·*Original Article*·

Levels of oxidative stress parameters and the protective effects of melatonin in psychosis model rat testis

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Abstract

Aim: To evaluate the effects of melatonin on antioxidant enzyme levels and histopathologic changes in dizocilpine (MK-801)-induced psychosis model rat testis. **Methods:** A total of 24 adult male Wistar-Albino rats were divided into three groups with 8 in each. Group I was used as control. Rats in Group II were injected with MK-801 (0.5 mg/kg body weight i.p. for 5 days). In addition to MK-801, melatonin (50 mg/kg body weight i.p. once a day for 5 days) was injected into the rats in Group III. The testes were harvested bilaterally for biochemical and histopathological examinations. Antioxidant enzyme activities, malondialdehyde, protein carbonyl and nitric oxide (NO) levels in testicular tissues were analyzed using spectrophotometric analysis methods. Histopathological examinations of the testes were also performed. **Results:** MK-801 induced testicular damage, which resulted in significant oxidative stress (OS) by increasing the levels of antioxidant enzymes. The malondialdehyde, protein carbonyl and NO levels were increased in testicular tissues of rats. Treatment with melatonin led to significant decrease in oxidative injury. Administration of melatonin also reduced the detrimental histopathologic effects caused by MK-801. **Conclusion:** The results of the present study showed that MK-801 cause OS in testicular tissues of rats and treatment with melatonin can reduce the harmful effects of MK-801. *(Asian J Androl 2008 Mar; 10: 259–265)*

Keywords: testis; dizocilpine; psychosis; oxidative stress; melatonin; antioxidant enzymes

1 Introduction

The neuropathology and psychopathology of schizophrenia is still poorly understood, which might be attributed to the paucity of adequate animal models for this disorder [1, 2]. The N-methyl-D-aspartate (NMDA) receptor hypofunction theory is the most widely used animal model for schizophrenia [3]. Noncompetitive NMDA-receptor antagonists produce a psychotic state, which includes both positive and negative symptoms of schizophrenia [3, 4]. Among the NMDA antagonists, MK-801 (dizocilpine, 5R, 10S-[4]-5-methyl-10, 11-dihydro-5H-dibenzo [a,d] cyclohepten-5, 10-imine hydrogen maleate) is the most potent agent that is used in psychosis models [3, 4].

Lower testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels have been reported in patients with schizophrenia impairments in hypothalamo-pituitary-gonadal axis functions [5–7]. However, no studies has investigated the relationship

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between this organic disorder and oxidative stress (OS) exerted on testicular tissues.

Oxidative injury develops when the production of reactive oxygen species (ROS) and/or free radicals exceeds the activity of the natural antioxidant defense mechanism in the body [8, 9]. Free radicals are produced as byproducts of normal cellular metabolism and can cause irreversible cellular damage by mitochondrial dysfunction, peroxidation of membrane lipids and oxidation of proteins [8, 9]. Mammalian spermatozoa are very rich in polyunsaturated fatty acids and they are very susceptible to attack by ROS [10, 11]. Repairing the harmful effects of OS on reproductive tissues with antioxidant agents might have great therapeutic potential in the future because of improvements in knowledge regarding the role of OS in infertility [10].

The pineal hormone melatonin (N-acetyl-5-methoxy-tryptamine) attracted much interest after its powerful antioxidant potential was proven by several *in vivo* and *in vitro* studies [12, 13]. The most basic function of melatonin is its antioxidant action. Other important physiological activities include the control of circadian rhythms, regulation of seasonal reproduction cycles and enhancement of the immune system [12, 13]. Binding sites for melatonin were detected in the reproductive systems of different species, so it seems reasonable to assume that melatonin exerts its actions through direct interaction with the steroidogenic cells of the reproductive organ [14, 15].

Two major questions prompted us to conduct such a study are how can experimentally induced psychosis affect testicular tissues by oxidative injury and what is the effect of melatonin against this effect? The aim of the present study is to address the biochemical and histopathological changes in the testes of rats after MK-801 administration and to evaluate the protective effects of melatonin.

2 Materials and methods

2.1 Animals

In total, 24 male Wistar-Albino rats weighing between 300 g and 350 g (age 3 months) were used for the experiments. They were housed in plexiglass cages with three animals per cage at the laboratory animal research center of our institute and fed with commercial food pellets and tap water provided *ad libitum* throughout the study. The animal rooms were windowless with automatic temperature (22 ± 2ºC) and lighting controls (12 h:12 h Light:Dark cycle).

