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# The Oxidative Stress May be Induced by the Elevated Homocysteine in Schizophrenic Patients

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**Abstract** The mechanisms of oxidative stress in schizophrenic patients are not fully understood. In the present study, we investigated the effect of elevated level of homocysteine (Hcys) on some parameters of oxidative stress, namely thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation in plasma, the level of carbonyl groups in plasma proteins, as well as the amount of 3-nitrotyrosine in plasma proteins isolated from schizophrenic patients. Patients hospitalised in I and II Psychiatric Department of Medical University in Lodz, Poland were interviewed with special questionnaire (treatment, course of diseases, dyskinesis and other EPS). According to DSM-IV criteria all patients had diagnosis of paranoid type. They were treated with antipsychotic drugs (clozapine, risperidone, olanzapine). Mean time of schizophrenia duration was about 5 years. High-performance liquid chromatography was used to analyse the total level of homocysteine in plasma. Levels of carbonyl groups and 3-nitrotyrosine residues in plasma proteins were measured by ELISA and a competition ELISA, respectively. The lipid peroxidation in plasma was measured by the level of TBARS. Our results showed that in schizophrenic patients the amount of homocysteine in plasma was higher in comparison with the control group. We also observed a statistically increased level of biomarkers of oxidative/nitrative stress such as carbonyl groups or 3-nitrotyrosine in plasma proteins from schizophrenic patients. Moreover, our experiments indicate that the correlation between the increased amount of homocysteine and the oxidative stress exists. Considering the data presented in this study, we suggest that the elevated Hcys in schizophrenic patients may stimulate the oxidative stress.

**Keywords** Schizophrenic disorders · Oxidative stress · Carbonyl group · 3-nitrotyrosine · Homocysteine

## Introduction

In schizophrenic patients dysregulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) metabolism, as detected by abnormal activities of critical antioxidant enzymes and other indicators—lipid peroxidation in plasma, red blood cells, blood platelets, and cerebrospinal fluid is observed [1–3]. Such abnormalities have been associated with tardive dyskinesia, negative symptoms, neurological signs and poor premorbid function. Li et al. [4] also suggest that excess ROS formation may play a critical role in the etiology of schizophrenia. A cell membrane dysfunction caused by lipid peroxidation can be secondary to a free radical–mediated pathology and may contribute to specific aspects of schizophrenic symptomatology and complications of its treatment. Our earlier studies by using different specific biomarkers of oxidative stress, including activity of platelet antioxidative enzyme—superoxide dismutase (SOD) revealed that in blood platelets from schizophrenic patients oxidative stress occurs [3]. We have presented that suppressed SOD activity in blood platelets from schizophrenic patients is

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associated with enhanced ROS generation and lipid peroxidation when compared with healthy control [3]. We also observed, that the level of isoprostanes (indicators of oxidative stress) in schizophrenic patients in acute period of psychosis is extremely high compared with control group [5], and our results indicate that in schizophrenic patients increased production of isoprostanes reflects oxidative stress and oxidative damage of lipids.

The modification of proteins plays an essential role in the pathogenesis of various diseases, including vascular complications, inflammatory and mental disorders. Our earlier results showed that the level of biomarkers of oxidation/nitration proteins in plasma of schizophrenic patients (in acute period of psychosis) is distinctly higher than in plasma of healthy subjects [6]. Moreover, in schizophrenic patients reduced status of plasma total antioxidant capacity was observed [7]. Our earlier results reported the changes of the level of low-molecular-weight thiols such as glutathione, cysteine and cysteinylglycine (which are physiological free radical scavengers) in plasma from schizophrenic patients, whereas the level of homocysteine (Hcys) was significantly elevated in plasma of schizophrenic patients in acute period of psychosis [6]. Because on the basis of various observations, it is proposed that Hcys may act as an oxidant in the model system in vitro and in vivo [8, 9], the aim of our present study was to explain the effect of the elevated Hcys on the selected parameters of oxidative stress (carbonyl groups and 3-nitrotyrosine levels in proteins and thiobarbituric acid reactive substances (TBARS)—a biomarker of lipid peroxidation) of plasma from schizo phrenic patients. Oxidative/nitrative changes in proteins include carbonyl groups formation and 3-nitrotyrosine generation. Nitration of tyrosine residues by nitric oxide—derived species results in the accumulation of 3-nitrotyrosine in proteins. Tyrosine nitration is also a biomarker of oxidative damage induced by peroxynitrite. Other protein modification mediated by free radical is protein carbonylation, which is a non enzymatic addition of aldehydes or ketones to specific amino acid residues.

