Potential role of some oxidant/antioxidant status parameters in prefrontal cortex of rat brain in an experimental psychosis model and the protective effects of melatonin

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Abstract. – **OBJECTIVES:** The etiology of schizophrenia is unknown. However, some of the neuropathological changes in schizophrenia may be the result of increased free radical-mediated or reactive oxygen species (ROS) mediated neurotoxicity. Melatonin is a hormone produced especially at night in the pineal gland; additionally is a highly important antioxidant. The aim of this study is to indicate the contribution effect of the neuropathophysiology of schizophrenia and protective effects of melatonin against this oxidative damaged. MK-801 induced selective neurotoxicity has been proposed as an animal model for psychosis.

MATERIALS AND METHODS: 21 healthy adult and male Wistar albino rats were divided into three groups. MK-801 was given intraperitoneally for 5 days in experimental psychosis group. Melatonin was given to the treatment group for 6 days by intraperitoneally. In control group, saline was given in the same way. At the 7th day of the experiments, rats were killed by decapitation. Brains were removed and prefrontal part of the brain was divided for biochemical analyses.

RESULTS: Some antioxidant enzymes, malondialdehyde and protein carbonyl analyses were made by spectrophotometric methods. SOD, GSH-Px, XO activities and malondialdehyde, protein carbonyl and NO levels were found to be increased significantly in prefrontal cortex of MK-801 group (p < 0.0001) compared to the control group. In melatonin treated rats, prefrontal tissue malondialdehyde and protein carbonyl levels were decreased significantly in comparison with MK-801 group (p < 0.0001).

CONCLUSIONS: MK-801 may induce oxidative stress in prefrontal cortex of rats. This experimental study provides some evidences for the protective effects of melatonin on MK-801-induced changes in prefrontal rat cortex.

Introduction

The N-methyl-D-aspartate (NMDA) receptor antagonists, such as phencyclidine (PCP) and MK-801 (dizocilpine maleate) have been shown to exacerbate psychotic symptoms in schizophrenia and have been proposed as a model both for the positive and the negative symptoms of schizophrenia[1]. MK-801, an uncompetitive blocker of the opened ion channel of NMDA receptor was shown to be one of the most neurotoxic NMDA receptor antagonists[2]. Findings demonstrate that a protracted NRhypo state can trigger neuronal injury throughout many corticolimbic brain regions[3]. Pharmacologic or genetic models of NMDA receptor hypofunction has significant potential as animal models of the pathophysiology of schizophrenia and as preclinical screening paradigms for the identification of mechanistically novel antipsychotic drugs[4]. Therefore, determining the mechanisms of neurotoxicity of NMDA antagonists in animals may help clarify the mechanisms of schizophrenialike psychosis in humans. On the other hand, there is great evidence that reactive oxygen species (ROS) are involved in membrane pathology in the central nervous system (CNS) and may play a role in neuropsychiatric disorders including schizophrenia[5]. Melatonin is a hormone (N-acetyl-5 methoxytryptamine) produced especially at night in the pineal gland. Its secretion is stimulated by dark and in-

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hibited by light. Melatonin is a highly important antioxidant. Melatonin possesses strong antioxidant activity by which it protects cells, tissues and organs from the oxidative damage caused by ROS, especially the hydroxyl radical ($\cdot$OH), which attacks DNA, proteins and lipids and causes pathogenesis. Out with its well-known efficacy in sleep induction and circadian rhythm modulation[6], melatonin's role in the etiology, course and treatment of schizophrenia has received relatively little attention[7]. Melatonin has a number of potential effects relevant to the context of schizophrenia, including its etiology, pathophysiology and on the prevention of the metabolic and other side effects induced by anti-psychotics[8,9]. Both dopamine receptor supersensitivity and oxidative stress-induced neurotoxicity in the nigrostriatal system are apparently implicated. Melatonin is a potent antioxidant and attenuates dopaminergic activity in the striatum and dopamine release from the hypothalamus.

As far as we know, there is no experimental study concerning the effect of melatonin treatment in experimental psychosis model. Also, there is no study addressed on the involvement of free radical damage in experimental psychosis model of rat and, furthermore, there is no study in experimental psychosis model on the antioxidant mechanisms of melatonin treatment.