2.2 Groups and procedures

The animals were divided into three groups with 8 rats in each. The rats in Group I received daily i.p. injections of 0.9% saline for 5 days. Daily MK-801 (0.5 mg/kg body weight, i.p. for 5 days) were injected into the rats in Group II. Group III rats received additional daily melatonin injections 60 min before MK-801 administration. Melatonin (Sigma, St. Louis, MO, USA) was dissolved in 0.9% saline and given to all rats in Group III with a dosage of 50 mg/kg body weight, once a day i.p. for 5 days. The rats were killed by decapitation 1 h following the last drug administration. The testes of all the rats were harvested bilaterally and weights of all testes were determined. One of the testis was stored at –70ºC pending biochemical studies and the other testis of each rat was fixed in 10% formalhyde solution for histopathological analysis. International standarts for the care of laboratory animals were followed and the study protocol was approved by the local ethical commitee.

Testis tissues were homogenized in 5 mL of ice-cold tris-HCl buffer (50 mmol/L, pH 7.4) containing 0.50 mL/L Triton × 100. The homogenization procedure (IKA Ultra-Turrax t 25 Basic, Staufen, Germany) was carried out for 2 min at 5 000 × *g*. Homogenate, supernatant and extracted samples were prepared and the following determinations were made on the samples using commercial chemicals (Sigma, St. Louis, MO, USA). All of the procedures were performed at 4ºC.

2.3 Superoxide dismutase (SOD) activity determination

Total (Cu–Zn and Mn) SOD activity was determined according to the method of Sun *et al.* [16], including a modification made by Durak *et al.* [17]. The principle of SOD activity determination method was based on the inhibition of nitroblue tetrasolium reduction by the xanthine-xanthine oxidase system as a superoxide radical generator. One unit of SOD was defined as the enzyme activity causing 50% inhibition in the nitroblue tetrasolium reduction rate. The SOD activity was expressed as units per miligram tissue protein (U/mg prot).

2.4 Catalase (CAT) activity determination

CAT activity was determined according to Aebi's method [18]. The essentials of CAT activity determination method were based on the determination of the rate

constant of the H_2O_2 decomposition rate at 240 nm. The CAT activity results were expressed as k (rate constant) per gram protein (k/g prot).

2.5 Glutathione peroxidase (GSH-Px) activity determination

GSH-Px activity was measured using the method of Paglia and Valentine [19]. It was measured by the enzymatic reaction, which was initiated by addition of H_2O_2 to the reaction mixture containing reduced glutathione, nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase. The change in the absorbance at 340 nm was monitored using a spectrophotometer and the enymatic activity was given as international units per gram tissue protein (U/g prot).

2.6 Malondialdehyde (MDA) level determination

MDA level was determined using Wasowicz's method [20], which was based on the reaction of MDA with thiobarbituric acid at 95–100ºC. MDA or MDA-like substances and thiobarbituric acid react together to produce a pink pigment with an absorbtion maximum of 532 nm. The results were expressed as nanomoles per gram wet tissue protein of testis (nmol/g wet tissue) according to standart graphics, which was prepared with serial dilutions of standart 1,1,3,3-tetramethoxypropane.

2.7 Protein carbonyl (PC) level determination

The PC contents were determined spectrophotometrically (GBC Cintra 10 E UV/Visible spectrophotometry, Melbourne, Australia) with the reaction of the carbonyl group with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone [21]. The results were given as nanomoles of protein carbonyl per milligram of protein.

2.8 Nitric oxide (NO) determination

Tissue nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as an index of NO production. Quantitations of NO_2^- and NO_3^- were based on the Griess reaction, in which a chromophore with a strong absorbance at 540 nm was formed by reaction of nitrite with a mixture of naphthlethylenediamine and sulphanilamide [22]. The results were expressed as μmol per gram wet tissue (μmol/g wet tissue).

2.9 Histopathological examination

The testicular tissues were embedded in paraffin wax. The paraffin blocks were cut into pieces with 5 μm thickness. Five slides were prepared for each testis and stained with hematoxylin-eosin stain. Randomly selected fields of the slides were examined with a light microscope under × 20 magnification.

2.10 Statistical analysis

Data were analyzed by using SPSS Version 15.0 for Windows software (SPSS, Chicago, IL, USA), and given as mean ± SEM. $P < 0.05$ was regarded as statistically significant.

