

Long-Term Methylphenidate Treatment Causes Increased Superoxide Dismutase Activity and Unchanged Lipid Peroxidation in Rat Brain

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ÖZET:

Uzun süreli metilfenidat uygulamasının rat beyinde antioksidan sistem ve lipid peroksidasyonu üzerine etkileri

Amaç: Metilfenidat (MP) dikkat eksikliği hiperaktivite bozukluğu olan çocuk ve ergenlerin tedavisinde yaygın olarak kullanılmaktadır. Ancak, uzun süreli tedavide MP'nin beyin üzerine olumsuz etkilerinin olup olmadığı halen sorgulanmaktadır. Bu çalışmada MP'nin beyin üzerine toksik etkilerini değerlendirmek amacıyla, antioksidan sistem ve lipid peroksidasyonu araştırıldı.

Yöntem: Çalışma grubundaki sıçanlara (n=10) 10 mg /kg dozda MP orogastrik entübasyonla 8 hafta boyunca hergün verilirken, kontrol grubundaki sıçanlara (n=10) aynı sürede orogastrik entübasyon ile distile su verildi. Çalışma sonunda sıçan beyinlerinde CuZn-süperoksit dismutaz (CuZn-SOD), Se-glutatyon peroksidaz (Se-GPx) ve malondialdehid ölçüldü.

Bulgular: Çalışma grubundaki sıçan beyinlerinde CuZn-SOD aktiviteleri, kontrol grubuna oranla daha yüksekti (p < 0.01). Ancak, beyin Se-GPx aktiviteleri ve MDA düzeyleri arasında anlamlı farklılık yoktu (p > 0.05). Sonuç: Uzun süreli MP verilmesi sıçan beyinlerinde antioksidan enzimleri etkilemekte; CuZn-SOD aktivitesini 1.2 kat artırmaktadır. Buna karşın, Se-GPx aktivitesini ve MDA düzeylerini anlamlı düzeyde etkilememektedir.

Sonuçlar: MP uygulaması sonucu oluşan beyindeki serbest radikallerin CuZn-SOD aktivitesini artırılarak yeterince nötralize edildiği ve lipid peroksidasyonuna neden olmadığını düşündürmektedir.

Anahtar sözcükler: Metilfenidat, antioksidan sistem, malondialdehid

Klinik Psikofarmakoloji Bülteni 2006;16:79-83

ABSTRACT:

Long-term methylphenidate treatment causes increased superoxide dismutase activity and unchanged lipid peroxidation in rat brain

Objective: Methylphenidate (MP) is commonly used to treat children and adolescents with attention-deficit/hyperactivity disorder. However, MP still poses a number of questions of whether it has toxic effects of long term treatment on the brain. In the present study, the effect of MP was investigated on the antioxidant system and lipid peroxidation with the purpose of evaluating its toxic effects on the brain.

Methods: MP at a dose of 10 mg/kg via orogastric intubation was administered to rats (n=10) daily for 8 weeks, whereas control rats (n=10) were given distilled water via orogastric intubation. CuZn-superoxide dismutase (CuZn-SOD), Se-glutathione peroxidase (Se-GPx), and malondialdehyde (MDA) were measured in rat brains.

Results: Brain CuZn-SOD activities were higher in the study group when compared with the controls (p < 0.01), but there were no significant differences in brain Se-GPx activities and MDA levels of the groups (p > 0.05).

Conclusions: Long-term MP treatment, which seems to interact with antioxidant enzymes, causes 1.2 fold increase in the activity of CuZn-SOD. However, it does not noticeably influence the activity of GPx, and the level of MDA in rat brains. -It seems possible that free radicals by MP administration neutralize sufficiently by increasing SOD levels and does not cause lipid peroxidation in the brain.

Key words: Methylphenidate, antioxidant system, malondialdehyde

Klinik Psikofarmakoloji Bülteni 2006;16:79-83

INTRODUCTION

Amphetamine-like stimulants, particularly methylphenidate (MP) are commonly prescribed for children diagnosed as having attention-deficit hyperactivity disorder (1). Although MP has been widely studied, it still poses a number of questions of whether it has adverse effects on the brain during long term

treatment. The toxicity of amphetamine and related drugs, especially toxic effects on central monoamine-containing neurons, was discovered a long time ago (2). However, little is known about possible adverse effects of chronic MP use on the brain in exhaustive search of the current literature. Only four studies have investigated its neurotoxic potential (3-6). In these studies, animals

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Kabul tarihi / Date of acceptance:
15 Mart 2006 / March 15, 2006

treated with MP did not develop long-lasting changes in regional brain catecholamine axon markers.

The brain is especially susceptible to free radical injury because of its elevated metabolic rate and rich lipid composition. Therefore, any toxicity on the brain can be predicted by evaluating antioxidant system and lipid peroxidation (7). Reactive oxygen species (ROS) or oxygen-free radicals, especially superoxide anion radical, hydroxyl radical and alkylperoxyl radical, are potent initiators of lipid peroxidation. The overproduction of free radicals can be detoxified by the endogenous antioxidants causing their cellular stores to be depleted. Malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) are currently considered to be basic markers of oxidative stress. MDA is one of the end-products of the peroxidation of membrane lipids, whereas enzymes SOD and GPx belong to the natural antioxidants (8).