This study aims to highlight the relevance of alterations in melatonin in the etiology and maintenance of schizophrenia. It is proposed that its adjuvant use will prevent many side effects of typical and atypical antipsychotics that contribute to decreased longevity and quality of life. We hypothesized that the damaging effect of ROS might have an important role in MK-801 induced model of psychosis and melatonin may affect the oxidant/antioxidant status of brain by stabilizing the membranous structures of cell and, thus, has therapeutic effect for schizophrenia.

Materials and Methods

Animals and Drug Treatment

Healthy adult and male Wistar albino rats (n=21) were divided randomly into three groups. Food and water were provided *ad libitum* throughout the treatment. The rats were kept in the room temperature (20-22ºC), the humidity level was 40-50% and light was 12h day/12h night cycle. The rats in the group I (n=7) were used as the control. The rats in the group II as experimental psychosis model were injected MK-801. The rats in the group III as treatment group (n=7), received melatonin while exposed to MK-801. MK-801 (5R, 10S-(4)-5-methyl-10, 11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine hydrogen maleate) was supplied by Sigma (St. Louis, MO, USA) and dissolved in 0.9% saline. MK-801 was prepared daily. MK-801 was injected intraperitoneally (IP) at the dose of 0,5 mg/kg/day once a day for 5 days. Melatonin (50 mg/kg/day) was given to the treatment group for 6 days IP. Pretreatment with melatonin was started one day before MK-801 treatment.

In control group (n=7), saline was given in the same way. During 5 experiment days, administration of MK-801 was performed 30 minutes after melatonin application. Control rats were given isotonic saline solution (an equal volume of MK-801) by the rout of intraperitoneal injection. At the 7th day of the experiment, rats were killed by decapitation and prefrontal cortex (PFC) was removed immediately for the biochemical analyzes. International standard for principles of laboratory animal care (NIH publication No: 86-23, revised 1984) were followed as well as specific national laws where applicable. The approval of the local Ethical Committee was obtained.

Biochemical Analyses

For biochemical analyses, PFC tissue was separated from the whole brain tissue and then stored at –70 ºC until the analysis. After weighing the PFC tissues, homogenization (homogenizer: IKA Ultra-Turrax t 25 Basic, Stanfen, Germany) was carried out for 2 min at 13,000 rpm in four volumes of ice-cold tris-HCl buffer (50 mM, pH 7.4) containing 0.50 ml L^{-1} Triton X-100. All procedures were performed at 4 ºC. Homogenate, supernatant and extracted samples were prepared as described elsewhere[10] and the following determinations were made on the samples using commercial chemicals supplied by Sigma (St Louis, MO, USA). Protein measurements were made in the samples according to Lowry et al[11].

Determination of Superoxide Dismutase (SOD) Activity

The principle of the total (Cu-Zn and Mn) superoxide dismutase (t-SOD) (EC 1.1.15.1.1) enzyme activity method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by $O_2^{\cdot-}$ generated by the xanthine/XO system[12]. Activity

was assessed in the ethanol phase of supernatant from brain tissue after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume supernatant and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. Tissue SOD activity was also expressed as units per milligram protein (U mg prot^{-1}).

Determination of GSH-Px Activity

GSH-Px (EC 1.6.4.2) activity was measured by using the method of Paglia and Valentine[13]. The enzyme reaction in the tube, which contained NADPH, reduced glutathione (GSH), sodium azide, and glutathione reductase, was initiated by the addition of H_2O_2 and the change in absorbance at 340 nm was monitored by a spectrophometer. Results were expressed as units per mg brain protein.

Determination of XO Activity

XO (EC 1.2.3.2) activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm, according to Prajda and Weber's[14]. A calibration curve was constructed by using 10-50 mU/ml concentrations of standard XO solutions (Sigma X-1875). One unit of activity was defined as 1 μmol of uric acid formed per minute at 37 °C, pH 7.5, and expressed in units per g protein (U/g prot).

