

SALIVARY GLUTATHIONE IN BIPOLAR DISORDER: A PILOT STUDY

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ABSTRACT

Background: Glutathione (GSH) is an important cellular antioxidant and its levels are decreased in some studies of bipolar patients. Saliva provides a simple and feasible means of measuring GSH but has not yet been applied to the study of bipolar disorder. The purpose of the study was to compare salivary levels of GSH and oxidized glutathione (GSSG) in bipolar patients and healthy controls.

Methods: Saliva was sampled from 22 medicated, euthymic patients with bipolar disorder and 20 healthy controls. GSH and GSSG were measured using an enzyme kinetic essay.

Results: GSH and GSSG were significantly higher in saliva from bipolar patients relative to controls. The ratio of GSH:GSSG was unchanged. There was no correlation between the measured clinical characteristics of the patients and GSH levels.

Limitations: The main limitation of the study was the small sample size. Patients were medicated which may have influenced saliva production and hence GSH levels. In addition, salivary GSH may not reflect GSH status in tissues more directly involved in the pathophysiology of bipolar disorder.

Conclusion: Salivary GSH can be readily measured in bipolar patients. Relative to controls, salivary levels of GSH and GSSG were increased in bipolar patients but their ratio was unchanged. The origin and significance of these change requires further study.

Key Words: Bipolar disorder; glutathione; oxidative status; saliva sampling

1. Introduction

Easily accessible biomarkers could be of great value in the diagnosis and management of mood disorders. Oxidative stress has been implicated in the pathophysiology of bipolar disorder and several measures of this process, often utilising enzymes measured in blood, have been explored in bipolar patients (Berk et al., 2011; Rosa et al., 2014).

Glutathione (GSH) is a major endogenous free radicle scavenger, and its lessened availability can increase vulnerability to cellular oxidative stress (Berk et al., 2011). A number of studies have measured GSH concentrations in blood and brain in patients with bipolar disorder but the findings have been somewhat inconsistent. For example, GSH levels in blood plasma and red cells have been reported both as being lowered and unchanged (Raffa et al., 2012; Rosa et al., 2014; Tunçel et al., 2015). Limited post-mortem work has found lowered GSH in cerebral cortex from bipolar patients (Gawryluk et al., 2011), though this has not been confirmed in magnetic resonance spectroscopy studies which are able to measure GSH concentrations in the living brain (Lagopoulos et al., 2013; Godlewska et al., 2014)

Recently we have described a novel method of analysing GSH in saliva using an enzyme kinetic assay (see Ngamchuea et al., 2016a; 2017). The determination is rapid and accurate and the stability of GSH in saliva is greater than in plasma due to decreased GSH degradation (Ngamchuea et al., 2016b; 2017). Salivary GSH therefore offers the possibility of an easily accessible biomarker for bipolar patients. The aim of the present study was to carry out a pilot study examining GSH levels in saliva in patients with bipolar disorder compared to a group of healthy controls.

2. Experimental procedures

2.1 Study setting

Participants with bipolar disorder were patients of the Oxford Health NHS Foundation Trust who were identified by their clinical teams as not being acutely unwell. They were approached by research facilitators attached to clinical teams and asked if they would like to participate in the study which involved a clinical assessment and the donation of a saliva sample. Healthy control participants were recruited by advertisement. All participants gave full, written informed consent to the study which was approved by the West of Scotland Research Ethics Service (16/WS/0141).

2.2 Patients and controls.

We recruited 22 patients with bipolar disorder and 20 healthy controls (Table 1). All participants were assessed for DSM-5 diagnoses using the Standard Clinical Interview for Diagnostic and Statistical Manual for Mental Health Disorders—Fifth Edition) (First et al., 2016). Current mood was assessed using the Young Mania Rating Scale (YMRS) (Young et al., 1978) and the Hamilton Depression Ratings Scale (HAM-D; Hamilton, 1960). Healthy controls were required not to have any significant DSM-5 disorder. Nineteen of the 22 patients were taking medications for the treatment of bipolar disorder. Eight were taking lithium, 5 lamotrigine and 3 valproate while 11 were taking atypical antipsychotic agents and 6 selective serotonin reuptake inhibitors (SSRIs). Only 4 patients were taking a single psychotropic agent. In the absence of previous data on salivary GSH estimations in psychiatric disorders, we considered the present investigation a pilot study and did not carry out a formal power analysis for sample size.