## Materials and Methods

### Materials

Sheep anti-nitrotyrosine polyclonal antibodies were from Oxis (Portland, USA). Biotyninylated anti-goat/mouse/rabbit antibody and streptavidin-biotynylated horseradish peroxidase were from DAKO (Glostrup, Denmark). All other reagents were of analytical grade and were provided by commercial suppliers.

**Table 1** Clinical characteristics of patients with schizophrenia and healthy volunteers subjects

|  | Patients with schizophrenia ($n = 19$) | Control subject ($n = 19$) |
| --- | --- | --- |
| Sex, M/F | 11/8 | 15/4 |
| Age (years) | 30.4 ± 3.2 | 30.0 ± 3.1 |
| Duration of illness (years) | 8.4 ± 4.3 | NS |
| PANSS | 68.3 ± 13.6 | NS |
| PANSS-positive symptom scores | 11.2 ± 4.1 | NS |
| PANSS-negative symptom scores | 19.7 ± 5.8 | NS |

### The Criteria of Schizophrenic Patients Inclusion

The studied population of schizophrenic patients comprised 19 patients between the ages of 25–36 years (mean 30, 4 ± 3.2) who were hospitalized in I and II Psychiatric Department of Medical University in Lodz, Poland. All subjects were interviewed with special questionnaire (treatment, course of diseases, dyskinesis and other extrapyramidal syndromes) and according to DSM-IV criteria [10] all patients had diagnosis of paranoid type. They were treated with antipsychotic drugs (clozapine, risperidone, olanzapine) during therapy. They did not use addictive antidepressants or mood stabilizers. Mean time of schizophrenia duration was 5 years. Table 1 present clinical characteristics of patients with schizophrenia.

### The Criteria of Volunteers Inclusion

Blood samples were taken from 19 healthy volunteers (males and females) aged between 25 and 35 years (mean 30 ± 3.1). Blood samples were taken from healthy subjects without psychiatric, neurological or somatic disorders and history of head injuries, allergy and lipid or carbohydrate metabolism disorders, untreated with drugs. Healthy subjects did not use addictive substances and antioxidant supplementation, their diet was balanced (meat and vegetables), lived in similar socio-economic conditions. Subjects with significant medical illness were excluded.

### Qualification Questionnaire

Structured medical interviews were carried out, considering both mental and physical status, medical history, alimentary habits and substance use. The following exclusion criteria were applied: any somatic disorders, especially circulatory diseases, disorders of lipid metabolism and diabetes, malnutrition and neurological diseases serious head injuries mental disorders either in volunteers or their

families use of any medications or addictive substances, unbalanced diet antioxidant supplementation psychiatric examination (using the M.I.N.I.—Mini International Neuropsychiatric Interview [11]) neurological and somatic examinations. Laboratory tests: lipid panel (total cholesterol, LDL, HDL, triglycerides) and glucose.

The protocol was passed by the Committee for Research on Human Subjects of the Medical University of Lodz number RNN/899/2000.

All patients and volunteers included in the study have been informed about aims of the study and methods implemented and expressed their written informed consent for participation in this study.

### Isolation of Plasma

Human blood from schizophrenic patients and healthy volunteers was collected into sodium citrate (5 mM final concentration) and immediately centrifuged (3,000×*g*, 15 min) to get plasma.

### Evaluation of Lipid Peroxidation Level

Samples of plasma (from schizophrenic patients and healthy volunteers) were transferred to an equal volume of 20% (v/v) cold trichloroacetic acid in 0.6 M HCl and centrifuged at 1,200×*g* for 15 min. One volume of clear supernatant was mixed with 0.2 volume of 0.12 M thiobarbituric acid in 0.26 M Tris at pH 7.0 and immersed in a boiling water bath for 15 min. Absorbance at 532 nm was measured and results were expressed as nmoles of TBARS [12].

