Elevated levels of glutathionyl haemoglobin as an oxidative stress marker in patients with major depressive disorder

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Background & objectives: Oxidative stress has been implicated in the pathophysiology of major depressive disorder (MDD), but biomarkers to assess oxidative stress in patients with MDD have yielded ambiguous results. Glutathionyl haemoglobin (GS-Hb) has been reported as a stable and potential biomarker for oxidative stress in various clinical conditions. The objective of the study was to evaluate GS-Hb as a potential biomarker of oxidative stress in patients with MDD through its quantification and to compare the levels of GS-Hb in age- and gender-matched healthy controls.

Methods: The levels of GS-Hb were estimated using liquid chromatography coupled to electrospray ionization mass spectrometry in patients diagnosed with MDD and in a subset of patients after six weeks of treatment with selective serotonin reuptake inhibitors (SSRIs).

Results: GS-Hb levels in drug-naïve patients with MDD (n=26) were significantly elevated compared to matched healthy controls (n=17). GS-Hb levels were not significantly different between MDD patients with and without co-morbid anxiety disorders. There were no significant differences in GS-Hb levels following six weeks of treatment with SSRIs compared to baseline.

Interpretation & conclusions: Compared to controls, GS-Hb level in patients with MDD was significantly elevated, suggestive of increased oxidative stress associated with MDD. However, six weeks of antidepressant treatment was not sufficient to modify the alterations in antioxidant/oxidant system. Further studies need to be done with a large sample of MDD patients with a longer duration of antidepressant treatment.

Key words Antidepressants - glutathionyl haemoglobin - major depressive disorder - mass spectrometry - oxidative stress

Increased oxidative stress has been implicated in the pathophysiology of major depressive disorder (MDD)¹. Major depression is a common psychiatric condition and considered as the fourth leading cause of disability worldwide². In addition, major depression is associated with chronic medical conditions including coronary artery disease and diabetes mellitus³.

Decreased levels of vitamin C⁴ and superoxide dismutase (SOD)⁵ and elevated levels of reactive pro-
oxidants have been reported in patients with major depression compared to age- and gender-matched healthy controls. Other studies have reported an increased concentration of lipid peroxidation products such as F2-isoprostanes, conjugated dienes and malondialdehyde (MDA) in patients with MDD. In addition, patients with recurrent depression showed significantly lower levels of glutathione peroxidise (GPx) and SOD compared to first-episode depression patients. These observations indicated an increased oxidative stress in patients with MDD. Bilici et al have shown a significant reduction in the levels of oxidative stress markers in MDD after treatment with antidepressants while others did not find any significant difference after treatment.

S-glutathionylation of proteins is an example of oxidative post-translational modification, which is known to protect irreversible thiol oxidation of proteins by reactive oxygen species (ROS). Under the condition of oxidative stress, ROS is destroyed by the oxidation of reduced glutathione, an antioxidant present in the living cells. In addition, the elevated levels of oxidised glutathione might undergo thiol exchange with free and accessible cysteine residues of proteins resulting in glutathionylated proteins (GSSPs). Elevated levels of glutathionyl haemoglobin (GS-Hb) have been found in several clinical conditions associated with oxidative stress such as chronic renal failure, iron deficiency anaemia, type 2 diabetes mellitus and Friedreich’s ataxia.

In the present study, the levels of GS-Hb were measured in drug naïve patients with MDD and compared with that in healthy controls. Furthermore in a subset of MDD patients, changes in the level of GS-Hb were determined after treatment with antidepressant drugs belonging to selective serotonin reuptake inhibitor (SSRI) class.

