Original Paper

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Plasma Norepinephrine and Dopamine Levels in Prepubertal Male Children with Attention-Deficit Hyperactivity Disorder do not Change with 8 Weeks of Methylphenidate Treatment

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ABSTRACT:

Plasma norepinephrine and dopamine levels in prepubertal male children with attention-deficit hyperactivity disorder do not change with 8 weeks of methylphenidate treatment

Objectives: The aim of this study was to determine plasma norepinephrine and dopamine levels at baseline and after 8 weeks of stimulant treatment in pre-pubertal male children with ADHD.

Methods: The study group consisted of 50 children (6-12 years old) diagnosed with ADHD. The control group comprised students from a primary school within the epidemiological catchment area of the clinic and was matched for class and age to the ADHD patients. The Kiddie Schedule for Affective Disorders and Schizophrenia for School Aged Children-Present and Lifetime Version (K-SADS-PL) was used to diagnose ADHD and allowed comorbidities. Mental retardation was ruled out with the Wechsler Intelligence Scale for Children-Revised (WISC-R) and impaired functioning. We evaluated disorder severity at the time of assessment using the Clinic Global Impression Scale (CGI). The DuPaul ADHD Rating Scale-IV (ARS) was also used. All patients were treatment-naive. The parents were advised to use OROS methylphenidate daily with no drug holidays during weekends. No rescue medications (immediate-release methylphenidate) were allowed. The dose started at 18 mg and was titrated to 54 mg in 4 weeks to yield average doses of 1 mg/kg/day. Baseline and endpoint plasma DA and NE were measured.

Results: Baseline plasma NE and DA levels had no statistically significant differences between ADHD patients `and controls (232.0±67.3 versus 232.2±65.3 pg/mL and 169.3±48.4 versus 186.9±40.5 pg/mL). Plasma NE levels in all ADHD subgroups decreased with 8 weeks of stimulant treatment, while changes in DA levels were more complex. Plasma DA levels decreased with treatment in the ADHD-inattentive subgroup but were elevated in the hyperactive/impulsive and combined subgroups. There were no statistically significant differences between ADHD subgroups for these variables. Endpoint NE levels were correlated with endpoint DA levels. There were no statistically significant differences between ADHD subgroups. Plasma NE levels were not related to symptom severity or treatment response. In contrast, baseline DA levels were negatively correlated with ARS total scores.

Conclusions: We found no statistically significant differences between plasma levels of NE and DA in a pre-pubertal male sample with ADHD and controls. Plasma DA and NE levels were correlated at both baseline and the endpoint. Although there was a signal that baseline DA levels may correlate with ADHD symptoms as evaluated via ARS, this was not true for endpoint analyses. Because this negative correlation disappeared after treatment, this finding about baseline DA levels may also be evaluated as an early treatment neuromarker. The negative results could also be explained by our focus on plasma. The recent consensus is that urinary levels of NE and DA may be more informative in patients with ADHD and that concurrent evaluation of multiple neurotransmitter systems (i.e., neuropeptide Y and NE) may be more informative. Further studies may benefit from concurrent measurements of plasma and urinary levels of catecholamines and their metabolites.

Keywords: ADHD, catecholamines, norepinephrine, dopamine, stimulants

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INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is one of the most common neurodevelopmental disorders of childhood and is characterized by developmentally inappropriate and impairing inattention, hyperactivity, and impulsivity[1]. Worldwide prevalence in childhood is estimated to be 5.3%, and impairing symptoms continue to adulthood in a significant group of patients[2,3]. Children with impairing inattention and hyperactivity benefit from dopaminergic agents, and catecholaminergic neurotransmitters (e.g., norepinephrine, serotonin, and dopamine) affect various components of attention, locomotor activity, reward sensitivity, and impulsivity. These observations have led to early efforts to evaluate the roles of these neurotransmitters in the pathogenesis of ADHD[4,5]. Multiple lines of converging evidence from genetics, neurophysiology, neuropsychology, neuroscience, and neuropharmacology support the role of these neurotransmitter systems in ADHD, although their interactions with other neurotransmitter systems, gender, and environmental risk factors are still not fully clarified[6-8].

Despite extensive evaluations of cerebrospinal fluid (CSF), blood, and urine samples from patients of variable age groups, the search for reliable and replicable biomarkers of ADHD has proven unsatisfactory[9-15]. Some studies suggest that diet, exercise and stress may also have short-term effects on plasma catecholamine levels in children and adolescents with ADHD[14,15]. Upon evaluating the results, some have proposed that the dopamine transporter genotype (DAT1) or methylphenidate response may be used as biomarkers, while others have underlined the importance of sample heterogeneity in studies[9-15]. A recent meta-analysis reported that 33.8% of the 210 studies evaluated had focused on the main metabolites and enzymes of the monoamine pathway and that norepinephrine (NE), 3-methox, 4,hydroxyphenylethylene glycol (MHPG), and monoamine oxidase (MAO) were found to be significantly different between cases and controls. NE, MHPG, and MAO results were also significant for symptom severity and treatment response[16]. These results suggest that further studies on biomarkers of ADHD are needed, preferably with more homogenous samples and controlling for confounders.

