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ORIGINAL ARTICLE

Increased mitochondrial and cytosolic antioxidant enzymes in manic episodes

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Abstract
Background and objectives: An increasing number of studies have been conducted to investigate the role of oxidative stress in the pathophysiology of bipolar disorder (BD). The aim of our study was to detect the levels of plasma malondialdehyde and the gene expression of antioxidant enzymes in BD patients during manic period and to assess the changes of these markers during the subsequent remission period.
Methods: This study involved 20 drug-free, hospitalized patients with BD type I who met the manic episode diagnostic criteria according to DSM-5, as well as 20 age-sex matched healthy controls. As a marker of lipid damage, malondialdehyde plasma levels were measured by the thiobarbituric acid method; mRNA expression levels of catalase (CAT), glutathione peroxidase (GPx), glutathione synthetase (GS), superoxide dismutase (SOD1) and mitochondrial superoxide dismutase (SOD2) were measured by real-time quantitative PCR from peripheral whole blood samples.
Results: In a manic episode, the expression levels of CAT, GPx, GS and SOD2 were higher in the patients than the healthy controls. With treatment, only the GPx levels decreased significantly. The malondialdehyde and SOD1 levels did not differ between the patients and controls, and in the patient group, they did not differ between the mania and remission periods.
Conclusion: This is the first study that has assessed the mRNA expression levels of antioxidant enzymes in patients with BD type I. The data from this study suggests that there is an increase in the expression of antioxidant enzymes as a response to oxidative stress in BD, and that the gene expression levels of these antioxidant enzymes could be potential biomarkers for manic phase of the disorder.

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Introduction

Bipolar disorder (BD) is a chronic disease that consists of repeating mania and depression episodes.[1] There is increasing evidence from recent studies about the role of oxidative stress in the etiology of BD.[2–6] These studies about oxidative stress in BD have also been intensified concerning the activities of antioxidant enzymes and the measurement of the lipid peroxidation end-products.[7]

Oxidative stress has been started to be defined as a disruption of redox signaling and control, despite it was previously described in terms of a balance between pro-oxidants and antioxidants.[8] As major components of oxidative stress, free radicals grow out in all of the tissues of the body during primarily oxidative phosphorylation in the mitochondria. Reactive oxygen species (ROS) are typically synthesized as a by-product of cellular respiration in the electron transport chain. If electrons are released from complexes of the transport chain prematurely, they transform molecular oxygen into superoxide radicals, and as a result of this biochemical reaction, various reactive oxygen species get synthesized. Enzymes like superoxide dysmutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) have the ability to save the cells from oxidative injury.[9,10]

Oxidative stress has been suggested to have an important role in BD pathophysiology recently. Some studies report increased peripheral markers of oxidative stress in BD and some studies report changes in the levels of antioxidant enzymes and increased lipid peroxidation end-products.[5,11–14] Significant increases in antioxidant enzymes, DNA damage, protein carbonyl ingredients attributable to oxidative stress, increases in lipid peroxidation end products and nitric oxide levels were detected in some other studies.[2,14–16] However, the results of these studies are contradictory. Cytosolic SOD activity was found to be high in an acute episode of BD dissimilar to a euthymic phase.[11,13] Similarly, increases were reported in cytosolic SOD activity in patients during manic episodes that were not treated.[17] However, other studies did not find any differences between the SOD activities of the patient group and that of the healthy control group; also, some other research showed decreases in the cytosolic SOD activity in BD.[18,19] Nevertheless, most studies have reported an increase in antioxidant enzyme levels.

There are clinical studies that showed that mood stabilizers decrease oxidative stress markers in peripheral samples. These studies proved the therapeutic effects of mood stabilizer treatments that show antioxidant properties. Evidences show that present mood stabilizer treatments have neuroprotective effects against oxidative stress by arranging various antioxidant pathways in the cell.[4,17,20–22] Another aspect of the role of oxidative stress in BD is the fact that antioxidant compounds like N-acetylcystein (NAC) have been shown to be beneficial in BD treatment.[23–27] The typical antipsychotics have been found to reduce the antioxidants and increase the oxidants; in contrast, the atypical antipsychotics have been found to increase the antioxidants and reduce the oxidants.[28–32]