Material & Methods

The study was conducted in the St. John’s Medical College and Hospital, St. John’s National Academy of Health Sciences (SJNAHS), Bengaluru, India, during the period of July 2014 to August 2016. The study was approved by the institutional ethical review board. Consecutive patients who were diagnosed with recurrent or first-episode depressive disorder as per the International Classification of Diseases-10 (ICD-10) guidelines were recruited into the study after obtaining informed consent (n=26). Patients with comorbid substance dependence syndrome and/or bipolar disorder and coexisting chronic medical conditions were excluded. Healthy controls were recruited among the volunteer staff and students of SJNAHS (n=17). The mental health status of healthy controls was assessed using the Kessler Psychological Distress Scale (K-10). Those with no history of any psychiatric illness and use of any psychotropic medication and who scored less than the recommended cut-off score of 20 on K-10 were enrolled in the study as healthy controls.

Psychiatric diagnosis was confirmed using the Mini-International Neuropsychiatric Interview (MINI-Plus). The 17-item Hamilton Depression Rating Scale (HDRS) was used to assess the severity of depression. Samples were collected from a subset of patients after six weeks of treatment with antidepressant to assess changes in GS-Hb levels in response to treatment. All patients in the study were treated with specific serotonin reuptake inhibitor (SSRI) class of antidepressants. Eight patients received sertraline in the dose range of 25-75 mg/day, and three patients were treated with escitalopram (dose range 10-15 mg/day).

Sample collection and processing: All first-episode MDD patients were drug naïve, while patients with recurrent depressive disorder had at least two weeks of washout period for antidepressant medication at the time blood sample was collected. Five millilitres of blood was collected into a vacutainer tube containing EDTA as anticoagulant, the plasma was separated. In a subset of patients, the samples were again collected after the treatment with antidepressant medication for six weeks. The red blood cells were washed with 0.9 per cent NaCl (aqueous) thrice before lysis with eight volumes of ice-cold distilled water. The haemolysate was centrifuged at 13,400 g for 10 min at 4°C to remove the erythrocyte membranes. The haemolysate was immediately dialysed against 10 mM ammonium acetate, pH 7.4 and stored at −120°C until mass spectrometric analysis.

Liquid chromatography (LC) electrospray ionization mass spectrometry (ESI-MS) based relative quantification of glutathionyl haemoglobin: Electrospray ionization mass spectra (ESI-MS) were acquired in positive ion “V” mode using a Q-Tof Synapt HDMS (Waters, UK). The haemolysate (3 µg) was injected into a C18 RP column (Eclipse, 4.6 mm × 150 mm, 5 µm) in a Shimadzu ultra performance liquid chromatography (LC, Shimadzu, Japan). The different globin chains were separated using a linear gradient of two per cent increase in acetonitrile per minute containing 0.1 per cent acetic acid with a flow
rate of 0.2 ml/min. The ESI-MS data were acquired over the mass range of 600-1600 m/z, with capillary voltage of 3 kV using a source temperature of 120°C and desolvation gas temperature of 350°C. The ESI-MS was mass calibrated using sodium iodide. The charge state mass spectra were deconvoluted, and the peak intensity for each globin chain was extracted. The relative percentage of GS-Hb with respect to normal beta globin and its adducts (glutathionyl beta and glycated beta) was calculated from the deconvoluted spectra using the following formula:

\[
\text{GS-Hb} \, (\%) = \frac{\text{Glutathionylated-} \beta}{\text{Glutathionylated-} \beta + \text{normal-} \beta + \text{Glycated-} \beta} \times 100
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**Statistical analysis:** The GS-Hb values in patients with MDD were compared with healthy controls using Mann-Whitney U-test. The HDRS score and GS-Hb values measured before and after treatment with antidepressants were compared using the Wilcoxon signed-rank test. Spearman’s rank correlation was used to measure the correlation between the change in GS-Hb values and change in HDRS score following six weeks of treatment with antidepressants (OriginPro v 8.0, Origin Lab, USA).

**Results & Discussion**

Oxidative stress is involved in the pathophysiology of MDD in multiple ways. Activation of oxidative and nitrosative pathways has been shown to damage membrane lipids and DNA of the neuronal cells altering neuronal membrane fluidity and integrity. This results in activation of the apoptotic pathway leading to cell death. An increased level of apoptosis has been observed in blood leucocytes and neuronal cells of MDD patients compared to healthy controls. Furthermore, MDD has also been reported to be associated with chronic inflammation. Thus, increased oxidative stress in MDD may be a result of chronic inflammation.