Determination of NO Levels

NO has very short half-life. The oxidation products of NO, nitrite (NO_2^-) and subsequently nitrate (NO_3^-), serve as an index of NO production. The method for measuring plasma nitrite and nitrate levels was based on the Griess reaction[15]. Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite+nitrate) was measured by spectrophotometry at 545 nm after conversion of nitrate to nitrite by copperized cadmium granules. A standard curve was established from nitrite standards to analyze unknown sample concentrations. Results were expressed as micromoles per g wet tissue (μmol/mg wet tissue).

Determination of ADA Activity

Adenosine deaminase activities (ADA; E.C.3.5.4.4) were estimated spectrophotometrically by the method of Giusti[16] based on the direct measurement of the formation of ammonia, produced when AD acts in excess of adenosine. Results were expressed as units per g protein.

Determination of CAT Activity

Catalase (CAT, EC 1.11.1.6) activity was measured according to the method of Aebi[17]. The principle of the assay is based on the determination of the rate constant k (dimension: s^{-1}, k) of H_2O_2 decomposition. By measuring the absorbance changes per minute, the rate constant of the enzyme was determined. Activities were expressed as k (rate constant) per g protein.

Determination of Thiobarbituric Acid-Reactive Substance (TBARS) Level

The tissue TBARS level was determined by a method[18] based on reaction with thiobarbituric acid at 90-100°C. In the TBA test reaction, malondialdehyde (MDA) or MDA-like substances and TBA react to produce a pink pigment with an absorption maximum at 532 nm. The reaction was performed at pH 2-3 and 90°C for 15 min. The sample was mixed with two volumes of cold 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water-bath for 10 min. After cooling, the absorbance was read at 532 nm. Results were expressed as nmol per g wet tissue, according to the standard graphic prepared from measurements with a standard solution (1,1,3,3-tetramethoxypropane).

Determination of Tissue Protein Carbonyl Content

The carbonyl contents were determined spectrophotometrically [Shimadzu UV-160 A, Tokyo, Japan)] by a method based on reaction of carbonyl group with 2, 4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone[19]. 2,4-dinitrophenylhydrazine was the reagent originally used for proteins subjected to metal-catalyzed oxidation. The results were given as nanomoles of carbonyl per milligram of protein.

Statistical Analysis

Biochemical data were analyzed by using SPSS® for Windows computing program (SPSS Inc., Chicago, IL, USA).

For biochemical variables, significant differences between the groups were determined using a non-parametric Mann-Whitney U test and followed Post Hoc multiple comparisons were done with LSD. p value less than 0.016 with Bonferroni ($p < 0.05/3 = 0.016$) was accepted as significant. Results were presented as mean ± standard error of mean (SEM).