### Determination of 3-Nitrotyrosine in the Plasma Proteins by a C-ELISA Method

Detection of 3-nitrotyrosine-containing proteins by a competition ELISA (C-ELISA) method in plasma (from schizophrenic patients and healthy volunteers) was performed according to the procedure of Khan et al. [13] as described previously [14]. The nitro-fibrinogen (at concentration of 0.5 µg/ml and 3–6 mol nitrotyrosine/mol protein) was prepared for use in the standard curve. The linearity of the C-ELISA method was confirmed by the construction of a standard curve ranging from 10 to 500 nM nitrotyrosine-fibrinogen equivalent. The concentrations of nitrated proteins that inhibit anti-nitrotyrosine antibody binding were estimated from the standard curve and are expressed as nitro-Fg equivalents. The amount of nitrotyrosine present in fibrinogen after treatment with peroxynitrite (at final concentration of 1 mM) was determined spectrophotometrically (at pH 11.5, $\varepsilon_{430nm} = 4,400$ M$^{-1}$ cm$^{-1}$).

### Detection of Carbonyl Groups in the Plasma Proteins by ELISA Method

Detection of carbonyl groups by ELISA method (using anti-DNP antibodies) in plasma (from schizophrenic patients and healthy volunteers) was carried out according to a method described by Buss et al. [15] as described previously [14]. Human plasma proteins reacted with dinitrotrophenylhydrazine (DNP) and then proteins were non-specifically adsorbed to an ELISA plate. The peroxynitrite treated-fibrinogen (10 nmol of carbonyl groups/mg of fibrinogen) was prepared for use in the standard curve. The linearity of the ELISA method was confirmed by the construction of a standard curve ranging form 0.1 to 10 nmol carbonyl groups/mg of fibrinogen. The amount of carbonyl groups present in fibrinogen after treatment with peroxynitrite (at final concentration of 1 mM) was determined spectrophotometrically as described Levine et al. [16].

### Determination of Homocysteine in Plasma

The classical technique High-performance liquid chromatography (HPLC) has been used to analysis of homocysteine from human plasma (from schizophrenic patients and healthy volunteers). HPLC analysis was performed with a Hewlett-Packard 1100 Series system according to Głowacki et al. [17] and Bald et al. [18].

### Statistical Analyses

All the values in this study were expressed as mean ± SD. In order to eliminate uncertain data, Grubbs test was performed. The statistically significant difference between the control group and schizophrenic patients was done by Mann–Whitney test using StatSoft Inc. "Statistica" v. 6.0. Regression line was calculated by means of the least-squares method.

## Results

Using HPLC method we determined in human plasma the levels of homocysteine. Our studies have shown that the level of homocysteine in plasma from schizophrenic patients was significantly higher (about 55%) than in plasma obtained from healthy volunteers (Table 2). We have also observed that the level of different biomarkers of oxidative stress in plasma from schizophrenic patients differs from their level in plasma obtained from healthy volunteers (Table 2). The level of carbonyl groups (determined by ELISA method) in plasma proteins from schizophrenic patients was significantly higher than the

**Table 2** Changes of the total level of homocysteine and the level of selected biomarkers of oxidative stress in plasma of healthy subjects and schizophrenic patients

| | The total level of homocysteine ($\mu$M) | The level of carbonyl groups in plasma proteins (nmol carbonyl groups/mg of plasma proteins) | The level of 3-nitrotyrosine in plasma proteins (nmol 3-nitrotyrosine/mg of plasma proteins) | The level of TBARS (nmol TBARS/ml of plasma) |
|---|---|---|---|---|
| Healthy subjects | $6.163 \pm 0.508$ | $0.178 \pm 0.017$ | $0.0075 \pm 0.0013$ | $1.109 \pm 0.083$ |
| Schizophrenic patients | $13.010 \pm 1.082$ ($P = 1.96 \times 10^{-6}$) | $0.482 \pm 0.065$ ($P = 3.1 \times 10^{-4}$) | $0.044 \pm 0.0072$ ($P = 3.6 \times 10^{-6}$) | $1.655 \pm 0.105$ ($P = 1.6 \times 10^{-4}$) |