2.3 GSH estimation

Saliva samples were collected in using salivettes (Sarstedt Ltd, Nümbrecht) which participants were asked to chew gently for one minute. The mean (\pm SEM) time of sampling was somewhat later in the bipolar patients than in the healthy controls (13.30h \pm 55 mins vs 12.00h \pm 42 mins; $p= 0.021$, unpaired t-test). Participants were asked not to eat or drink for one hour prior to saliva collection. The salivettes were centrifuged at 1000g for two minutes and the saliva stored at -20°C for no more than seven days before assay. Assays were carried out blind to diagnosis.

Total glutathione (GSH plus GSSG) concentrations were determined using the Tietze enzymatic kinetic assay (Tietze et al., 1969; Rahman et al., 2006) together with the method of standard addition (Ngamchuea et al., 2016). All solutions were made in 0.1 M phosphate buffer at pH 7.5 containing 10 mM EDTA. First, 50 μL of the saliva sample, 30 μL of 1.68 mM DTNB and 30 μL of 40 $\mu\text{L}/3.0\text{ mL}$ GR were added respectively to a cuvette containing 350 μL of the phosphate buffer. After 30 seconds, 30 μL of 0.80 mM NADPH was then added. The absorbance at 412 nm was then recorded for 60 seconds. The above procedures were repeated for each sample but with increasing GSH concentrations added to the assay to perform standard additions.

The concentrations of oxidized glutathione (GSSG) were measured using the modified Tietze method (Ngamchuea et al., 2017). First, reduced glutathione (GSH) was masked by the reaction with benzoquinone added at the time of measurement. The benzoquinone-treated samples were then subjected to Tietze measurements as described above. The result yields the concentrations of GSSG. GSH concentrations

were calculated from the subtraction of GSSG concentrations from the concentration of total glutathione.

2.4 Data Analysis

The assay provided measures for GSH, GSSG and their combination. We also calculated the ratio of GSH:GSSG which is an indicator of oxidative status. According to the Kolmogorov-Smirnov test, these data were not normally distributed and were analysed with the Mann-Whitney Test (two tailed). Demographic data and ratings on the mood scales were assessed with unpaired t-tests (two tailed). Correlations were carried out using Spearman's rank-order correlation.

3. Results

3.1 Demographic data

Patients and controls were well matched for age and gender (Table 1). Twelve of the patients met DSM-5 criteria for Bipolar 1 disorder, the remainder had Bipolar 2 disorder. Most patients were mildly symptomatic in terms of depressive symptomatology on the HAM-D, though none met criteria for Major Depressive Episode. There was no significant manic symptomatology as detected on the YMRS. As often reported, smoking was more frequent in the bipolar patients than controls (Table 1).

3.2 GSH values

GSH, GSSG and their combination were significantly higher in patients than controls (Table 2) but the ratio of GSH:GSSG did not differ between the two groups. The various GSH measures were not significantly influenced by smoking or lithium treatment (all Mann-Whitney p values > 0.05) There were no significant correlations

between years of illness and the GSH measures or between the GSH measures and scores on the mood rating scales. There was no significant correlation between time of saliva sampling and GSH values either in the bipolar patients or controls, or in both groups combined (all Spearman p values > 0.05).

4. Discussion

In this pilot study of salivary GSH in bipolar patients, we found raised salivary levels of GSH and its oxidised form GSSG compared to healthy controls. However the ratio of GSH:GSSG was unchanged. Since the latter has been suggested to represent a measure of oxidative status (Berk et al., 2011), our results suggest that in saliva at least there does not appear to be evidence for acute oxidative stress in medicated bipolar patients in a relatively euthymic state.

The increase in salivary GSH and GSSG was unexpected because previous studies of GSH in other biological media in bipolar patients have either reported no significant change or a decrease in GSH levels. No studies of salivary GSH in bipolar patients have been reported previously, as far as we aware. Rosa et al. (2014) reported in 50 medicated bipolar patients that plasma GSH levels were decreased while those of GSSG were increased. A similar finding was obtained by Raffa et al. (2012) studying GSH in the red cells of 30 medicated bipolar patients. However, Tunçel et al. (2015) found a tendency to increased plasma GSH levels in medicated bipolar patients though in a smaller sample size ($n= 19$). The measurements of GSH and GSSG in plasma however are likely to be undermined due to the fast rate of degradation *in-vitro* (Ngamchuea et al., 2016b; 2017).