Distributions of continuous variables were tested using the Kolmogorov-Smirnov test. All groups showed a normal distribution, so parametrical statistical methods were used to analyze the data. One-way ANOVA test was performed and all parameters were significant. Therefore, *post hoc* multiple comparisons were done with Tukey Honestly Significantly Different (HSD) Test.

3 Results

The enzyme activities in testicular tissues of all groups and testes weights are presented in Table 1 and the statistical comparison of these results between the groups are presented in Table 2. The mean testicular weights in Groups I, II and III rats were 1.317 ± 0.045 g, 1.077 ± 0.017 g and 1.198 ± 0.034 g, respectively. The mean testicular weight was significantly decreased in the MK-801 group when compared to the control group ($P < 0.001$), but it was significantly increased in the MK-801+ melatonin group (Group III) when compared to Group II ($P < 0.05$). No difference was observed between mean testicular weights of Group III and that of control group ($P > 0.05$). There was a significant increase in SOD activity and reduction in CAT activity after MK-801 injection ($P < 0.001$). However, no difference could be observed in SOD and CAT enzyme activities in melatonin treated rats (Group III) when compared to the controls ($P > 0.05$). GSH-Px activity was reduced in Group II ($P > 0.05$). Melatonin treatment caused further reduction in GSH-Px activity in Group III, but no difference was observed ($P > 0.05$). When GSH-Px activity occured, the differences between all groups were not statistically significant ($P > 0.05$). The tissue MDA levels were higher in the MK-801 group ($P < 0.001$), but not in the melatonin+MK-801 group (I *vs.* III, $P > 0.05$). Significantly increased tissue PC levels by MK-801 injection ($P < 0.001$) were reduced to a level closer to controls by melatonin in Group III (I *vs.*

Table 1. Biochemical parameters and statistical comparisons between the groups. The values are expressed as mean ± SEM and $P < 0.05$ is considered significant. MK-801, dizocilpine; Mel, melatonin; TW, testes weight; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; PC, protein carbonyl; NO, nitric oxide; *n*, number of rats.

	Group I-control (*n* = 8)	Group II-MK-801 (*n* = 8)	Group III-MK-801+Mel (*n* = 8)	F	P
TW (g)	1.317 ± 0.045	1.077 ± 0.017	1.198 ± 0.034	12.291	0.001
SOD (U/mg prot)	0.055 ± 0.005	0.090 ± 0.015	0.049 ± 0.009	4.341	0.023
CAT (*k*/g prot)	0.260 ± 0.031	0.145 ± 0.009	0.250 ± 0.009	10.337	0.001
GSH-Px (U/g prot)	1.226 ± 0.172	1.119 ± 0.158	0.807 ± 0.161	1.760	0.191
MDA (nmol/g wet tissue)	0.879 ± 0.041	2.741 ± 0.324	1.641 ± 0.223	16.743	0.001
PC (nmol/mg prot)	0.362 ± 0.051	0.798 ± 0.087	0.459 ± 0.068	28.206	0.001
NO (μmol/g wet tissue)	0.038 ± 0.002	0.053 ± 0.002	0.042 ± 0.001	18.980	0.001

Table 2. Multiple comparisons of the biochemical parameters between groups I, II and III. $P < 0.05$ is considered significant. TW, testes weight; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; PC, protein carbonyl; NO, nitric oxide; *N.S.*, none significant.

P values	TW (g)	SOD (U/mg prot)	CAT (*k*/g prot)	GSH-Px (U/g prot)	MDA (nmol/g wet tissue)	PC (nmol/mg prot)	NO (μmol/g wet tissue)
I *vs.* II	0.001	0.001	0.001	*N.S.*	0.001	0.001	0.001
I *vs.* III	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*
II *vs.* III	0.001	0.029	0.002	*N.S.*	0.002	0.06	0.001

III, $P > 0.05$). Tissue NO level was significantly increased in Group II ($P < 0.001$) and lowered by melatonin administration in Group III ($P > 0.05$) (Tables 1 and 2).

Histopathological examination of the tissues showed that the regular course of spermatogenesis and normal tubular epithelium was affected by administration of MK-801 (Figure 1A). Atrophy of the tubular structures, degenaration and disorganization of the tubular epithelium and degenerated germinal cells in lumina of the tubules were seen in the MK-801 group (Figure 1B). The administration of melatonin reduced these harmful effects There was marked reorganization of cellular elements and tubular structures in this group. Melatonin administration reduced the degenerative changes in cellular and tubular structures in more than 90% of the inspected areas of the histological sections (Figure 1C).