The studies about oxidative stress in BD have investigated the serum antioxidant levels till today. Conflicting results showing different serum enzyme levels have been reported. This may be a result of the variable production of enzymes under influence of numerous factors. Thus, the serum levels of these enzymes may not be showing the real tendency in BD on antioxidant enzyme production. According to a literature search, no previous human studies have examined the peripheral blood antioxidant gene expression. We hypothesized that the gene expression of the antioxidant enzymes and plasma malondialdehyde (MDA) levels that show lipid peroxidation will be altered in BD patients. In this study, the mRNA expressions of cytosolic and mitochondrial superoxide dismutase (SOD1, SOD2), catalase (CAT), glutathione synthetase (GS), glutathione peroxidase (GPx) were examined in the peripheral blood of adult, drug-free BD patients during manic and remission periods and a healthy control group. We selected the studied possible biomarkers focusing on evidence based on findings in peripheral blood.[2,11,12,14–16,33] Despite the fact that studies about oxidative stress have not yet explained the pathophysiology of BD, these investigations could resolve this issue by finding markers specific to the disorder that can be searched in blood samples and following the response to treatment with biological markers.

Materials and methods

Patients

This study included 20 medication-free patients aged 18–65 years who had been diagnosed with BD-I in the past and who were currently experiencing an acute manic episode. Other inclusion criteria for the patient and control groups were as follows: having a body mass index (BMI) within a normal range (18.5–25 kg/m2), having a diet similar to that of the other participants (i.e. rich in carbohydrates) and not performing physical exercise. All the patients and healthy controls stated that they were not on a special diet and did not participate in strenuous exercise activity.

Exclusion criteria for the patient and the control groups were as follows: alcohol and substance (except nicotine) abuse or dependence, the presence of systemic diseases (including cardiovascular and endocrine diseases), usage of any antioxidant agents (e.g. vitamins E, C and NAC), the presence of epilepsy or severe neurological disorders and the presence of infectious diseases. For both medical and neurological diseases, exclusion was based on the results of general medical, neurological examinations and routine laboratory results. Patients who used psychotropic drugs were also excluded. Purification periods for psychotropic drugs were two weeks for oral drugs and one month for parenteral drugs; for participants who used mood stabilizer drugs, their blood levels had to be 0.

The control group consisted of 20 medication-free, healthy volunteers who had no current, past or first-degree family history of a major psychiatric disorder. The participants were matched for age, gender, smoking, dietary and exercising habits.

This study was performed at the Medical Faculty of Trakya University from October 2014 to April 2015. A semi-structured form was used to detect several sociodemographic data and clinical variables. The diagnosis of BD was carried out using the Structured Clinical Interview for DSM-IV-Axis I (SCID-I). The patients had to fulfill criteria for

Table 1 Primers of antioxidant enzymes.

SOD1	Forward (5′-GTTCGGTGACAACACCAATG-3′)
	Reverse (5′-GGAGTCGGTGATGTTGACCT-3′)
SOD2	Forward (5′-TCTGAAGAAGGCCATCGAGT-3′)
	Reverse (5′-GCAGATAGTAGGCGTGCTCC-3′)
CAT	Forward (5′-TACGAGCAGGCCAAGAAGTT-3′)
	Reverse (5′-ACCTTGTACGGGCAGTTCAC-3′)
GS	Forward (5′-TGGGACCAGCAAGTAAAACC-3′)
	Reverse (5′-TCGCGAATG TAGAACTCGTG-3′)
GPx	Forward (5′-TTCCCGTGCAACCAGTTTG-3′)
	Reverse (5′-TTCACCTCGCACTTCTCGAA-3′)

SOD1, SOD2, superoxide dismutase 1, 2; CAT, catalase; GS, glutathione synthetase; GPx, glutathione peroxidase.

manic episodes according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5). Symptoms were assessed using the Young Mania Rating Scale (YMRS) and the Hamilton Depression Rating Scale (HDRS).

Approval of this research was given by the ethics committee of the Medical Faculty of Trakya University. All patients or family members were given a written informed consent form before entering the study.