Results

Effects of MK-801 on Gross Behavior

Locomotor activity of the rats like sniffing of the floor and wall, circling, head weaving and ataxia (inability to maintain body posture and body rolling) increased within a few minutes after intraperitoneal injection of MK-801 (0.5 mg/kg). MK-801 induced severe ataxia. Whereas the decreased locomotor activity was observed on the behaviors of rats by pretreatment with melatonin. However, ataxia and circling behaviors of rats continued in melatonin pretreatment group. Moreover, behaviors of the rats changed by time. Hence, the issue of behavioral changes in melatonin+MK-801 group compared to MK-801 group needed further investigation.
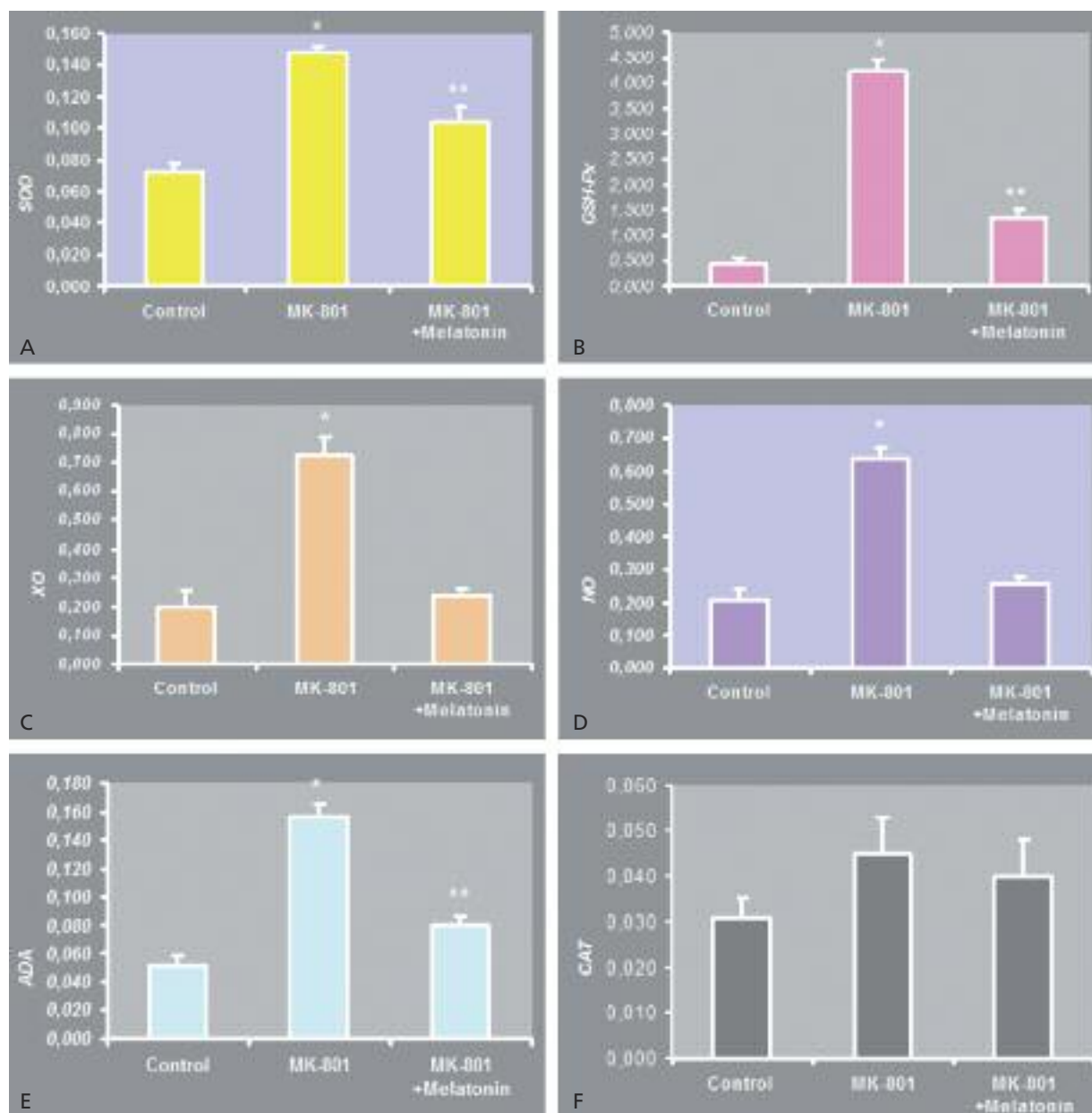

Figure 1. SOD (A), GSH-Px (B), XO (C), NO (D), and ADA (E) enzymes activities in PFC of rat brain. MK-801 group had significantly higher values in the corresponding group ($p < 0.0001$) than those of the control and melatonin treated groups. CAT (F) enzyme activity did not significantly change in MK-801 group compared to the control and melatonin groups. **A**, SOD (*U/mg protein*) enzyme activity in PFC of the rat brain. **B**, GSH-Px (*U/g protein*) enzyme activity in PFC of the rat brain. **C**, XO (*U/g prot*) enzyme activity in PFC of the rat brain. **D**, NO (*µmol/g wet tissue*) levels in PFC of the rat brain. **E**, ADA (*U/g prot*) enzyme activity in PFC of the rat brain. **F**, CAT (*k/g prot*) enzyme activity in PFC of the rat brain.