4 Discussion

Determination of oxidative injury and structural changes in the testes of schizophrenic rats, rather than behavioral and biochemical changes in the brain, are the main focus of the present study. To our knowledge, this is the first study to investigate the biochemical and his-
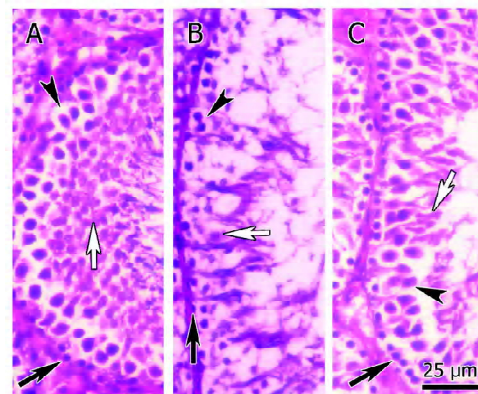

Figure 1. Representative histologic sections of testicular tissues of rats (H&E, original magnification × 20). (A): Control group (Group I): normal testicular histology. (B): MK-801 treated group (Group II): seminiferous tubular and cellular elements are degenerated and disorganized. (C): MK-801+melatonin (Mel) treated group (Group III): the degeneration in tubular and cellular structures was restored by Mel administration (arrowhead, spermatocytes; white arrow, spermatogenetic cell lines; black arrow, spermatogonia).

topathological effects of melatonin in experimentally-induced psychosis model rat testis. Involvement of free radicals in the cell membrane pathologies of the central

nervous system, such as defects in polyunsaturated fatty acid synthesis, oxidation of proteins, lipid peroxidation (LPO) and damage to DNA, have been shown in other studies [23]. Free radical induced oxidative damage to spermatozoa has gained considerable attention for its role in inducing poor sperm function and infertility [10, 24, 25]. Most of the harmful effects on sperm functions and motility have been attributed to elevated levels of ROS and reactive nitrogen species in these studies [10, 24, 25]. Zini and Schlegel [9] show the cascade of oxidative events that cause oxidative injury and lipid peroxidation in androgen deprived rat testes.

OSrefers to a condition that is associated with an increased rate of cellular damage. It is induced by oxidant substances commonly known as ROS [24]. The main highly reactive ROS that have potential implications in reproductive biology are the superoxide anion ($\cdot O_2^-$), the hydroxyl radical ($\cdot OH$), the hypochlorite radical ($\cdot OHCl$), and the hydrogen peroxide (H_2O_2) [10, 12, 13]. Lipid membranes of the cells are the major target of highly reactive ROS. Normally, a balance is maintained between the amount of ROS produced (pro-oxidants) and that scavenged by a cell (antioxidant). Cellular damage arises when this equilibrium is disturbed [25]. The ROS disrupts cellular functions and cellular integrity in OS conditions by peroxidation of lipid membranes and consequently causes cellular damage [10, 12]. Three major enzymatic systems have been shown in the seminal plasma: GSH-Px, SOD and CAT [10, 14, 25, 26]. CAT, which prevents ROS damage, has been found in both human spermatozoa and seminal plasma. Similarly, a selenium containing glutathione enzyme scavenging system exists in the spermatozoa of several mammalian species, including human beings [25, 26]. This system might act directly as an antioxidant and inhibitor of LPO, which will help spermatozoa to combat oxidative insults [10, 24, 25]. Vitamin C, vitamin E, beta carotene, albumin and biomolecules (glutathione and ubiquinol) are nonenzymatic antioxidant agents that have also been used as antioxidants in several studies [11, 14, 15, 24].

The testicular tissues are very sensitive to ROS effects. Additionally, spermatozoa are particularly susceptible to LPO because of the high concentration of polyunsaturated fatty acids in their plasma membrane [10, 24–27]. This high concentration of polyunsaturated fatty acids in the sperm membrane is required to give the plasma membrane fluidity, which is needed for sperm motility and participation in the events associated with

fertilization [27]. The ROS production by a sperm is a normal physiologic process and ROS is produced by a variety of semen components, including immotile or morphologically abnormal spermatozoa, leukocytes, and morphologically normal but functionally abnormal spermatozoa [10, 24–27]. LPO is a well established index of OS, and LPO of sperm membrane is considered the key mechanism of ROS-induced sperm damage [10, 14, 25, 27]. In previous related human studies it has been shown that OS is associated with a reduction in sperm motility, viability and defects in sperm-oocyte fusion [24, 27]. Defective sperm function is the most prevalent cause of male infertility, and it is difficult to treat [10, 24, 27]. As a result, understanding the role of OS in the pathophysiology of human sperm function has become an increasingly important subject in human male infertility studies.