Laboratory data

Blood samples were obtained using vacutainer tubes from the patients and the controls between 08:00 and 10:00 a.m., and the samples were centrifuged at 3000 rpm for 15 min and stored at −80° C for further analysis. The samples from the patients were taken the day after their admittance to the inpatient clinic. Blood samples were taken again when the patients fulfilled the remission criteria as defined by their total score on the YMRS of <4.[34]

Determination of gene expression levels

The mRNA expression levels were measured using the quantitative real-time polymerase chain reaction (qRT-PCR). The total RNA was isolated from the whole blood using the PureLink® RNA Mini Kit (Life Technologies, USA) according to the manufacturer's instructions. The extracted RNA concentrations were measured by the Qubit® Fluorometer (Life Technologies, USA). The first strand of cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA). The c DNA synthesis was performed using a thermal cycler Applied Biosystems® Veriti®. The gene expression levels of the antioxidant enzymes were analyzed by qRT-PCR using the SYBR® Select Master Mix (Life Technologies, USA) on an ABI Step One Plus Real-Time PCR system. PCR Cycling: 2 min at 50 °C and 10 min at 95 °C, 40 cycles at 95 °C for 15 s, and 60 °C for 60 s. Glyceraldehydes 3-phosphate dehydrogenase (GADPH) [Forward (5′-AATTCCGATCTTCGACATGG3-′) Reverse (5′-GAAAAAGCGGCAGTCGTAAT-3′)] was used as the housekeeping gene. The primers used in the gene expression are shown in Table 1. The comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method was used for the gene expression analysis of data measured by PCR.[35] Ct (threshold cycle) indicated the fractional cycle number at which the amount of amplified target reached a fixed threshold. ΔCt corresponded to normalized Ct values and was equal to the difference in cycle thresholds between the target (gene expressions of predetermined molecules) and reference genes (housekeeping genes). GAPDH was used as the housekeeping gene in our research. ΔΔCt corresponded to the difference between the expression level of the average ΔCt of the patient group and that of the control group. $2^{-\Delta\Delta Ct}$ denoted the fold change in the expression level change of the following measures of the patient group compared to the control group. All analyses were performed in the same laboratory.

Determination of plasma MDA levels

Lipid peroxidation levels were determined by measuring the end product of lipid peroxidation MDA. Plasma MDA values were calculated using the extinction coefficient of the MDA-thiobarbituric acid complex at 532 nm. Levels of plasma MDA were measured by the thiobarbituric acid (TBA) method, which was modified from Young et al.'s[36] method. Peroxidation was measured as the production of MDA, which in combination with TBA forms a pink chromogen compound whose absorbance at 532 nm was measured. The MDA results were expressed as nmol/ml.

Statistical analysis

Data were analyzed using the SPSS 20.0 version for Windows statistical software. The Kolmogrov–Smirnov test was used to compare the observed cumulative distribution functions; our sample presented abnormal distribution. Non-parametric statistical methods were used to analyze the data, and groups were compared using Mann–Whitney *U* and Wilcoxon tests. Categorical variables were analyzed with a chi-square test, and the relationship between the biochemical and clinical variables was assessed using spearman correlation. The results were expressed as the means ± standard deviation (SD). A p value of less than 0.05 was used to indicate statistical significance.

Results

Demographics

Twenty inpatients with BD in a manic episode ($n = 20$) and healthy volunteers ($n = 20$) were included in this study. None of the subjects in either group had comorbid diseases. The mean ages of the patient and control groups were similar (31.75 ± 8.23 and 31.85 ± 7.31, respectively; $p = 0.968$). Each group was composed of 14 males and 6 females. The average BMI of the patients and controls was within the normal range (24.3 kg/m^2 ± 3.2 kg/m^2, 24.7 kg/m^2 ± 3.0 kg/m^2) and was similar between the two groups ($p = 0.669$). In the patient group, the time to reach remission was 9.3 ± 1.5 weeks. The clinical characteristics of the BD patients and the drugs used during remission are included in Table 2.

Table 2 Demographics and clinical characteristics of BD and control group.

Variables	Patients ($n=20$)	Controls ($n=20$)	p
Age (years)	31.75 ± 8.23	31.85 ± 7.31	0.968
Gender (male/female)	14/6	14/6	1.000
Education, years	11.10 ± 2.90	11.70 ± 3.32	0.547
Duration of illness	7.40 ± 7.32	–	
The number of manic episodes	3.10 ± 2.57		
The number of depressive episodes	1.00 ± 0.97		
Hospitalization number	2.85 ± 2.85	–	
BMI (kg/m^2)	24.33 ± 3.25	24.76 ± 3.01	0.669
Current smoking (yes/no)	16/4	14/6	0.716
YMRS at admission	30.95 ± 8.33	–	
YMRS at the end of the study	2.10 ± 1.11		
Psychotic features	17	–	
Drug use at the end of the study			
Lithium	10 (50)		
Valproat	10 (50)		
Risperidone	6 (30)		
Ketiapine	6 (30)		
Olanzapine	4 (20)		
Aripiprazole	3 (15)		
Haloperidol	1 (5)		

Values are given as mean ± standard deviation or number (%). BMI, body mass index; YMRS, Young Mania Rating Scale.
*$p < 0.05$.