Some of the damages to the central neural system that are observed following amphetamine and methamphetamine (METH) administration are known to be linked to increased formation of free radicals. METH-induced dopaminergic neurotoxicity is related to an increase in the generation of both reactive oxygen (hydroxyl, superoxide, peroxide) and nitrogen (nitric oxide) species (9). However, as far as we know, there are no data concerning the effects of chronic MP administration on the antioxidant system and lipid peroxidation in the brain. The current study performed to determine the activity of antioxidant enzymes including CuZn-SOD and Se-GPx to assess the effects on ROS, and MDA level as an indicator of oxidative damage or lipid peroxidation in rat brain after long term MP administration.

MATERIALS AND METHODS

Animals and Drug Administration

A total of 20 four-week-old Wistar male rats (120 ± 15 g) were housed in wire-topped plastic cages, as five animals per cage. Control and experimental rats received a standard diet of rodent chow (12-15 g/d) and water ad libitum. All rats were kept on an alternating 12-hour-light and 12-hour-dark cycle. The temperature inside the chambers was 22°C (± 2) with

relative humidity from 40 to 60%.

All experiments were performed at the same time every day and in the light period (9.00-11.00 AM) during 8 weeks. The experiments have been carried out according to rules in the Guide for the Care and Use of Laboratory Animals adopted by National Institutes of Health (USA) and the Declaration of Helsinki. This study was approved by the Ethics Committee of Gülhane Military Medical Academy Research Center.

After seven-day acclimatization period, the rats were randomly assigned to two groups of 10 rats per group. The study group was administered 10 mg/kg/d of MP, whereas the control group was administered distilled water. The dosage of MP administration to rats is similar to that of Gerasimov et al (10).

Ten mg tablets of MP (Ritalin[®], 10 mg tablets) were dissolved and homogenized in sterile distilled water via centrifugation providing 20 mg of MP per 10 ml. In the study group, 0.5 ml of the solution containing MP 1 mg was administered once a day via orogastric intubation for 8 weeks between 9.00 AM and 11.00 AM to each rat. In the control group, distilled water (0.5 ml) was given to each rat via orogastric intubation. The aim of intubation by using orogastric applicator was to prevent any drug loss due to uncontrollable conditions.

Tissue sampling

Three rats died (2 from the study group and 1 from the control group) because of the traumas during application of the orogastric apparatus and the study was completed with 17 rats.

At the end of 8 weeks, animals were decapitated and their brains were removed. The brain tissues were immediately frozen after the sampling and kept at -70°C until chemical analysis was performed. Tissue samples were weighed analytically. Then nine fold concentrated 1.15% KCl solution was added to the tissues and homogenized in glass homogenizer in ice. The homogenised samples were centrifuged at $+4^\circ\text{C}$ at $4400 \times g$ for ten minutes. The supernatant was used for the analysis.

Laboratory methods

At the end of 8 weeks, CuZn-SOD, Se-GPx and MDA

levels in the whole brain tissue of the rats were measured.

CuZn-SOD activity measurement. CuZn-SOD activity in brain homogenate was measured by the method described in our previous study (11). Briefly, each homogenate was diluted 1:400 with 10 mM phosphate buffer, pH 7.00 25 mL of diluted homogenate was mixed with 850 mL of substrate solution containing 0.05 mmol/L xanthine sodium and 0.025 mmol/L 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride in a buffer solution containing 50 mmol/L CAPS and 0.94 mmol/L EDTA pH 10.2. Then, 125 mL xanthine oxidase (80 U/L) was added to the mixture and absorbance increase was followed at 505 nm for 3 min against air. 25 mL of phosphate buffer or 25 mL of various standard concentrations in place of sample were used as blank or standard determinations. CuZn-SOD activity was expressed in U/g tissue protein (determined by the method of Lowry et al.) (12).

Se-GPx activity measurement. GPx activity in brain homogenate was measured by the method described in our previous study (11). The reaction mixture was 50 mmol/L tris buffer, pH 7.6 containing 1 mmol/L of Na₂EDTA, 2 mmol/L of reduced glutathione, 0.2 mmol/L of NADPH, 4mmol/L of sodium azide and 1000 U of glutathione reductase. 20 mL of homogenate and 980 mL of reaction mixture were mixed and incubated for 5 min at 37°C. Then the reaction was initiated with 8.8 mmol/L, H₂O₂ and the decrease in NADPH absorbance was followed at 340 nm for 3 min. Enzyme activities were reported in U/g tissue protein.

TBARS level measurement. Determination of lipid peroxidation in the brain tissue was performed by measuring thiobarbituric acid reactive substances (TBARS). Briefly, 10% (w/v) tissue homogenate was mixed with sodium dodecyl sulfate, acetate buffer (pH 3.5), and aqueous solution of thiobarbituric acid. After heating at 95°C for 60 min, the red pigment was extracted with n-butanol-pyridine mixture and determined by the absorbency at 532 nm (13). TBARS levels of brain homogenate were expressed as nmol/mg protein wet weight.