In the present study, administration of MK-801 induced significant changes in the activity of antioxidant enzymes (SOD, CAT) in rat testes when compared to control rats ($P < 0.001$). Relatively stable GSH-Px levels in both MK-801 and melatonin groups ($P > 0.05$) might be explained by the collaborative scavenging activity of the three antioxidant enzymes against ROS and/or free radicals. However, melatonin almost completely reversed these alterations in the enzyme activities and brought the levels of all three enzymes closer to control levels ($P > 0.05$). These results indicate that melatonin has a primary role in mediating the scavenger action in such an OS condition.

The tissue MDA and PC levels, which are the indicators of LPO and protein denaturation, respectively, also increased significantly after MK-801 injection in this study. Melatonin administration brought the tissue levels of these denaturation end-products closer to the control levels ($P > 0.05$), which also supported the antioxidant activity of melatonin.

The tissue NO increase in the MK-801 group might be attributed to the injury of testicular tissue in relation to abundant ROS production and consequent migration of macrophages and polymorphonuclear leucocytes to the region [10]. NO radicals have been found to regulate multiple biological functions in inflammation and in mediating many cytotoxic and pathological events [10, 24]. NO has a bimodal effect on sperm motility whereby low concentrations of NO enhance sperm motility, whereas high concentrations of NO decrease it [10, 24]. This effect might be due to the dual nature of NO, which is a signal transduction molecule at low concentrations, while

being cytotoxic at higher concentrations [10, 24]. Increased tissue NO levels in the MK-801 group ($P < 0.001$) were reversed by melatonin and the resultant NO levels in the melatonin treated group were not significantly different from the controls.

The synthesis and secretion of melatonin is regulated in the hypothalamic suprachiasmatic nucleus by a circadian clock. Because of the presence of an electron rich aromatic ring system and *O*-methyl-and *N*-acetyl residues in its structure, melatonin functions as an amphibilic, electron donor substance. This structural property forms the basis for its antioxidant power in various subcellular compartments [13]. The protective actions of melatonin against OS ranges from direct scavenging activity of ROS to control or modulate many processes that might induce a redox imbalance between prooxidant and antioxidant species [12–14, 28]. The direct scavenging activity of melatonin against the hydroxyl radical (·OH), which is by far the most aggressive reactive oxygen radical known, was discovered by Tan *et al*. [12]. Melatonin has been used as an effective antioxidant agent in almost all pursuing studies about free radicals [13, 28, 29].

MK-801 acts by blocking glutamatergic-NMDA-receptor complex in the brain. However, the expression of functional glutamate transporters in the rat testis has also been reported [30]. According to previous related studies, it appears that glutamate pathway might play a role in the spermatozoal functions and integrity of spermatogenesis in the rat testis [30]. Additionally, it has been proposed that the presence of glutamate receptors in testes will provide researches insight in terms of the discovery and development of drugs that will be useful for the treatment of a variety of disorders associated with testicular malfunction in human beings [30]. Although the precise mechanism of OS as a result of MK-801 toxicity has not been clarified in the present study, the histologic and biochemical analysis results support that an OS injury occured in testicular tissues of rats. In the present study, it was difficult to determine whether the testicular toxicity was due to schizophrenia per se, or simply a side effect of MK-801. In a previous study, we studied the levels of testis oxidative stress parameters after establishing a MK-801 induced psychosis model and showed the protective effects of caffeic acid phenethyl ester, which is the active component of propolis from honeybee hives and has powerful antioxidant, anti-mitotic, antiviral and antiinflammatory properties [31].

Although our results suggest that OS exerted on testicular tissues by MK-801 was reversed by melatonin, further studies are strongly recommended to determine the mode of actions of these agents on the male reproductive system.

In conclusion, the present results demonstrate that administration of MK-801 produces OS injury in rat testes. Melatonin seems to be a highly promising antioxidant agent, which protected testicular tissues from this injury. Further investigations are warranted to provide data regarding the general toxicity and effects of psychotic state on the male reproductive system.

Acknowledgment

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