The results are representative of independent experiments in triplicate and expressed as a mean $\pm$ SD. The statistical analysis of difference between the tested groups was done using Mann–Whitney test
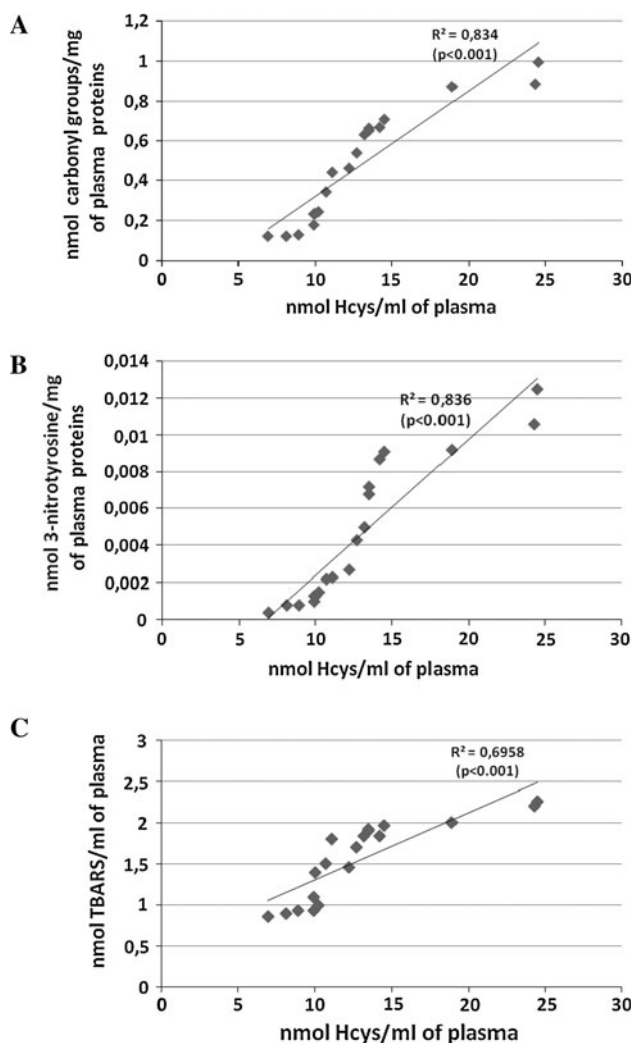


**Fig. 1** The correlation between the selected parameters of oxidative stress [the level of carbonyl groups (**a**), the level of 3-nitrotyrosine (**b**), the level of TBARS (**c**)] and the total level of homocysteine in plasma obtained from schizophrenic patients. Regression line was calculated by means of the least-squares method

determined by a competition C-ELISA method was also higher than in control group (Table 2). We have observed the same process when we measured the lipid peroxidation (Table 2).

The correlation between the increased amount of Hcys and changes in the level of various biomarkers of oxidative stress in plasma from schizophrenic patients is presented in Fig. 1.

## Discussion

L-Homocysteine is an endogenous amino acid, containing the free thiol group, which in healthy cells is involved in methionine and cysteine synthesis/resynthesis. Indirectly, Hcys participates in methyl, folate, and cellular thiols metabolism [19]. Approximately 80% of total plasma Hcys is protein bound, and only a small amount exists as a free reduced Hcys (about 0.1 $\mu$M). The majority of the unbound portion of Hcys is oxidized to form dimers (homocystine) or combined with cysteine to form mixed disulphides [20, 21]. Elevated concentrations of homocysteine in human tissues, defined as hyperhomocysteinemia have been correlated with some diseases, such as cardiovascular, neurodegenerative, and kidney disorders. The elevated level of homocysteine has been repeatedly observed in patients with schizophrenia [6, 22–24]. Molecular-genetic studies revealed tha association between schizophrenia and polymorphisms of two genes—methylenetetrahydrofolate reductase and cystathionine-beta-synthase involved in the conversion of homocysteine to methionine and cysteine, respectively [23]. Haidemenos et al. [25] also observed that patients in chronic schizophrenia had increased the amount of plasma homocysteine compared to control, but this increase in plasma homocysteine is not related to plasma folate and vitamin B$_{12}$ levels. In the Tunisian population, hyperhomocysteinemia in schizophrenia seems to be linked to vitamin B$_{12}$ deficiency, likely caused by a lack of dietary animals proteins [22]. Results of Kim and Moon [24] showed that Korean schizophrenic patients with high serum

