Title:

A longitudinal proton magnetic resonance spectroscopy study investigating oxidative stress as a result of alcohol and tobacco use in youth with bipolar disorder

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Abstract

Alcohol and tobacco have been suggested to be "aggravating factors" for neuroprogression in bipolar disorder (BD), however the impact of these substances on the underlying neurobiology is limited. Oxidative stress is a key target for research into neuroprogression in BD and in accordance with this model, our previous cross-sectional studies have found that risky alcohol and tobacco use in BD is associated with increased oxidative stress, investigated via *in vivo* glutathione (GSH) measured by proton magnetic resonance spectroscopy (^{1}H-MRS) in the anterior cingulate cortex (ACC). What remains unknown is whether the negative impact on GSH levels can be modified as a result of limiting alcohol and tobacco use.

Thirty BD patients were included in the study. ^{1}H-MRS and tobacco and alcohol measures were conducted at baseline and follow-up assessments (15.5 +/- 4.6 months apart). Pearson's correlations were performed between percentage change in ACC-GSH and changes in alcohol/tobacco use. Regression analyses were then conducted to further explore the significant correlations. An increase in GSH was associated with a decrease in alcohol consumption ($r = -0.381$, $p < 0.05$) and frequency of tobacco use (-0.367, $p = 0.05$). Change in alcohol consumption, tobacco use and age were significant predictors of change in ACC-GSH ($F(3, 26) = 3.69$, $p < 0.05$). Due to the high comorbidity of alcohol and tobacco use in the sample, the individual effects of these substances on GSH levels could not be determined.

This study offers longitudinal evidence that changing risky drinking patterns and tobacco use early in the course of BD is associated with improvements in antioxidant capacity, and therefore may be specific targets for early intervention and prevention of neuroprogression in BD.

Keywords: bipolar disorder, alcohol, longitudinal, glutathione, ^{1}H-MRS, anterior cingulate cortex

Introduction

Substances of abuse, such as alcohol and tobacco are suggested to be aggravating factors for neuroprogression of bipolar disorder (BD) (Berk, 2009, Kapczinski et al., 2008). Accordingly, both substances are found to significantly impact illness trajectory, with increased rates of mood episode recurrence and severity (Rakofsky and Dunlop, 2013, Salloum et al., 2002, Waxmonsky et al., 2005), worsening general function (Cardoso et al., 2008), increased morbidity (Farren et al., 2012), poorer response to treatment (Berk et al., 2008), lengthier stays in hospital (Dodd et al., 2010) and increased risk of suicide (Oquendo et al., 2010). Despite the increased understanding of the clinical impact of these modifiable environmental exposures (Berk et al., 2011, Berk et al., 2013) knowledge of the neural interaction between alcohol/tobacco and BD pathophysiology remains limited. Thus, research in this area is necessary to enable a better understanding of how these factors contribute to poorer outcomes for patients and to help identify neurobiological risk factors responsible for the heightened susceptibility of risky drinking and tobacco use in this population.

Oxidative stress is a key target for research into the neuroprogression of BD (Berk et al., 2011). Accordingly, ethanol and cigarette smoke have a demonstrated propensity to stimulate the formation of reactive oxygen species (ROS) resulting in oxidative stress (Li and Wang, 2004, Mendez-Alvarez et al., 1998, Nordmann et al., 1990, Zhang et al., 2007). Neural tissue is especially prone to such stress due to its high consumption of oxygen and resultant production of ROS, easily oxidisable substrates such as lipids with unsaturated fatty acids and relatively low activity of antioxidant defence molecules (Halliwell, 1992; Dringen, 2000; Halliwell, 2006). Previously, we have addressed the impact of alcohol and tobacco use on oxidative stress in BD by cross-sectionally examining the brain's primary antioxidant, glutathione (GSH), measured *in vivo* via proton magnetic resonance spectroscopy (^{1}H-MRS) (Chitty et al., 2013, 2014b). We found that increased/more frequent alcohol and tobacco use were both negatively associated with GSH levels in the anterior cingulate cortex (ACC), and that this effect was specific to BD and not to matched controls. Although we were unable to untangle the individual effects of alcohol and tobacco use on GSH levels due to high comorbidity of their use in our sample, these findings

support the notion that people with BD have an increased propensity for oxidative stress when consuming tobacco and risky levels of alcohol, highlighting the importance of the early identification and treatment of BD patients who may be susceptible to their use. It is unknown whether these initial negative impacts on GSH levels can be modified as a result of limiting alcohol and tobacco use, and hence whether these aggravating factors should be specific targets of early intervention and prevention of neuroprogression in BD.