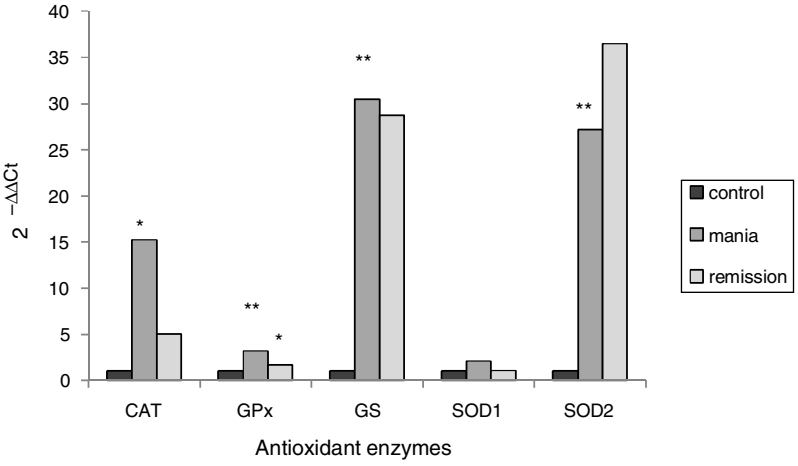

Figure 1 Gene expression levels of antioxidant enzymes, mean of $2^{-\Delta\Delta Ct}$.
CAT, catalase; GPx, glutathione peroxidase; GS, glutathione synthetase; SOD1, SOD2, superoxide dismutase 1, 2. *$p < 0.05$, **$p < 0.001$.

Table 3 Gene expression levels of antioxidant enzymes.

Patients in manic phase	CAT	GPx	GS	SOD1	SOD2
Mean ± sd	15.23 ± 38.56	3.21 ± 1.93	30.47 ± 36.91	2.08 ± 2.26	27.18 ± 46.08
Median	3.874	3.015	16.031	1.400	12.837
p values	0.022*	<0.001**	<0.001**	0.682	<0.001**

CAT, catalase; GPx, glutathione peroxidase; GS, glutathione synthetase; SOD1, SOD2, superoxide dismutase 1, 2.
The gene expression levels of controls are 1 (according to $2^{-\Delta\Delta Ct}$ method) and not represented in the table.
p values are calculated by using Mann–Whitney test.

Comparison of mania and controls

The bipolar patients in a manic phase had higher mean gene expression levels of SOD2, CAT, GPx and GS than the healthy subjects. The gene expression levels of SOD1 showed no significant difference between the two groups (Table 3, Fig. 1). The gene expression levels of antioxidant enzymes had no significant association with the YMRS scores (for CAT, $r = 0.360$; for GPx, $r = 0.230$; for GS, $r = 0.231$; for SOD1, $r = 0.064$; for SOD2, $r = 0.241$) ($p > 0.05$). The plasma levels of MDA showed no significant difference between the two groups (manic group = 0.113 ± 0.180 nmol/ml, control group = 0.064 ± 0.023 nmol/ml; $p = 0.935$).

Comparison of mania and remission

Twenty bipolar inpatients received follow-up appointments, in which the gene expression levels of their antioxidant enzymes and the plasma levels of their MDA were measured after treatment. The patients' (16 men and 4 women) mean hospitalization duration was 36 ± 7.82 days. The YMRS scores at baseline were 30.95 ± 8.33, and 2.10 ± 1.11 at the end point. We found significantly decreased changes in the expression levels of GPx in bipolar patients who had received treatment ($p = 0.003$), while other markers (SOD1,2, CAT, GPx, GS) showed no significant changes after treatment (Table 4, Fig. 1). The plasma levels of MDA at baseline were 0.113 ± 0.180 nmol/ml and 0.073 ± 0.030 nmol/ml at the end point, showing no significant change after treatment ($p = 0.654$).

Discussion

The most important finding in this study is that bipolar patients in a manic phase had significantly higher gene expression levels of SOD2, CAT, GPx and GS than the healthy controls. The expression levels of GPx mRNA decreased during the remission period compared to the levels in the manic state. It can be supposed that this dissimilarity formed as a compensating mechanism against the oxidative stress that increases during a manic episode.

