Effect of Lipid Peroxidation and Protein Carbonylation on Altered Na\(^+\)/K\(^+\)-ATPase Activity in Newly Diagnosed Schizophrenia Patients

**ABSTRACT**

**Background** Alteration of Na\(^+\)/K\(^+\)-ATPase activity is found in several neuropsychiatric disorders including schizophrenia. Effect of oxidative stress has been put forward as a plausible mechanism for this, although with variable results.

**Aim** To assess the effects of oxidative stress induced membrane lipid damage and cellular protein alteration on Na\(^+\)/K\(^+\)-ATPase activity in newly diagnosed schizophrenia patients.

**Settings and Design** It was undertaken as a hospital-based horizontal, observational case control study in a tertiary care urban hospital involving 46 cases and 50 controls spanning a period of 1 year.

**Materials and Methods** RBC membrane Na\(^+\)/K\(^+\)-ATPase levels were measured by the NADH/ATP coupled kinetic assay method. Serum thiobarbituric acid reacting substances (TBARS) and protein carbonyl (PC) values were measured by spectrophotometric techniques. Tissue protein was estimated by Lowry’s method.

**Statistical Analysis** After testing for normal distribution of the data obtained, independent t test, Pearson’s correlation analysis and multiple linear regression analyses were performed to analyse the difference between mean values, strength of association between parameters and predictive values of oxidative stress parameters on the Na\(^+\)/K\(^+\)-ATPase activity, respectively.

**Results** Membrane Na\(^+\)/K\(^+\)-ATPase activity was significantly lower with significantly higher values of serum TBARS and PC in cases \((p < 0.001)\). Although, both PC and TBARS showed significant negative correlation with Na\(^+\)/K\(^+\)-ATPase activity, it was found to be dependent significantly on the serum TBARS values only.

**Conclusion** The study indicates that although, the increased oxidative stress in schizophrenia damages both membrane lipids and tissue proteins significantly, the compromised Na\(^+\)/K\(^+\)-ATPase activity is more crucially dependent on membrane lipid damage.

**KEYWORDS** Na\(^+\)/K\(^+\)-ATPase, TBARS, protein carbonylation, lipid peroxidation

INTRODUCTION

Schizophrenia is a psychiatric disorder, characterized by delusions, hallucinations and disorders of formal thought process, persistent bizarre behaviour with or without alterations in affective behaviour signifying its heterogeneity. The heterogeneous psychiatric phenotypes are found in all age groups encompassing the childhood, adolescent and adult ages affecting about 2.4 million people in the USA and about 50 million people worldwide. In India, its prevalence varied from 2.49 to 2.6 per thousand. Different studies have suggested its prevalence a little higher among the males e.g., a ratio of 1.4 by McGrath et al. and 1.5 by Dube et al. Although, the morbidity risk for schizophrenia over the total lifespan appears to be equal among both genders i.e., 1.6%, its overall mortality tends to be higher in males thus resulting in higher prevalence of this disorder among women. Till now several phenomenology, clinical and psychosocial variables in schizophrenia have been investigated. Among them are the increased association of the disease with certain blood group antigens, lymphocytic abnormalities, abnormal expression of serum proteins, increased urinary levels of catecholamines a hyperdopaminergic state in certain brain areas, deranged redox balance etc. that have been associated with schizophrenia damages both membrane lipids and tissue proteins significantly, the compromised Na\(^+\)/K\(^+\)-ATPase activity is more crucially dependent on membrane lipid damage.
put forward as different biochemical abnormalities contributing to the development of the disease. Most of these are linked to different genetic predispositions suggesting this disease to be a polygenic disorder. Different biochemical abnormalities and neuroimaging techniques indicated close links between the cognitive impairment of schizophrenia and the deranged network between the prefrontal cortex and its connections with other brain areas along with an altered glucose metabolism in the regions of basal ganglia, medial temporal regions and the left thalamic regions. Genome wide association studies (GWAS) have indicated several genes to be implicated in schizophrenia, the strongest association being with the xMHC region (6p21.32–p22.2) and at least ten other loci as evident from the GWAS.