The aim of the present study was to examine longitudinal changes in GSH and its relation to self-reported changes in alcohol use patterns and tobacco use among BD patients. We examined different components of risky drinking behaviour (e.g. alcohol consumption, problem use or the development of dependence) in order to investigate the specific aspects of alcohol use that are associated with any corresponding changes in GSH levels. Additionally, we assessed the impact of tobacco use on these relationships. We hypothesised that a reduction in risky drinking and smoking would be associated with an increase in GSH concentration, reflecting a relaxation of oxidative stress.

Patients and Methods

Participants

The study was carried out in accordance with the Declaration of Helsinki, and approved by the University of Sydney ethics committee. Participants gave written informed consent before participation. The sample consisted of the 57 BD participants from our cross-sectional study (Chitty et al., 2014b) plus an additional recruitment/inclusion of 20 patients, giving a total of 77 participants in the study.

Participants were recruited as part of a wider Youth Mental Health cohort study (Hermens et al., 2011, Lee et al., 2013, Scott et al., 2013), with referral from psychiatrists with a diagnosed bipolar illness using DSM-IV criteria (APA, 2000) as follows: bipolar I (n = 19), bipolar II (n = 30) or bipolar spectrum with family history of BD (n =21) or bipolar NOS (n = 7), defined as an illness pattern consisting of periods of both elevated and depressed mood consistent with a bipolar spectrum disorder (Angst, 2007). Participants were asked at baseline

whether they would be interested in being contacted for a follow-up assessment. Thirty-six of these patients were followed up after 11 months and all measures were repeated. Researchers gave no instruction regarding alcohol or tobacco use before or during the follow-up period. Recorded changes at follow-up reflect changes in patients' self-reported alcohol and tobacco habits. Likewise, researchers did not enquire about reasons for any change in substance use at follow-up.

Exclusion criteria for all participants were medical instability, history of neurological disease, medical illness known to impact cognitive and brain function, intellectual disability and insufficient English for assessment. All participants were asked to abstain from drug or alcohol use for 48 hours prior to testing and informed that they may be asked to under-take an alcohol breath test and/or a saliva drug screen if the researcher had reason to believe the participant was under the influence or intoxicated. Patients' usual psychotropic medication regimens were not interrupted in any way.

Measures

Clinical and self-report measures

Participants underwent a clinical interview including the Hamilton Depression Rating Scale (HDRS; (Hamilton, 1967), the Brief Psychiatric Rating Scale (BPRS; Overall and Gorham, 1962) and the Young Mania Rating Scale (YMRS; Young et al., 1978).

Participants completed the Alcohol Use Disorders Identification Test (AUDIT) in self-report format. The AUDIT was developed from a World Health Organisation (WHO) collaboration as a screening instrument for hazardous and harmful alcohol consumption (Saunders et al., 1993). The tool differs from other screening tests as it emphasizes identification of hazardous drinking rather than long-term dependence and focuses primarily on recent symptoms and behaviours (Babor et al., 2001), making it more appropriate for youth cohorts many of whom will be initiating their drinking habits or will be risky drinkers rather than alcohol dependent. The AUDIT is made up of 10 questions, with possible scores ranging from zero (abstinence) to 40.