Conflicting results were detected in clinical studies that investigated antioxidant enzyme levels in BD. Different and conflicting data in the literature could be a result of racial differences, diet properties, lifestyles that influence oxidative stress, differences between disease episodes, treatment differences and procedures used in studies.[12,14,18] Regarding the studies comparing patients with BD in a manic episode and healthy subjects, some show no increase, some show a decrease and others show no change in serum or erythrocyte SOD activities at all.[11,13,17,37]

Detecting no difference between the SOD activities of patient groups and healthy subjects, despite expectations that those of the patient groups would increase, can be interpreted as an indication that the changes at transcriptional levels were not actualized at the protein level. Since there is no data about differences between the gene expression levels of these enzymes in BD, in the current study, we aimed to investigate the SOD1 and SOD2 gene expression levels in order to fill this empty space in the literature. There are studies in which cytosolic SOD levels were detected in BD patients that were similar to that of the control group.[4,38] There were no significantly important differences between the SOD2 levels in a study in which postmortem prefrontal cortex samples of BD patients, schizophrenia patients and healthy subjects were evaluated.[39] Furthermore, there were no differences between the SOD1 and SOD2 levels of the postmortem dorsolateral prefrontal cortex cross-sections from BD patients, schizophrenia patients and healthy subjects.[40]

In most of the studies, SOD enzyme levels were not evaluated separately as cytosolic or mitochondrial. In our study, it was discovered that mitochondrial SOD gene enzyme levels were higher in manic patients than in healthy subjects. There were no statistically significant differences in cytosolic SOD levels between the two groups. The increase that was detected in the mitochondrial SOD expression level, which is the first antecedent of antioxidant defence, shows that oxidative stress may be increasing during the acute episode of the disease; antioxidant enzymes prevented this from happening in our study.

Increases were detected in the CAT gene expression levels during manic episodes compared to the controls in our study. This increase can be interpreted as a compensating response that is promoted synchronously with the increase in SOD expression that grows as the first defence step against oxidative stress. Some studies showed that there were increases or decreases in serum CAT levels during BD manic episodes.[11,14,17,19,38]

The GPx expression levels were higher in manic patients when compared to the controls. This increased levels may have a relation with increased expression of mitochondrial SOD that removes H2O2. A positive correlation was found between gene expression levels of SOD2 and GPx. Variable results were found in studies investigating GPx activity. For example, GPx activity was found significantly high during euthymic phase by Andreazza et al.[11] GPx levels were also found significantly higher in BD patients during depressive phase when compared to the controls.[38] However, some studies found unchanged GPx activity between BP patients and healthy controls.[12,14,41]

The significant increase in GS expression levels in the manic patients when compared to the control group may be due to the fact that glutathione synthesis plays an important role in the defence mechanism, which is composed of antioxidant enzymes against oxidative stress. Also, some studies show decreases in the glutathione levels of BD.[19,41] When BD patients in early or late stages were compared with each other in order to evaluate the effect of BD duration on antioxidant enzymes, there were no significant differences between the two groups regarding the gene expression levels of their antioxidant enzymes. However, an increase in antioxidant enzymes during the late stage was detected in antioxidant enzymes.[42] This difference between this study and our study could be a result of the shorter disease duration of the sample that composes our study.

In the current study, the antioxidant gene expression levels of the patients who were in manic episode and subsequent remission phase were found to be similar except for GPx. No differences were detected between the SOD, CAT and GPx levels before and after treatment in the patient group,[43] the fact that there were no differences in our study either could be interpreted as a result of continu-

Table 4 Changes in the gene expression levels of antioxidant enzymes and plasma MDA levels after teatment in bipolar patients.

Variable ($n = 20$)	CAT	GPx	GS	SOD1	SOD2	MDA(nmol/ml)	YMRS scores
Baseline	3.874	3.015	16.031	1.400	12.837	0.061	30.95 ± 8.33
Endpoint	2.524	1.042	4.323	0.195	10.572	0.070	2.10 ± 1.11
p value	0.650	0.003*	0.281	0.155	0.943	0.654	<0.001*

Median values are given in the table. SOD1, SOD2, superoxide dismutase 1, 2; CAT, catalase; GS, glutathione synthetase; GPx, glutathione peroxidase; MDA, malondialdehyde; YMRS, Young Mania Rating Scale.
* $p < 0.05$.

ing oxidative stress during a remission phase and continuing the compensatory response at the level of gene expression. In contrast, the CAT, GPx and MDA levels were found to show significant change after mood stabilizer treatment in BD patients.[4] Also, their TBARS and SOD levels decreased significantly after six months of lithium treatment for BD patients during depressive episodes.[38] In our study, patients were on different antipsychotic drugs, in addition to lithium and valproate. It was discovered that typical antipsychotics were increasing oxidants and decreasing antioxidants; conversely, atypical antipsychotics were decreasing oxidants and increasing antioxidants.[28,30,31,44] Ninety-five percent of the manic patients in our study were on atypical antipsychotic treatment; from the results, it can be supposed that atypical antipsychotic drugs had a mixed effect on their gene expression level changes during their remission phase.