GSSPs have been reported to act as a protective mechanism against irreversible oxidative modification of important protein thiols. In the present study, glutathionylated haemoglobin was chosen as a marker of oxidative stress in patients with MDD. The relative level of GS-Hb with respect to normal globin chains and their adducts was calculated from the deconvoluted spectra of ESI-MS analysis (Fig. 1). The levels of normalized GS-Hb were found to be significantly elevated in patients with MDD (n=26), median (Q1, Q3)=8.34 per cent (6.52, 10.16), compared to healthy controls (n=17),

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**Fig. 1.** Electrospray ionization mass spectra of intact alpha and beta globin chains of haemoglobin obtained from a patient with major depressive disorder: The charge states (parenthesis) of Hbα, Hbβ, glutathionylated haemoglobin beta (GS-Hbβ) and glycated haemoglobin beta (Gly-Hbβ) are labelled. The deconvoluted mass spectra of Hbβ, GS-Hbβ and Gly-Hbβ are shown as inset.
median (Q1, Q3)=5.73 per cent (4.62, 6.47) (P=0.01) (Table). The GS-Hb values obtained for patients with MDD having co-morbid anxiety disorders (n=10), median (Q1, Q3)=7.93 per cent (6.72, 11.43), were not statistically significantly different from patients with MDD not having any co-morbid anxiety disorders (n=16), median (Q1, Q3)=8.63 per cent (6.48, 9.75). In a subset of patients (n=11), GS-Hb values were estimated at six-week post-treatment with antidepressant medication. The severity of depression assessed by HDRS showed a significant reduction in post-treatment samples, median (Q1, Q3)=6 (5, 8), compared to pre-treatment samples, median (Q1, Q3)=19 (15, 20) (P=0.001). Although GS-Hb values decreased in post-treatment samples, median (Q1, Q3)=7.68 per cent (5.46, 9.26), compared to pre-treatment samples, median (Q1, Q3)=8.07 per cent (6.54, 10.71), the reduction observed was not significant. However, a significant positive correlation was observed between the changes in GS-Hb levels before and after treatment with changes in the severity of depression on HDRS (correlation coefficient 0.747; P=0.0081). Fig. 2 depicts the scatter plot of the comparison between GS-Hb levels and HDRS scores estimated before and after treatment with antidepressants.

The observed increase in GS-Hb levels in drug-naïve MDD patients compared to healthy controls was in agreement with previous reports of elevated oxidative stress in patients with MDD. Bilici et al.\textsuperscript{10} reported a significant decrease in plasmatic GPx and MDA in erythrocyte and plasma after three months of treatment with fluoxetine, fluvoxamine, sertraline and citalopram. Similar results were reported by other investigators with a decrease in oxidative stress following treatment with SSRI group of antidepressant drugs\textsuperscript{25,26}. However, some investigators did not find any difference in markers of oxidative stress following treatment\textsuperscript{11,27}. These conflicting results may be related to different classes of antidepressants used in different studies. Duration of treatment may also be a factor with negative findings being more common in studies with shorter duration of treatment (6-8 wk)\textsuperscript{28}.

The strength of the study was that psychiatric diagnosis of all patients was arrived at using semi-structured psychiatric interview schedule, which also allowed to capture co-morbid psychiatric conditions. Limitations of our study included moderate sample size and relatively short duration of treatment with antidepressant medication. In addition, the presence or absence of significant life stressors in both patients and controls was not considered. The relationship between GS-Hb levels and commonly used inflammatory markers also needs to be explored.

GS-Hb, a marker of oxidative stress, was significantly elevated in patients with MDD compared to healthy controls. The observed lack of significant difference in GS-Hb levels between pre- and post-
treatment groups might be due to a relatively shorter duration of intervention with antidepressant medication. Future studies need to explore the association of GS-Hb with MDD in a large sample group and longer duration of treatment with antidepressants.

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Conflicts of Interest: None.

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