The AUDIT can be further broken down into sub-scores, which were also calculated at each timepoint. The consumption sub-score assesses hazardous alcohol use (e.g. frequency and amount of drinking), the dependence sub-score is comprised of symptoms associated with dependence (e.g. morning drinking and impaired control over drinking) and the problems sub-score addresses harmful alcohol use (e.g. alcohol related injuries, blackouts, guilt after drinking and concerns of others). A score of 6 – 7 on the consumption sub-score may indicate a risk of self-related harm. The dependence score assesses symptoms associated with dependence, a score of 4 or more in this sub-score plus a total AUDIT score of 20 or more indicates almost certain dependency. Any score on the problems sub-score is indicative of problem drinking.

Frequency of tobacco use was assessed using baseline and follow-up answers from the WHO Alcohol, Smoking and Substance Involvement Screeening Test (WHO-ASSIST) (Edwards et al., 2003), in which participants were asked to indicate how often in the previous three months they had used tobacco products with ordinal options: never, once or twice, monthly, weekly or daily/almost daily.

Current self-reported symptoms were assessed using the depression anxiety stress scale (DASS; Lovibond and Lovibond, 1995)) and the Kessler-10 (K-10), a psychological distress scale (Kessler et al., 2002).

^{1}H-MRS data acquisition and processing

As per previously published protocols (Hermens et al., 2012, Lagopoulos et al., 2013), participants were scanned on a 3Tesla GE Discovery MR750 MRI (GE Medical Systems, Milwaukee, WI). Firstly, a 3D sagittal whole-brain scout was undertaken for orientation and positioning of scans (TR=50ms; TE=4ms; 256matrix; no averaging, z=5mm thickness). Next a T1-weighted Magnetization Prepared RApid Gradient-Echo (MPRAGE) sequence producing 196 sagittal slices (TR=7.2ms; TE=2.8ms; flip angle = 10°; matrix 256x256; 0.9mm isotropic voxels) was acquired for the purpose of localization of the ACC. A 2 x 2 x 2 cm single voxel was placed midline on the ACC (for example spectra and voxel placement please see (Chitty et al., 2014a). Spectroscopy data was acquired using PRESS (TE=35ms, TR=2000ms, 128 averages)

along with two chemical shift-selective imaging pulses for water suppression. All spectra were shimmed to achieve full-width half maximum (FWHM) of <13Hz and visually inspected by independent raters. Spectra with the following features were excluded: Cramer–Rao Lower Bound greater than 20%, poor spectral morphology (spectra that do not adequately represent normal spectra morphology), poor spectral fit (LCModel line fitting that does not adequately represent the raw data), large variation in residuals, poor signal-to-noise ratio and presence of artefact.

To obtain reference spectra used to determine GSH quantification, six phantom solutions containing varying concentrations of GSH (0 - 7.0 mM) were prepared with physiological brain concentrations of creatine, glutamate and glutamine in a phosphate buffer. All solutions were kept at 37 °C, and GSH linear dependence was calculated as $R^2 = 0.994$.

Data were transferred offline for post processing using the LCModel software package (Provencher, 1993). All spectra were quantified using a GAMMA-simulated PRESS TE 35 basis set of 15 metabolites (including GSH) and incorporated macromolecule and baseline fitting routines. Absolute GSH concentration was determined using the ensuing reference spectral calibration curve for the ACC.

Following this, the coordinates of the acquired ACC voxels for each participant were obtained using the SAGE (Spectroscopy Analysis GE) software package and the reconstructed acquisition voxels for all participants were corrected for grey matter (GM) content. GM correction was achieved by segmenting each participant's structural image into GM, white matter and cerebrospinal fluid using the FAST4 algorithm as implemented in FSL (Zhang *et al*, 2001) and volume fractions were calculated. All subsequent statistical analyses were conducted on GM corrected absolute GSH concentration.

Statistical analyses

Statistical analyses were carried out using SPSS for Windows 21.0 (SPSS Inc., Chicago, Illinois, USA). Differences in age, symptoms, alcohol, tobacco and medications, and GSH concentrations between baseline and follow-up were determined using paired t-tests for string

variables or McNemar tests for nominal data (Field, 2013). Alpha was set to 0.05.

