prepared for every index patient and the siblings were screened by measuring serum ceruloplasmin, urinary and serum Cu, slit lamp examination for KF ring, liver function test and ultrasound abdomen. The siblings of neurologic WD patients were considered to have WD based on low serum ceruloplasmin (<20 mg/dl), high urinary Cu (>40 μg/24 h) and additional findings such as KF ring and evidence of liver dysfunction on biochemistry and/or ultrasound. DNA sequencing study for the diagnosis of WD is not available locally.

Mini Mental State Examination (MMSE) was used for the evaluation of cognitive functions (Crum et al., 1993). The severity of neurologic WD was based on activities of daily living (ADL) and the sum score of five signs (dysarthria, tremor, ataxia, rigidity/bradykinesia and chorea/athetosis). Each neurologic sign was given a score of 0 to 3; zero being none and three being severe. The severity was categorized into the grade-I (mild), grade-II (moderate, sum score of 2–7 and patient was independent for ADL) and grade-III (severe, the sum score of >7 and dependent for ADL) (Grimm et al., 1991; Kalita et al., 2010). The severity of dystonia was assessed using Burke–Fahn–Marsden (BFM) score (Krystkowiak et al., 2007). Liver dysfunction was considered if there was raised liver enzyme (transaminases), coagulopathy (deranged international normalized ratio/prolong prothrombin time) or ultrasound evidence of chronic liver disease (CLD).

2.1. Investigations

5-ml of venous blood was drawn from the antecubital vein in an ethylene diamine tetra-acetic acid (EDTA) and plain vial. Plasma was separated from the EDTA vial and serum from the plain vial and stored at −80 °C until analyzed. GSH, MDA and TAC levels were analyzed monthly whereas cytokine and glutamate levels were analyzed about one year later. Serum Cu and 24 hour urinary Cu levels were measured by an atomic absorption spectrophotometer. Blood counts, hemoglobin, blood sugar, blood urea nitrogen, serum creatinine, protein, albumin, calcium, alkaline phosphatase, bilirubin and transaminases were measured. Coagulation profile was also evaluated. Ultrasound abdomen was performed to evaluate evidence of CLD. Ultrasound evidence of CLD included decrease in liver size, coarse echotexture and irregular margin with or without raised portal vein pressure (Rumack et al., January 5, 2011). Serum ceruloplasmin was assayed by its oxidase activity with o-dianisidine dihydrochloride following the method of Schosinsky et al. (1974). The serum free Cu was calculated by subtracting three times the serum ceruloplasmin level (mg/dl) from the total serum Cu level (mg/dl). Cranial MRI was done using a 3 T MRI scanner and axial sections were obtained in T1, T2, and FLAIR sequences. The location of abnormal signal intensity and number of MRI lesions were noted.

GSH, TAC and MDA levels were measured in 38 WD patients and 64 healthy age (patients vs. controls − 16.9 + 5.9 vs. 17.4 ± 4.4 years; P = 0.78) and gender (M:F, 49:15; P = 0.63) matched controls by a spectrophotometer.

2.2. Glutathione (GSH) assay

Plasma GSH was measured by a spectrophotometer at 412 nm according to the method described by Tietze (1969). Plasma was added to 10% trichloroacetic acid (TCA) and allowed to stand at 4 °C for 2 h. This mixture was centrifuged at 2000 ×*g* for 15 min and the supernatant was added to Tris–HCl buffer (0.4 M, pH 8.9) containing EDTA (0.02 M). Finally 5,5-dithiobis {(2-nitrobenzoic acid) (DTNB) (0.01 M)} was added to the mixture which was reduced into the yellow product, 5-thio-2-nitrobenzoic acid (TNB) that was measured by spectrophotometer at 412 nm. A standard curve of reduced glutathione (GSH) was plotted to determine the amount of glutathione in the plasma sample.

2.3. Total antioxidant capacity (TAC) assay

Serum TAC was measured by the method described by Koracevic et al. (2001). In this method, the hydroxyl radical is produced by the Fenton reaction, reacts with benzoate resulting in the release of TBARS (thiobarbituric acid reactive substances), which is bright yellowish-brown in color. Upon addition of a serum sample, the oxidative reactions are initiated by the hydroxyl radicals present in the reaction mixture, and are suppressed by the antioxidant components of the serum, which prevents the color change and thereby provides an effective measure of the total antioxidant capacity of the serum. This was measured by spectrophotometer at 532 nm and the inhibition of color development was defined as TAC.

2.4. Lipid peroxidation (LPO) assay

LPO was measured by assessing malonodialdehyde (MDA) level, which is the end product of LPO (Janero, 1990). Plasma was mixed with EDTA, ascorbate (10 mM) and $FeSO_4$ (16.7 mM) and incubated at 37 °C for 60 min. The reaction was stopped by adding ice-cold 10% trichloroacetic acid (TCA). The mixture was centrifuged at 2000 ×*g* for 10 min and supernatant was aspirated. The supernatant mixed with equal volume of 0.67% thiobarbituric acid (TBA) and was kept in a boiling water bath for 15 to 20 min. MDA level was determined with the absorption coefficient of MDA–TBA complex at 532 nm using spectrophotometer.

2.5. Cytokine assay

Interleukin (IL-6, IL-8 and IL-10) and tumor necrosis factor-alpha (TNFα) were measured in serum using a flow cytometer with bead-based immunoassay (BD Biosciences, US). Cytokines were measured in 29 WD patients and 44 healthy age (patients vs. controls − 16.9 + 5.3 vs. 17.2 ± 4.4 years; P = 0.75) and gender (M:F, 22:33; P = 1.00) matched controls.

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