An optimum Na+/K+-ATPase activity is essential to maintain the normal neuronal excitability as it maintains the normal membrane polarization. This pump is a supramolecular structure composed by separate alpha and beta subunits. Among the alpha subunits, alpha 1 is found in prefrontal cortex, alpha 2 in the glial cells and alpha 3 exclusively in the neuronal tissues. Abnormalities of Na+/K+-ATPase activity have been reported to be associated with several neuropsychiatric disorders like seizures, bipolar disorders, spongiform encephalopathy, Alzheimer’s disease etc. Na+/K+-ATPase activity has been suggested to play a major role in the pathogenesis of schizophrenia. In experimental animals, MK-801-induced schizophrenia was found to be reversed by antipsychotic treatment along with a significant increased Na+/K+-ATPase activity. Recent studies involving mass spectrometry have reported disrupted activity of Na+/K+-ATPase pump and linked it with the derangement in the Na+ dependent glutamate transporters found in schizophrenia. However, the alteration in the role of sodium pump in schizophrenia has not been consistent. Transcription factors like Sp4 opposes the function of alpha 1, complements the functions of alpha 3 and acts parallel with the beta subunit of the sodium pump. Accordingly some studies proposed a possible increase in its activity in prefrontal cortex in animal models for development of schizophrenia.

Oxidative stress-induced derangement in redox balance has been found to be associated with several neuropsychiatric disorders like bipolar disorders (BDs), major depression, obsessive compulsive disorder (OCD) and schizophrenia and in some of them like BD it has been clearly reported to be directly associated to the decrease in Na+/K+-ATPase pump activity thus signifying a direct effect of oxidative stress on its activity. Treatment with MK 801, a chemical inducing schizophrenia like symptoms in animal models, was found to cause substantial increases in the lipid peroxidation marker thiobarbituric acid reacting substances (TBARS) in zebra fish brain along with a significant decrease in the Na+/K+-ATPase activity that was successfully prevented by antipsychotic therapy with haloperidol, olanzapine or clozapine.

In cultured cells also an oxidative stress-induced reduction in Na+/K+-ATPase activity was found to be reversed by protective molecules like beta fibroblast growth factor (bFGF). A direct role of oxidative stress has been suggested in reducing the Na+/K+-ATPase activity in patients suffering from homocysteinuria that was successfully prevented by pre-treatment with vitamin E and C. On the other hand, studies have also reported that Na+/K+-ATPase activity can be increased by oxidative stress in the cardiac myocytes. Thus, although the role of oxidative stress on the regulation of Na+/K+-ATPase activity in schizophrenia is evident from several studies, its contribution or interference with the pump activity is incongruent so far. Furthermore, relative effects of the oxidative stress-induced cellular protein damage and lipid peroxidation on the Na+/K+-ATPase pump activity is also not consistently reported. Under these conditions, we hypothesised that there should be a compromised Na+/K+-ATPase activity in schizophrenia subjects in our population that might be directly dependent on lipid peroxidation and cellular protein damage due to oxidative stress. Accordingly, we designed the present case control study to find out the changes in Na+/K+-ATPase activity in newly diagnosed schizophrenia patient and whether this change, if any, showed any significant dependence on the cellular protein damage or membrane lipid peroxidation both or in isolation.

**MATERIALS AND METHODS**

**Study design**

The present study was undertaken as a cross-sectional, observational hospital-based study spanning duration of 1 year from August 2014 to August 2015. Newly diagnosed cases of schizophrenia patients were selected on convenience basis from the outpatient Department of the Psychiatry of a tertiary care medical college & hospital following prefixed inclusion and exclusion criteria. Inclusion criteria included the following: (1) cases diagnosed as schizophrenia by DSM IV-R criteria and (2) patients in the age group of 17–60 years for both males and females with no gender discrimination. Exclusion criteria included the following: (1) patients suffering from any neuropsychiatric disorder other than schizophrenia, (2) schizoaffective patients or any mixed type of psychiatric disorder, (3) patients with any drug addiction, chronic smoking habit or alcohol addiction and (4) patients suffering from any metabolic disorder like diabetes mellitus, any endocrinological diseases like disorders of the pituitary, thyroid or adrenal gland, malignant disorders or any chronic inflammatory conditions were also excluded from the study.

Control subjects were selected from the age matched healthy female subjects attending the patients to the psychiatry OPD. First degree relatives were not considered.