In order to test the primary hypotheses, that decreased drinking and smoking will be associated with an increase in GSH, we calculated change scores for AUDIT total and subscores, ASSIST tobacco frequency item and GSH concentrations. Raw change scores were used for AUDIT and ASSIST and percentage change was used for GSH concentration to take into account baseline levels. Values over 100 for "change-GSH" represent an increased concentration at follow-up, a positive value for "change-AUDIT" represents an increase in drinking from baseline to follow-up and a positive value for "change-tobacco use" indicate a higher frequency of tobacco use at follow-up. Pearson's correlations were performed between change-GSH and change-AUDIT and change-tobacco use; co-varying for age at baseline. A simple bootstrapping method based on 1000 samples was used to obtain bias-corrected and accelerated (BCa) 95% confidence intervals (Wright et al., 2011).

Hierarchical, robust regression analyses were then conducted to further explore the significant correlations. Predictors were entered in two blocks in order to assess the predictive value of change-AUDIT separately and then to see the influence of tobacco use on the model. Hence, any significant change-AUDIT subscores and age at baseline were entered into Block 1, and change-tobacco use at follow-up entered into Block 2. Multicollinearity of predictors was assessed by the variance inflation factor (VIF) with values greater than 10 considered as evidence of a strong linear relationship between predictors (Myer, 1990). Cook's distance was used to assess the impact of influential cases with a Cook's distance of greater than 1 considered a case with possible undue influence on the model (Cook & Weisberg, 1982). Standardized residuals were inspected to identify outliers, with values +/- 3 standard deviations from the regression line considered outliers (Field, 2013).

Results

Baseline characteristics

At baseline participants (n = 77) were aged 22.6 +/-3.3 years, duration of illness 7.5 +/- 3.9 years, years of education 13.0 +/- 2.1 years. Out of the 36 participants who were followed up,

six participants were excluded due to invalid spectroscopy data, leaving 30 participants with ACC-GSH data at two time points (the "follow-up sample"). Comparisons in baseline demographics, ACC-GSH and alcohol and tobacco use between the follow-up sample (n = 30) versus those who were only included at baseline (the "baseline-only sample" n = 47) are provided in supplementary Table 1. Notably, the baseline-only sample had significantly worse HDRS scores at baseline compared with their peers who returned for follow-up ($t(70) = 2.15$, $p < 0.05$). There were no other significant differences between the groups.

Follow-up sample characteristics

The follow-up sample (n = 30) included 22 (73.3%) females, aged 22.0 +/- 3.8 years; illness duration was 6.89 +/- 3.56 years, years of education 13.3 +/- 2.32. The mean +/- SD time between baseline and follow-up was 15.5 +/- 4.6 months (min: 11 months, max: 32 months) for MRI scans.

Clinical scores for at baseline and follow-up for the follow-up sample are shown in Table 1. Self-reported stress and anxiety, as measured by the DASS, as well as clinician ratings (BPRS and HDRS scores) were significantly improved at follow-up. There were no significant differences in YMRS, K-10 or DASS-depression ratings.

Alcohol, tobacco and medication use

Table 2 shows the mean scores (and SD) in alcohol, tobacco and medication use over time for the follow-up sample. There were no significant changes in alcohol use (as measured by AUDIT scores), tobacco use or medications between baseline and follow-up time points.

Longitudinal GSH

At the group level, there were no significant differences between ACC-GSH recorded at the baseline (1.35+/- 0.42 I.U.) as compared with the follow-up (1.37 +/- 0.33 I.U.) time-point ($t(29) = 0.17$, $p > 0.05$). Furthermore, the correlation between baseline and follow-up GSH was non-significant ($R = -0.06$, $p = 0.757$) (see Figure 1).

Correlations between change scores

The correlation matrix (ACC-GSH by four alcohol variables and one tobacco variable) is provided in Table 3. Change in ACC-GSH was negatively associated ($p < 0.05$) with change-AUDIT consumption score (see Figure 2). Change-tobacco use was negatively associated with change-GSH at trend level ($p = 0.05$). No other correlations between change-GSH and change-AUDIT/change-tobacco use were found.