level of carbonylation in plasma obtained from healthy volunteers (Table 2). In plasma proteins from schizophrenic patients the amount of 3-nitrotyrosine as

homocysteine levels may have the genetic defect of having low folate serum levels.

Increased concentration of homocysteine in the blood may not only be an independent risk factor for atherosclerotic disease, deep vein thrombosis and thromboembolism, and may promote also cerebrovascular diseases. Bleich et al. [26] and Sachdev et al. [27] showed a significant positive relationship between plasma homocysteine levels and brain atrophy. Brown et al. [28] suggest that elevated third-trimester homocysteine levels may increase schizophrenia risk through developmental effects on brain structure and function and/or through subtle damage to the placental vasculature that compromises oxygen delivery to the fetus. Results of Song et al. [29] showed that homocysteine metabolism and monoaminergic neurotransmitter systems are important in schizophrenia pathology. They hypothesized that the gene PNPO (pyridoxine 5′-phosphatase oxidase gene) might be a candidate for susceptibility to schizophrenia because PNPO encodes pyridoxamine 5′-phosphate oxidase (EC 1.4.3.5), a rate-limiting enzyme in pyridoxal 5′-phosphate (PLP, vitamin B(6)) synthesis. PLP is a metabolically-active form of vitamin B(6) and thus, is required as a co-factor for enzymes involved in both homocysteine metabolism and synthesis of neurotransmitters such as catecholamine. Moreover, some results suggest that association of homocysteine with schizophrenia may involve the glutamatergic system [30–32]. Homocysteine may act as an antagonist at the glycine site of the NMDA receptor (in the presence of normal or low glycine levels) or it may act as an agonist at the glutamate site of this receptor (when glycine levels are increased) [32]. Homocysteine may also enhance oxidative stress [31, 33]. Our earlier [3, 5] and present results (Table 2) or the results of Yao et al. [34] suggest that schizophrenia is characterized by abnormal oxidative stress. The present study provides more information about the mechanisms of oxidative stress in schizophrenic patients. The first time, our results showed that the elevated level of Hcys may play an important role in the oxidative stress in schizophrenic patients (in an acute period of psychosis; Fig. 1). Experiments presented here showed that the correlation between the increased amount of Hcys and the oxidative stress in plasma from schizophrenic patients exists (Fig. 1). It should be underlined that in our present study all schizophrenic patients were treated with second-generation anti-psychotic drugs, which do not induce the oxidative stress in different elements of blood, including blood platelets and plasma [35, 36]. However, typical antipsychotic drugs (chlorpromazine) may play a role as modifying factor for folate metabolism in chronic schizophrenic patients [37], but in our present experiments patients were treated with other antipsychotic drugs (clozapine, risperidone, olanzapine) during therapy. Moreover, results of Henderson et al. [38] did not show association with second-generation anti-psychotic drugs (clozapine, risperidone,

olanzapine) and Hcys levels in schizophrenic patients. Experiments of Neeman et al. [39] also demonstrated that plasma levels of amino acids, including Hcys in normal subjects and patients treated with these drugs did not differ significantly.

On the basis of various observations, it proposed that diet polyphenolic antioxidants can inhibit the oxidative stress induced by Hcys [8, 40], therefore the next step of future studies is to evaluate the role of different antioxidants in the oxidative stress in schizophrenic patients, which have the elevated Hcys.

**Conflict of interest** None.

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