During selection of both cases and control subjects it was considered that they were from the similar socio-economic status.
Informed consents were taken from all participants following the protocol and guidelines of the Helsinki declaration 1975, revised in 2000 and the work was started after obtaining written approval from the properly constituted institutional ethical committee.

**Measurement of study parameters**

1. **Measurement of Na⁺/K⁺-ATPase activity**
   
   Na⁺/K⁺-ATPase activity was measured from RBC membrane as RBC membrane sodium pump activity correlates well with the neuronal cell sodium pump activity. It was done in following steps as described by Noori et al (2009):)

   a. Preparation of RBC haemolysate: The packed red cells were extracted by centrifugation at 4°C, 450 g for 15 min and then resuspended and diluted in 25 volume of 0.011 mol/L Tris-HCl buffer at pH 7.4. The haemolysed cells were centrifuged for 30 min at 12,000 rpm at 4°C and the membrane pellet was resuspended in 30 ml of 0.011 mol/L Tris-HCl buffer. This centrifugation step was repeated three times. The final concentration of the membrane suspension was adjusted to about 4 mg protein/ml of Tris buffer for optimum measurement of the enzyme activity. The membrane suspension was stored at –20°C until the assay was performed.

   b. Measurement of Na⁺/K⁺-ATPase activity: The enzyme was assayed by linked enzyme system with the sequence of reactions which is based on an ATP-regenerating system, where the linear rate of NADH oxidation correlates to the hydrolysis of ATP. One unit (1 U) of ATPase represents 1 μmol of NADH oxidation per minute. The reaction mixture was put in an UV spectrophotometer and the baseline was established by measuring the extinction at 340 nm for 1–2 min. At the end of this time, 50 μl of suitably diluted sample was added and the reaction was initiated by adding 100 μl of ATP solution. Once a good linear rate was established, 100 μl of ouabain was added and the rate was measured after addition of the inhibitor. The Na⁺/K⁺-ATPase activity was taken to be the difference between these two rates (ΔA/minute).

   \[ \Delta A/\text{minute for Na}^+ / \text{K}^+ -\text{ATPase activity} = \Delta A/\text{minute before addition of Ouabain} - \Delta A/\text{minute after addition of ouabain} \]

   Final value was expressed in units or milli units (1 μmol/min = 1 unit).

   RBC membrane protein was measured by Lowry method using Folin-Ciocalteu reagent and the membrane Na⁺/K⁺-ATPase activity was finally expressed in IU/mg of tissue protein.

2. **Measurement of serum TBARS**

   Serum TBARS was measured by precipitating the proteins out of 0.5 ml of serum by 2.5 ml 10% trichloroacetic acid (TCA) and standing for 10 min. After adding 2.5 ml of 0.05 M H₂SO₄ and vortexing to mix well, 3.75 ml of thio-barbituric acid was added and the reaction mixture was kept in boiling water for 30 min. After cooling, the chromogen was extracted by mixing 4 ml of n-butanol, mixing by vortex and centrifugation at 3000 rpm for 10 min. The colour of the supernatant was measured at 532 nm in a spectrophotometer and the absorbance was converted into the TBARS concentration in nmol/ml by calibrating against a standard curve prepared with the 2.5, 5, 7.5, 10 and 12.5 nmol/ml of 1’1’3’3’ tetraethoxypropane obtained from Fluka, Germany.

**Data collection and analysis**

The data obtained were compared for the significance of difference between the mean values of case and control groups by independent student’s t-test. Strength of association between the study parameters in the case group was assessed by bivariate Pearson correlation study. The degree of predictive values of different study parameters on the Na⁺/K⁺-ATPase activity was analysed by multiple linear regression analysis. All statistical analyses were done with the SPSS 17 software for Windows. P value was considered significant at a level less than 0.05 for a 95% confidence interval.

**RESULT ANALYSIS**

On comparison of the data between case and control groups, results shown in Table 1 exhibited that the serum Na⁺/K⁺-ATPase activity of schizophrenia patients were significantly lowered. On the other hand, the oxidative stress-induced membrane lipid damage indicator TBARS, and the protein adduct indicator PC, were significantly higher in them (P < 0.001). These results indicated a significant amount of reduction in the Na⁺/K⁺-ATPase activity in newly diagnosed schizophrenia patients with a shift of the redox balance towards oxidative...
stress leading to considerable damage to the cellular proteins and membrane lipids.