Predicting change in ACC-GSH

Overall, the regression model was significant ($F(2,27) = 3.94$, $p < 0.05$) with change-AUDIT consumption score and age at baseline explaining 22.6% of the variance in change-GSH. Both age (B = -4.14, SE = 1.8, beta = -0.37, $p < 0.05$, BCa 95% CI – 7.34, -1.3) and change-AUDIT consumption (B = -8.31, SE = 4.0, beta = -0.37, $p < 0.05$, BCa 95% CI – 15.8, -1.7) were significant predictors of change-GSH, whereby a younger age at baseline and a decrease in alcohol consumption was associated with an increased percentage change-GSH. The addition of change-tobacco use did not result in a significant change in R^2, though the model remained significant ($F(3, 26) = 3.69$, $p < 0.05$) and explained 29.9% of the variance in change in ACC-GSH. Age at baseline (B = -3.83, SE = 1.8, beta = -0.35, $p < 0.05$, BCa 95% CI -7.14, -0.64) and change in tobacco use (B = -7.78, SE = 4.3, beta = -0.28, $p < 0.05$, BCa 95% CI – 13.8, 3.4) were significant predictors of increased GSH, with change in AUDIT consumption as a trend-level predictor (B = -6.78, SE = 4.1, beta = -0.30, $p = 0.097$, BCa 95% CI – 14.0, -0.24). VIF values were between 1.0 and 1.2. Cook's distance for all variables was less than 1, and standardized residuals were all within three standard deviations.

Discussion

The aim of the present study was to investigate the relationship between alcohol and tobacco use and change in ACC-GSH concentrations over time. Our results confirmed our hypotheses; firstly, that a decrease in alcohol consumption, as measured by the AUDIT was

associated with increased ACC-GSH and secondly, that a decrease in frequency of tobacco use was also associated with increased ACC-GSH over time, albeit with a p-value of 0.05.

These findings were further supported by a regression model, which identified that a decrease in alcohol consumption predicted an increase in GSH. When change in tobacco use was entered into the model change in tobacco use and age were significant predictors whereas alcohol consumption became only a trend-level predictor ($p = 0.097$). These results are similar to our cross-sectional study (Chitty et al., 2014b), which found that the negative relationship seen between AUDIT score and ACC-GSH loses its significance when controlling for smoking status. A previous ^{1}H-MRS study revealed that comorbid heavy alcohol use and smoking is associated with more pronounced and widespread neurometabolite impairments compared with heavy alcohol use alone, suggesting that these two substances may have additive neurobiological consequences (Durazzo et al., 2004). Thus, a reasonable interpretation of the present GSH findings is that both alcohol and tobacco promote the production of ROS; therefore one would expect a greater, combined oxidative effect. To our knowledge no studies have compared clinical outcomes in BD in those who use alcohol or tobacco separately vs. those who use these substances in conjunction. This lack of evidence is likely due to the same limitation faced here; that is, the high prevalence of comorbid alcohol and tobacco use in BD, hence the individual effects of each substance are difficult to tease apart without large sample sizes. Consequently it is unclear whether the compounded neurobiological effects of the alcohol-tobacco comorbidity is associated with a greater impact on neuroprogression of BD, nonetheless it would be logical to speculate that they do

Younger age at baseline was a predictor of increased percentage change-GSH at follow-up. This may suggest that the effect of alcohol and tobacco on GSH levels varies depending on age, with younger patients more resilient to decreases in anti-oxidant capacity. A rodent study has also implicated the role of age on GSH synthesis after acute ethanol exposure (Sommavilla et al., 2012) and many other rodent studies have identified developmental changes in antioxidant capacity (Hussain et al., 1995; Siqueira et al., 2005; Beiswanger et al., 1995). This collective evidence suggests that age most probably has a role to play in the effects that toxic substances

have on oxidative stress systems, highlighting the importance of early intervention of alcohol and tobacco risk management in BD.