The aim of this study was to determine baseline levels of plasma norepinephrine and dopamine in pre-pubertal male children with ADHD and the effect of 8 weeks of stimulant treatment on these levels. In accordance with previous reports, we posited that patients with ADHD and controls would differ significantly for plasma NE levels and that NE levels would also correlate with symptom severity and /treatment response. The analyses for DA were exploratory.

MATERIAL AND METHODS

We attempted to control the confounding effects of puberty, gender, and nutrition by enrolling only male pre-pubertal patients with BMI in the 50th percentile. Stressful life events experienced by children within the past 12 months were screened by parental reporting via the Stressful Life Events Screening Scale-20 (SLES-20), and only children with no stressful life experiences within the last 12 months were enrolled[17,18].

Study Center, Sampling, and Ethics

The study group consisted of children with ADHD between 6 and 12 years old. All consecutive referrals to the Dokuz Eylul University Medical Faculty Department of Child and Adolescent Psychiatry between June 1st 2012 and December 31st 2012 were included. Inclusion criteria were male gender, diagnosis with ADHD according to DSM-IV-TR criteria, and verbal consent along with the informed consent of parents. The exclusion criteria were past psychiatric treatment, chronic medical conditions (history of seizures, progressive/non-progressive neurological illnesses, etc.), a history of severe head trauma within the last year (resulting in loss of consciousness for any duration), mental

retardation (as defined by IQ < 70 in standardized testing with impaired daily functioning), pervasive developmental disorders, substance use disorders, psychotic and mood disorders, and experience of any stressful life experience within the past year.

Baseline neurological/physical examinations of all cases were conducted in the Department of Pediatrics to achieve consensus. International criteria by the World Health Organization (WHO) were used to calculate BMI percentiles, and all subjects were in the 50th percentile for their ages (http://www.who.int/growthref/who2007_bmi_for_age/en/, accessed on 01/01/2014). The research protocol was approved by the Dokuz Eylul University of Medical Sciences Research Ethics Committee (Date: 17/12/2009; No:165/2009). All of the study procedures were in accordance with principles listed in the Declaration of Helsinki and local laws and regulations.

Measures

Kiddie Schedule for Affective Disorders and Schizophrenia for School-Aged Children-Present and Lifetime Version (K-SADS-PL): K-SADS-PL is a widely used semi-structured diagnostic interview tool[19]. It inquires about current and past episodes of child and adolescent psychiatric disorders and allows a diagnosis to be made. The Turkish version of the K-SADS-PL was reported to have good test-retest and inter-rater reliability[20]. In the current study, K-SADS-PL was used to diagnose ADHD and other psychiatric comorbidities (enuresis, tic disorders, etc.).

Wechsler Intelligence Scale for Children-Revised (WISC-R): The WISC-R was designed to measure the intelligence quotient (IQ) of children between the ages of 6 and 16[21,22]. The Turkish version was previously standardized[22]. WISC-R was used to rule out mental retardation in this study.

Clinical Global Impression (CGI) Scale: CGI is a 7-item Likert-type scale that allows clinicians to evaluate the severity of a disorder at the time of assessment relative to the clinician's past experience with patients who had the same diagnosis. The scores range from 1 (normal, not at all ill) to 7 (extremely ill)[23]. In this study, CGI scales were completed by physicians in the outpatient department.

DuPaul ADHD Rating Scale-IV (ARS): This scale consists of 18 items that tap into symptoms of ADHD listed in the DSM-IV criteria. Each item has a 4-point scale of 0 to 3. Nine of the items are related to inattention, while the rest are related to hyperactivity and impulsivity[24]. This scale was previously used in ADHD studies conducted in Turkey[25]. In this study, a senior clinician (APA) who was blind to the evaluations and treatments of children with ADHD completed the questionnaires in the outpatient department according to parental reports at the baseline and after two months of methylphenidate treatment.

Methylphenidate dosage: The parents were advised to use OROS methylphenidate daily with no drug holidays during weekends. No rescue medications (i.e., immediate-release methylphenidate) were allowed. At each visit, the parents were asked about medication compliance, and patients who skipped their doses more than three times during the study period were deemed to be non-compliant and removed from analyses. Drug dosages were calculated from prescription refills.

Measurement of Plasma Dopamine (DA) and Norepinephrine (NE) Levels

For the measurement of baseline and endpoint plasma DA and NE, 4 cc of blood were drawn into straight tubes containing EDTA without anticoagulants. Baseline evaluations also included blood drawn for whole blood counts (2 cc) and for liver and thyroid function tests (10 cc). At both evaluation points, the participants had been fasting for the last 12 hours, and blood was drawn while participants were in a sitting position and had rested for the previous 15 minutes. Samples

were immediately transferred to the laboratory for analysis. To prevent destruction of amine groups, reduced glutathione (0.25 M) was added to the blood samples.