In Table 2, Pearson’s bivariate correlation analysis showed that decrease in the Na⁺/K⁺-ATPase pump activity was linearly associated with increases in TBARS and PC levels both with TBARS values showing a stronger degree of this association than the PC levels. Although the outcomes of the bivariate correlation analysis are true from both sides, our results suggested that lipid peroxidation may play a more critical role in reducing the Na⁺/K⁺-ATPase activity in schizophrenia patients. To ascertain it more significantly we carried out the multiple linear regression analysis considering the Na⁺/K⁺-ATPase activity as a dependent factor and serum TBARS and PC levels as its predictors.

Results of multiple linear regression in Table 3 revealed that the lipid peroxidation product TBARS showed a more significant negative predictive value (β = –0.701) than that of the PC adducts (β = –0.116) on Na⁺/K⁺-ATPase pump activity. Level of statistical significance (P < 0.001 and 0.098 for TBARS and PC adducts, respectively) also confirmed that decrease in the Na⁺/K⁺-ATPase activity was significantly dependent on the elevations in TBARS levels only.

**DISCUSSION**

In the present study, a significant reduction in the Na⁺/K⁺-ATPase activity in the schizophrenia patients in comparison to the control subjects indicated a close link between this neuropsychiatric disorder and a compromised stress and lipid peroxidation processes. It is already known that Na⁺/K⁺-ATPase is inhibited in schizophrenia by an excess of dopaminergic activity that is supposed to cause a substantial inhibitory effect on the Na⁺/K⁺-ATPase activity by several mechanisms like cAMP and protein kinase A (PKA) mediated phosphorylation of the alpha subunits and a decreased intracellular calcium concentration [Ca²⁺]. A significant role of oxidative stress on the Na⁺/K⁺-ATPase activity has been observed in our study which is associated with a significant increase in the levels of oxidative stress induced lipid peroxidation product TBARS in the schizophrenia patients (independent t-test, Table 1). In support of these findings, there are several studies which reported that the structural integrity of functional proteins.

**Table 1** Results of independent t-test showing comparison between the mean values of case and control groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Na⁺/K⁺ ATPase activity in IU/mg of tissue protein (Mean ± SD)</th>
<th>Serum TBARS in nmol/ml (Mean ± SD)</th>
<th>Serum protein carbonyl in nmol/mg of protein (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases (n = 60)</td>
<td>4.40 ± 2.19</td>
<td>5.64 ± 0.87</td>
<td>1.86 ± 0.34</td>
</tr>
<tr>
<td>Controls (n = 50)</td>
<td>28.99 ± 5.57</td>
<td>3.0 ± 0.63</td>
<td>1.19 ± 0.14</td>
</tr>
<tr>
<td>t value</td>
<td>–31.38</td>
<td>17.31</td>
<td>12.24</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*P value was considered significant at a level of P < 0.05 for a 95% of confidence interval.

**Table 2** Bivariate Pearson correlation analysis showing the strength of relationship between the study parameters in the case group (n = 46).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Na⁺/K⁺ ATPase activity in IU/mg of tissue protein</th>
<th>Serum TBARS in nmol/ml</th>
<th>Serum protein carbonyl in nmol/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>r value</td>
<td>1</td>
<td>–0.709</td>
<td>–0.014</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>0.116</td>
</tr>
</tbody>
</table>

*P value was considered significant at a level of P < 0.05 for a 95% of confidence interval.

**Table 3** Multiple linear regression analysis showing the relative dependence of Na⁺/K⁺-ATPase activity on oxidative stress parameters in the schizophrenia patients.