Effects of MK-801 on Biochemical Variables of PFC

Biochemical results were summarized in Figure 1 A-F and Table I. In this study, nitric oxide (NO), malondialdehyde (MDA) and protein carbonyl (PC) levels as well as total superoxide dismutase (t-SOD), xanthine oxidase (XO), catalase (CAT), adenosine deaminase (ADA) and glutathione peroxidase (GSH-Px) enzyme activities were measured in prefrontal cortex tissues of the rat brain. MK-801 group had significantly higher values of XO ($p < 0.0001$), NO ($p < 0.0001$), GSH-Px ($p < 0.0001$), and ADA ($p < 0.0001$), t-SOD ($p < 0.0001$) than those of the control group and melatonin treated group. There were no significant changes in the CAT enzyme activity in MK-801 group compared to the control group and the melatonin group. MDA and PC levels were found to be increased in the MK-801 group compared to the control and MK-801+melatonin groups ($p < 0.0001$). These results indicate that MK-801 can increase ROS and antioxidant enzymes activities in prefrontal cortex. On the other hand, melatonin may reduce the oxidative damage.

Discussion

The PFC is responsible for integrating cortical and subcortical inputs to execute essential cognitive functions such as attention, working memory planning and decision-making. Prefrontal cortical dysfunction has been detected in schizophrenia. Postmortem studies have shown that abnormalities in PFC are associated with schizophrenia[20]. There is increasing evidence that ROS plays an important role in the pathophysiology of schizophrenia. Excessive ROS generations are very important cell membrane. Major target of highly reactive ROS are membrane lipids, initiating the self-perpetuating process of lipid peroxidation, which disrupts the functional state and integrity of the membrane. Our previous neuropsychological studies have largely focused on the role of antioxidant properties CAPE on the PFC[21]. Thus, we want to investigate whether endogenous indices of oxidative stress change with treatment with melatonin in experimental schizophrenia model.

There are lots of evidence[7] proving that melatonin play an important role in the pathophysiology of schizophrenia. Monteleone et al[22] suggest that decreased nocturnal secretion of melatonin has been detected in drug-free as well as paranoid schizophrenic patients. Another group of researchers[23] found disrupted melatonin patterns in medicated schizophrenic patients. Usually decreased melatonin has been found in schizophrenic patients[24]. Melatonin is beneficial as a neuroprotective agent with its anticonvulsive, sedative, hypnotic properties and cortical dysplasia in the neonatal hypoxia-ischemia model[25]. Melatonin has also a potent antigenotoxic effect against cyclophosphamide-induced toxicity in mice[26], which may be due to the scavenging of free radicals and increased antioxidant status. Another study suggests[27] that piromelatine, a novel melatonin agonist, possess the effects of melatonin in attenuating the development of hypertension in adult spontaneously hypertensive rats. Lu et al[28] observed that high dose of melatonin can protect INS-1 cells from oxidative damage induced by intermittent hypoxia. Due to the high lipophilicity of melatonin and its low molecular weight, it easily passes through cell

Table I. Malondialdehyde (MDA) and protein carbonyl (PC) levels in the prefrontal cortex (PFC) of the rat brain.

	N	MDA (nmol/g wet tissue)	PC (nmol/mg prot)
I- Control	7	6.584 ± 1.352	0.206 ± 0.049
II- MK-801	7	20.635 ± 1.827	0.448 ± 0.092
III- MK-801+ Melatonin	7	10.864 ± 1.879	0.290 ± 0.037
***p* values**			
I-II		0.0001	0.0001
I-III		0.0001	0.008
II-III		0.0001	0.0001

Results are presented as mean ± standard error mean (SEM); N, number of rats, MDA and PC levels were found to be increased in MK-801 group compared to the control and MK-801+melatonin groups.

membranes and provides on-site protection against locally generated free radicals directly at DNA sites.