We found similar levels of plasma MDA between patient and control groups in our study. Our findings are discordant with the results of the meta-analysis,[9] which found that thiobarbituric acid reactive substances (TBARS) increase in BD. However, some recent studies did not find any difference between the TBARS levels in BD like our study did.[14,45,46] Plasma TBARS levels were found to be similar to that of the control group in BD type I and type II, this similarity was explained by the simultaneous CAT and GPx increase.[38]

The plasma MDA levels of manic episode patients that have gone into remission after mood stabilizer treatment were detected as similar to their levels before treatment in our study. Mood stabilizer drugs that were given to patients in various studies were reported to produce some effects that helped decrease oxidative lipid damage. In the studies that were done with rat models, in which mania episodes were induced by amphetamine, lithium treatment was reported to decrease lipid peroxidation.[47–49] Clinical studies have been shown to reduce the plasma TBARS levels of lithium treatment in BD patients.[17,38,50,51] The effect of only mood stabilizer treatment on oxidative lipid damage was considered in the inspected studies, but various antipsychotic treatments were given to patients for treatment of manic episodes. This situation may have had an effect on the MDA levels that were measured during remission episodes.

Bipolar patients are known to have higher rates of smoking than the general population.[52] Nicotine exposure was shown to increase cytosolic ROS levels and leads to oxidative stress in numerous studies on cultured cells. The levels and activities of antioxidant enzymes such as SOD, CAT, GR and GPx were reported to decrease following nicotine exposure.[53] The subjects with substance abuse and dependence were excluded in our BD and control groups but the subjects with nicotine abuse and dependence were not excluded. Nearly all of our manic patients (80%; $n = 16$) and controls (70%; $n = 14$) were smokers. Therefore, the effects of smoking on oxidative stress molecules were not ruled out in our study.

Our study has some limitations: First, only the manic episodes of BD were investigated. Also, this research used a small sample size, and obtained information regarding the patients' diet and exercise habits based on subjective self-reports. Additionally, another limitation was the variability of the antipsychotic treatments given to our manic episode patient group and the inability of excluding the drug's effects. We analyzed mood stabilizing drug blood levels before enrolling the participants into the study. However, analysing antipsychotic drug blood levels were impossible due to laboratory limitations.

Since there is no data about the differences between the gene expression levels of the enzymes in BD, we aimed to investigate the SOD1 and SOD2 gene expression levels in order to fill this empty space in the literature. A strong point of our research is the study population, since it comprised BD patients with severe mania and no psychotropic drug use. Also, it involved the subsequent remission period of the same patients and healthy controls.

Conclusions

Detection of increases in CAT, GPx, GS and SOD2 in the gene expression levels during a manic episode may be due to the activation of antioxidant systems against oxidative stress that increases in patients. Research shows that oxidative stress plays an important role in BD pathophysiology, and the drugs that decrease oxidative stress can give hope for future treatment modalities of BD. Further research needs to be conducted in bigger samples than ours concerning antioxidant gene expression in all episodes of BD with gene expressions. Investigation of these differences in groups in which disorder did not yet arise, but of higher risk for BD and additional research specifically about mitochondrial SOD, GS, GPx and CAT enzymes could provide further information about the role of the differences in BD etiopathogenesis; as a result, newer diagnosis, treatment and follow-up options could be developed.

To the best of our knowledge, this is the first study in the field of psychiatry to show gene expression changes in oxidative stress biomarkers of BD patients and treatment-related changes in the levels of these biomarkers. Follow-up appointments with the same patient group will be needed in order to verify these findings.

Ethical considerations

This study was approved by the local ethics committee of the Trakya University Faculty of Medicine (Project No. 2014/113). All procedures followed were in accordance with The Code of Ethics of the World Medical Association and with the Helsinki Declaration.

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Conflict of interest

The authors have no conflict of interest to declare.

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