A decrease in GSH was also apparent in the single study that examined brain GSH and GSSG levels in bipolar patients. Thus Gawryluk et al. (2011) reported lower levels of both GSH and GSSG in prefrontal cortex of post-mortem samples from 14 bipolar patients compared to matched controls; however, the ratio of GSH:GSSG was unaltered. MRS studies permit the measurement of GSH in brain cortical regions in vivo. However, two studies of bipolar patients (one of which assessed unmedicated participants) did not find any difference in GSH levels from healthy controls (Lagopoulos et al., 2013; Godlewska et al., 2014).

In the current study there was no significant correlation between the GSH measures and patient characteristics, so whether there might be useful clinical correlates of saliva GSH in bipolar patients is currently unclear and will require further study. Many psychotropic drugs have an effect to diminish saliva flow (Scully, 2003) which raises the possibility that the increase in GSH may reflect an effect of medication on saliva production. However, there were too few unmedicated patients in the current study for this possibility to be tested statistically.

The increase in GSH and GSSG we found in saliva in bipolar patients in the current study was unexpected and requires replication. If GSH levels are increased it may reflect the origin of saliva GSH which is likely to differ from that in blood. Indeed in a previous study we found no significant correlation between GSH measured in saliva and that in whole blood (Ngamchuea et al., 2016a). GSH in plasma has been regarded as possible overall index of the oxidative status of the organism (Jones, 2006); however, salivary GSH which is largely produced in the parotid gland may be more influenced by local conditions in the oral cavity (Nagler et al., 2002), being

lower, for example, in people affected by dental caries (Öztürk et al., 2008). One study has reported increased saliva GSH in smokers (Zappacosta et al., 1999) but such an effect did not appear to explain the increase in saliva GSH in bipolar patients in the current investigation.

It is also worth noting that, apart from its anti-oxidant effects, GSH may act as a neuromodulator of glutamatergic receptors. This is of interest in view of the glutamatergic abnormalities associated with bipolar disorder (Oja et al., 2000; Chin et al., 2006; Gigante et al., 2012) and suggests that simultaneous measurement of salivary GSH and glutamate would be of interest in bipolar patients.

5. Strengths and limitations

The strength of the study is its novel and rapid measurement of GSH in saliva in bipolar patients. The limitations include the small sample size and the fact that the patients were medicated.

6. Conclusions

Assay for salivary glutathione measurements in bipolar patients was simple and feasible, allowing easy, swift and non-invasive testing of both GSH and GSSG. The finding of increased GSH and GSSG in bipolar patients was statistically significant, but unexpected and requires replication. It is possible that saliva GSH is not representative of GSH levels in tissues more directly related to the pathophysiology of bipolar disorder, for example the central nervous system. Nevertheless, salivary

GSH may provide an indicator of local oxidative processes relevant to the health of patients receiving treatment for bipolar disorder.

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Table 1: Demographic and clinical data

| | Patients (n= 22) | Controls (n= 20) |
|-------------------------------|------------------|------------------|
| Age (years) ¹ | 38.7 ± 2.0 | 35.4 ± 2.1 |
| Gender (M:F) | 8:14 | 8:12 |
| Smoking (Y:N) | 8:14 | 2:18 |
| HAM-D ¹ | 8.6 ± 1.9 | 0.2 ± 0.1 |
| YMRS ¹ | 1.9 ± 0.5 | 0.0 ± 0.0 |
| Years of Illness ¹ | 14.2 ± 2.2 | N/A |
| Lithium treatment (Y:N) | 9:13 | N/A |

¹Mean ± SEM

Table 2: GSH and GSSG values in bipolar patients and controls

| | Patients (n= 22) | Controls (n= 20) | p value ² |
|------------------------------|------------------|------------------|----------------------|
| GSH ¹ (μM) | 1.7 (0.3-19.7) | 1.1 (0.1-3.3) | 0.022 |
| GSSG ¹ (μM) | 0.6 (0.1-4.6) | 0.4 (0.1-1.5) | 0.015 |
| GSH + GSSG ¹ (μM) | 2.2 (0.5-20.8) | 1.5 (0.2-3.5) | 0.008 |
| GSH:GSSG ¹ | 2.5 (0.2-18.1) | 2.0 (0.5-15.0) | 0.435 |

¹Median (and range); ²Mann-Whitney test (two tailed)