<table>
<thead>
<tr>
<th>Coefficienta</th>
<th>Model</th>
<th>Beta (β)</th>
<th>t value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>10.377</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum TBARS in nmol/ml</td>
<td>–0.701</td>
<td>–0.598</td>
<td>&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>Serum protein carbonyl in nmol/mg of protein</td>
<td>–0.014</td>
<td>–0.116</td>
<td>0.908</td>
<td></td>
</tr>
</tbody>
</table>

aNa⁺/K⁺-ATPase activity in IU/mg of tissue protein. *P value was considered significant at a level of P < 0.05 for a 95% of confidence interval.
lips in the synaptosomal membranes are essential for proper maintenance of this pump activity and even minute amounts of reactive oxygen species (ROS) can cause a functional impairment in its activity in the brain tissues. Thiol groups (-SH) of proteins are particularly susceptible to the oxidative stress induced lipid peroxidation that make the cysteine SH groups in the alpha subunit particularly vulnerable to the oxidative stress. ROS can also activate the NF-kB pathway and lower the [Ca\(_2\)] level thus lowering the sodium pump activity. Furthermore, dopamine excess in schizophrenia leads to generation of several quinone products that further accentuates the oxidative stress induced lipid peroxidation in the membrane and thereby cause significant damage to the Na\(^+\)/K\(^+\)-ATPase pump.

In addition to the membrane lipids, recent findings described significant increases in protein carbonyl products also in the serum of schizophrenia patients. Protein molecules represent significant amounts of oxidative stress susceptible molecules in the brain neuronal tissues. Their constituent amino acids are variably damaged by oxidative stress producing carbonyl adducts, oxidized thiol groups and production of nitrosotyrosines. These changes in the antioxidant enzymes further reduce their activities and aggravate the impaired redox balance in the tissues. Protein carbonyls are the markers of oxidative stress induced non-enzymatic addition of aldehyde or ketone groups to some amino acids like histidine, threonine, lysine, proline, cysteine and arginine. This helps in explaining the significant negative bivariate relationship of the Na\(^+\)/K\(^+\)-ATPase activity with increased lipid peroxidation product TBARS, and the cellular protein damage marker PC adducts in our case population (Table 2).

However, different mechanisms involved in the oxidative stress induced damage to the Na\(^+\)/K\(^+\)-ATPase pump herald a variable degree of effects of lipid peroxidation and cellular protein oxidation on its activity. In our present study, when the predictive values of both protein carbonylation and lipid peroxidation on the Na\(^+\)/K\(^+\)-ATPase activity were compared, the latter one was found to play a statistically significant negative predictive value on the pump activity only (\(\beta = -0.701, P < 0.001\), in Table 3). These observations signify that lipid peroxidation induced membrane damage plays a more crucial role in reducing the Na\(^+\)/K\(^+\)-ATPase activity in schizophrenia patients. This may be explicable from the fact that the sodium pump is an integral membrane protein lipid supramolecular structure that is susceptible to even minor amounts of oxidative stress products including hydroxyl radicals, superoxide anions, singlet oxygen, hypochlorous anion and hyperchlorite anion all of which are in close association with this membrane pump. Furthermore, increased dopaminergic activity in schizophrenia augments lipid peroxidation in the synaptosomal membranes. Apart from its alpha and beta subunits, its independent regulator protein FXYD domains situated inside the lipid bilayer membrane, are also susceptible to oxidative stress induced lipid peroxidation that leads to a comprehensive derange-ment in its structure and function as well. This has been evident in our study where a significant dependence (\(\beta = -0.701, P < 0.001\), in Table 3) of the Na\(^+\)/K\(^+\)-ATPase activity has been observed on the lipid peroxidation marker, TBARS without any such dependence on the cellular protein oxidation. Our findings are in close agreement with some recent findings that observed a decrease in Na\(^+\)/K\(^+\)-ATPase activity in animal model with rise in lipid peroxidation but without any effect of increased protein carbonyl products.

In conclusion, results of our study indicate that the compromised Na\(^+\)/K\(^+\)-ATPase activity in schizophrenia is crucially dependent on the degree of oxidative stress induced membrane lipid damage and although there is a significant increase in the oxidative stress-induced cellular protein damage, it does not have any significant predictive value on the Na\(^+\)/K\(^+\)-ATPase activity in biomembranes. These views strengthen the close interaction between the optimum activity of this pump with the surrounding lipid environment in the membrane along with the key link between the signaling pathways generated in the biomembrane with a proper maintenance of Na\(^+\)/K\(^+\)-ATPase activity. Our findings will help to understand the need for a stringent control over ROS generation in schizophrenia patients as well as to undertake further research to elucidate the detailed mechanism of the Na\(^+\)/K\(^+\)-ATPase activity and its regulation by structural and functional changes in the surrounding membrane.