Oxidative stress potentially attacks critical macromolecules such as DNA, RNA, lipids and proteins. SOD is a potent protective enzyme that can selectively scavenge O_2^- by catalyzing its dismutation to H_2O_2 and oxygen (O_2). The other antioxidant enzymes, CAT and GSH-Px, catalyzes the conversion of H_2O_2 to water and oxygen. Antioxidant enzymes t-SOD, CAT and GSH-Px have complementary activities in the antioxidative defense system. In this study, overall tissue activities of antioxidant enzymes were increased during the process of schizophrenia, which reflected the oxidative stress over the PFC of rat brain. We found increased GSH-Px activity and t-SOD activity in MK-801 group compared to melatonin treated group and control group. MK-801 and phencyclidine have also been shown to significantly increase cerebral blood flow and glucose utilization in brain regions, especially the limbic system[29-32]. Since ROS are by-products of normal metabolism, it is possible that hypermetabolism may produce oxidative stress in local brain areas. A microdialysis study[33] showed increased hydroxyl radical levels in the mouse posterior cingulate and retrosplenial cortex following administration of MK-801. Also, MK-801 has also been shown to increase the levels of several markers of oxidative damage in the rat PFC[34]. Increased antioxidant enzyme activity may reflect a preceding cellular oxidative stress or serves as compensatory mechanism. These results indicate that melatonin has a primary role in mediating the scavenger action in such an oxidative stress condition.

On the other hand, the level of MDA; which is the indicator of lipid peroxidation in the cells and the level of PC; which is the indicator of protein oxidation was also increased due to schizophrenia. In this study, MDA and PC levels were found to be increased in MK-801 group compared to control and MK-801+melatonin groups ($p < 0.0001$). Melatonin administration brought the tissue levels of these denaturation end-products closer to the control levels, which also supported the antioxidant activity of melatonin. In our previous studies, we have shown that some antioxidant enzymes (SOD, GSH-Px and CAT) and the products of lipid peroxidation (MDA) led to the increase of erythrocyte in different forms of schizophrenia[35]. We have also found that NO increased in the erythrocyte for the patients with schizophrenia[36]. In another study on schizophrenia, increased plasma XO activity and NO levels, decreased SOD activity, and unchanged GSH-Px activity were detected compared to control group. Plasma TBARS levels were increased in schizophrenic patients, especially in the residual subtype of schizophrenia. Also, we observed that NO levels in schizophrenic patients were significantly increased[37]. We studied the levels of testis oxidative stress parameters after MK-801 induced psychosis model and showed the protective effects of CAPE[38]. In the previous study oxidative stress (OS) exerted on testicular tissues by MK-801 was reversed by melatonin. Administration of MK-801 produces OS injury in rat testes and melatonin seems to be a highly promising antioxidant agent, which protected testicular tissues from this injury[39].

We assayed ADA and XO enzyme activities, as an indicator of DNA oxidation. ADA is an important enzyme participating in purine and DNA metabolism. XO is an enzyme that catalyzes the last step of the chain of reactions through which the urine bases are degraded into uric acid. XO produces large amounts of ROS, especially superoxide during the above-mentioned reaction. Hence, the increased XO activity may cause further tissue damage because of free radical-generating effect. This study indicates that XO and ADA activities were increased in the prefrontal cortex of rats in experimental psychosis model whereas melatonin significantly reduced XO and ADA activities. Also, these results indicate that MK-801 can increase ROS and antioxidant enzymes in PFC. On the other hand, melatonin may reduce the oxidative stress. This effect might possibly be explained that melatonin shows a potent scavenging capacity on superoxide anion medical, which is a product of XO and ADA reaction.

Nitric oxide has been recognized as a biological neural messenger molecule although it is best known as a toxic reactive free radical in the CNS. Increased oxidant end-products by the reactions of NO with other free radicals may probably contribute to the neuropathophysiology, and thereby psychopathology, of schizophrenia because of the preferential vulnerability of the brain to oxidative injury. In the latest study, the finding that the reduction of NO levels in prefrontal cortex in rats treated melatonin may have important implications on schizophrenia. This study showed that MK-801 group had significantly higher value of NO than that of the control group and melatonin treated group.

Conclusions

These results provide further evidence for psychosis associated with increase in oxidative stress indices, and more importantly, indicate that treatment with melatonin might have protective effects against oxidative stress. We may suggest that adding of melatonin to the standard neuroleptic treatment in schizophrenia may have beneficiary effects for prevention of cellular structures of CNS.

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Conflict of interest

The Authors declare that there are no conflicts of interest.

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