While there was no between-subjects difference in ACC-GSH levels from baseline to follow-up, there was also no within-subject correlation between baseline and follow-up GSH concentrations and inspection of Figure 1 suggests that there were substantial between-subject differences in the fluctuation of GSH across time points. This is likely due to differences in GSH consumption and *de novo* synthesis based on oxidative stress states (Dringen, 2000; Dringen and Hirrlinger, 2003). Whereby higher levels of alcohol and/or tobacco consumption promote the production of ROS (Nordmann et al., 1990; Zhong et al., 2012; Zhang et al., 2007), leading to higher GSH consumption (Janaky et al., 1993).

There were no significant correlations between ACC-GSH and change in dependent and problematic drinking as measured by the AUDIT. These findings indicate that specifically decreasing the *amount and frequency* of alcohol consumption is associated with improved antioxidant capacity in BD. This may suggest that problematic drinking and/or alcohol dependence is not associated with GSH or that changes in such drinking behaviours are not linked to potential changes in GSH synthesis or consumption. It is noteworthy that during adolescence and young adulthood, alcohol dependence is not common, whereas alcohol abuse patterns (i.e. binge/risky drinking) do emerge and are prevalent, hence alcohol consumption may be the most important indicator of future dependence patterns.

Limitations

There are several limitations to consider when interpreting these study results. Firstly, due to the sample size of the study only three predictors were possible to add to the regression model, limiting our assessment of other factors that may have predicted change in GSH-ACC. For example, participants showed significantly improved clinical outcomes at follow-up. While this is most likely a consequence of continued clinical care it may be related to improved neurobiological measures. Given that overall participants were significantly improved in clinical measures without significant overall change in ACC-GSH, it may not be that the two are related. However it does present a confounding factor in our analyses and one that should be

investigated further with a larger sample size for regression. Also our study has used multiple correlations to explore the relationship between GSH and change in alcohol and tobacco use and as a result the reader must be advised there is a risk of Type I error. Furthermore, quantifying GSH concentration via ^{1}H-MRS is a new technique, which limits our ability to make inferences about what changes in GSH are showing. To our knowledge there is no data available that shows the test-retest reliability and normal fluctuation of GSH levels measured via ^{1}H-MRS. Likewise, we have not collected longitudinal GSH control data, limiting our ability to speculate as to how much of this variation is random individual variation in GSH. In light of these limitations we advise the reader that these results are preliminary.

Alcohol status at baseline was significantly different between those in the baseline-only sample vs. those in the follow-up sample, with more risky drinkers in the baseline sample (as well as a higher mean HDRS). We speculate that those in the baseline-only sample with higher levels of drinking may have presented with decreased GSH at follow-up, given they maintained their risky drinking patterns. Further research into the effects of maintaining risky drinking over many years is necessary.

Participants were on an array of medications. Although there was no significant difference in patients that remained on or off medications, there is the possibility that some medications may have influenced ACC-GSH. For example, lithium has been shown to have neuroprotective effects (de Sousa et al., 2014). A larger sample size would allow us to look further into the effect of medications on this relationship.

Conclusions

It has been proposed that BD is a neuroprogresssive disorder partly associated with inadequately compensated metabolic stress, and that this neuroprogression has the potential for modifiable pathophysiology over the course of the illness (Berk et al., 2011, Post, 2007). Oxidative stress is implicated in this progression and acceleration of disease processes in BD (Berk et al., 2011), hence our cumulative results suggest that risky alcohol use may contribute to the neuroprogressive process and, indeed, may explain the heightened negative consequences documented in those with this cormorbidity. Importantly, the present study offers longitudinal

evidence that shows changing risky drinking patterns and tobacco use early in the course of illness is associated with improvements in ACC-GSH, and therefore may be early modifiable risk factors for heightened neuroprogression. In addition, augmentation of GSH has been identified as a targeted pathway for neuroprotection in BD (Berk et al., 2011). Thus, our results stress the importance of monitoring alcohol and tobacco use from first presentation of BD symptomology and importantly, further highlight alcohol and tobacco reduction as a first-line treatment avenue.

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