Statistical analysis

Results were expressed as standard error (of the

mean) (S.E.M.). Differences between the two groups were analyzed using Mann-Whitney U test. A significance level of $p < 0.05$ was considered to be statistically significant.

RESULTS

Brain CuZn-SOD activities were significantly higher in the study group when compared with the control group ($p = 0.006$), but there were no significant differences in brain Se-GPx activities and MDA levels of the groups ($p = 0.23$) (Table 1).

Table 1: Brain antioxidant enzyme activities and lipoperoxide levels in the study and the control groups.

Parameters	Control Group (n=9)	Study Group (n=8)	z	p
Se-GPx (U/g protein)	4.42±0.65	4.89±0.74	1.208	0.227
CuZn-SOD (U/g protein)	144.44±17.18	174.88±15.90	2.747	0.006*
MDA (nmol/mg protein)	0.63±0.16	0.64±0.17	0.290	0.772

Values are indicated by mean ± S.E., Se-GPx: Se-glutathione peroxidase; CuZn-SOD: CuZn-superoxide dismutase; MDA: malondialdehyde.

DISCUSSION

In this study, the significantly elevated SOD activity in rat brains administered 10 mg/kg MP for 8 weeks showed that this agent might have an effect of increasing reactive oxygen species (ROS). The imbalance between protective antioxidants and increased free radical production, leading to oxidative damage, is known as oxidative stress. SOD is the first step of the defense system against oxidative stress, which is an important free radical scavenging action. Therefore, SOD is an enzyme used extensively as a biochemical indicator of pathological states associated with oxidative stress. SOD catalyzes dismutation of the superoxide anion into hydrogen peroxide (14). In our study, a significant increase in SOD activity might be related to the increase of superoxide anion, which might be induced by MP.

In the antioxidant system, at first step, increased reactive oxygen would be converted into more hydrogen peroxide via increasing the activity of SOD. Hydrogen peroxide is one of the most active reactive oxygen species (15). At the second step of defense

system against oxidative stress, an excess of hydrogen peroxide must be efficiently neutralized by either GPx or catalase (16). GPx and catalase then independently convert hydrogen peroxide into water in this second step. So GPx protects cells against endogenously formed hydroperoxides (14). In our study, MP slightly increased the brain GPx levels, nevertheless this effect was not significant. This indicates that excess hydrogen peroxide might be neutralized efficiently. Moreover, GPx and catalase could compensate each other in case of one or the other is deficient. However, a limitation of this study is that catalase activity was not measured.

Several studies link the formation of reactive oxygen species (ROS) to the CNS neurotoxicity following administration of METH, which is an amphetamine stimulant. Increases in levels of various oxidation products following METH administration have been reported (17,18) suggesting a role for antioxidant mechanisms in reducing METH-induced toxicity (19). In support of this, METH-induced neurotoxicity is attenuated by concurrent treatment with antioxidants or free radical spin-trapping agents (20,21). Furthermore, METH toxicity is also reduced in mice that over-express the antioxidant enzyme CuZn superoxide dismutase (22). In our study, which is the first study that evaluates the effects of MP on antioxidant system, compatible with METH studies, we found significantly elevated CuZn-SOD activity in brains of rats which were administered MP when compared with the controls.

Increased free radical production can cause oxidative damage; an example of this process is oxidation of membrane-bound lipids and lipoproteins known as lipid peroxidation (23). MDA is one of the most frequently used indicators of lipid peroxidation. If there is an imbalance between the levels of free radicals and antioxidant enzymes, the amount of free radicals would increase and provoke lipid peroxidation. However, in our study, there was no

difference in MDA levels of rat brains between the two groups. This result indicates that MP at a dose of 10 mg/kg after 8 weeks would not cause lipid peroxidation as a predictor of neurotoxicity suggesting that ROS were being neutralized efficiently. However in METH studies, elevated TBARS levels have been indicated as an indicator of lipid peroxidation following METH administration (17,24).

A cause and effect relationship between ROS production following MP administration and subsequent nerve terminal damage remains unclear. In order to examine accurately and to be able to predict whether MP has neurotoxicity or not, it is also important to determine long-lasting changes of monoamine levels in the brain. In animal studies, MP did not cause long-lasting changes in regional brain catecholamine axon markers (3-6).

Our study had three limitations. First, we administered a single MP dose of 10 mg/kg per day, secondly, the number of the animals was low, and thirdly, we could not examine differently, the specific brain regions. The possible effects of MP on rat brain might be interpreted more accurately if different dosages were used, the number of the animal increased, and particularly regional examination of the brain was performed. Despite these limitations this study provides an informative research since as much as we screened the current literature, this study is the first to suggest an effect of MP on reactive oxygen species.

In conclusion, our results suggest that the long-term MP treatment at a dose of 10 mg/kg causes 1.2-fold increase in the activity of CuZn-SOD but does not noticeably influence the activity of GPx, and the level of MDA in rat brains. Further future experimental studies are definitely needed to examine issues related to the limitations of the current study. Furthermore, effects of MP on other organs, such as the liver and the bone tissue should also be examined.

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