The following components were added to a 1.5-mL micro-centrifuge tube: 10 mg of alumina, 125µL of 0.1M HCl, 250µL of plasma, 5µL of 5mM sodium metabisulfate, 50µL of 0.2N $HClO_4$ (containing 1ng/mL dihydroxybenzylamine-DHBA-), and Tris-tamponade (pH 8.6). These were mixed for 10 minutes, and then the mixture was centrifuged at 500g for 5 minutes and the supernatant was separated. The mixture was washed twice with 1mL of pure water and centrifuged at 500g for 5 minutes, and the supernatant was separated after each washing.

Elusion was made with 100µL of 0.1M $HClO_4$ (containing 0.1 mM sodium metabisulfate). A 20-µL injection was made into a high-performance liquid chromatography (HPLC) machine, which contained a pump, autoinjector, column oven, and electrochemical detector. Chromatographic separation was done using a 250X4.6mm C18 column with 5-µm particle diameter. The mobile phase was prepared by adjusting the pH to 3.1 using a phosphoric acid solution with 6.9g of sodium phosphate, 29.4g of sodium citrate, 0.2g of EDTA, 2.2g of diethyl-amine HCl, and 0.5g of 1-octanosulphonic acid, to which 12mL of acetonitrile and 5.5mL of dimethyl acetamide were added. The mobile phase flow rate was 0.4mL per min. A standard series was used for quantitative evaluation, and machine software was used for measurement of the peak area[26,27].

Treatment and Follow-up

All patients were treatment-naive. OROS-methylphenidate treatment was applied in a naturalistic way with daily dose individually titrated in accordance with the clinical response on the CGI scale. The starting dose was 18mg, which was titrated up to 54mg in 4 weeks to yield an average dose of 1mg/kg/day. Follow-up visits were conducted at baseline and at the 1st, 2nd, 3rd, 4th, and 8th weeks. Height, weight, blood pressure, side effects, CGI-S, and ARS were assessed at each visit.

Statistical Analyses

Statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL). Assumptions of normality were evaluated with the Shapiro-Wilk test. Descriptive analyses for normally distributed numerical variables included means with standard deviations, while ordinal variables and numerical variables with non-normal distributions were summarized as medians and inter-quartile ranges (IQR). Categorical variables are reported as percentages. The chi-square test and Fisher's exact chi-square test (when needed) were used for comparison of categorical variables.

Kruskal-Wallis analysis was performed to test for differences in the NE and DA levels for ADHD subtypes. Correlations between baseline and endpoint ARS and NE and DA levels were evaluated with Spearman Rank-Order analyses. Bonferroni correction was used for multiple comparisons. Statistical significance was determined at $p<0.05$. All of the tests were two-tailed.

RESULTS

Baseline plasma NE and DA levels did not have statistically significant differences between patients with ADHD and the controls (232.0±67.3 versus 232.2±65.3pg/mL and 169.3±48.4 versus 186.9±40.5pg/mL; respectively; Mann-Whitney U Test). Within the study period, 400 patients with probable ADHD applied to our clinic. Among these, 120 patients had previously received treatment for ADHD, 56 patients did not fulfill criteria for ADHD according to K-SADS-PL, 98 patients had entered puberty according to pediatric examination, 10 had comorbid epilepsy, 80 patients did not have BMI in the 50th percentile, 135 had experienced at least one stressful life event according to parental report within the past year, and the parents of 12 patients had declined study participation (there were over-

Table 1: Socio-demographic variables and Wechsler Intelligence Scale for Children-Revised (WISC-R) results of pre-pubertal male children with ADHD and controls

VARIABLES	ADHD (n=50) IQR	Controls (n=50) IQR	p*
Age (years)	8.8 (1.5)	8.8 (1.5)	1.00
Maternal Education (years)	6.8 (3.0)	8.5 (3.7)	0.02
Paternal Education (years)	7.5 (3.8)	8.9 (3.4)	0.06
Mean Family Income/Month (TL)	1204.0 (519.7)	1785.0 (1250.9)	0.05
WISC-R Verbal I.Q.	91.6 (12.0)	96.3 (13.2)	0.07
WISC-R Performance I.Q.	99.5 (17.0)	99.2 (12.4)	0.90
WISC-R Total I.Q.	95.1 (13.8)	97.6 (12.5)	0.20

*Mann-Whitney U Test, TL: Turkish Liras, I.Q. : Intelligence Quotient, IQR: Median

Table 2: Total methylphenidate doses received, baseline and endpoint levels of psychometric evaluations and norepinephrine/dopamine levels (after 8 weeks of treatment) of pre-pubertal male children with ADHD

VARIABLES	ADHD-IA (n=4, 8.0%) IQR	ADHD-HIP (n=10, 20.0%) IQR	ADHD-C (n= 36, 72.0%) IQR	p*
Total MPH dose received (mg)	1013.8 (169.4)	971.0 (121.2)	849.9 (198.8)	0.10
DA-Baseline (pg/mL)	167.4 (49.6)	141.9 (65.3)	177.1 (41.1)	0.39
DA-Endpoint (pg/mL)	161.5 (22.2)	163.6 (20.0)	187.8 (41.5)	0.12
NE-Baseline (pg/mL)	292.4 (70.3)	221.5 (91.2)	228.2 (57.6)	0.21
NE-Endpoint (pg/mL)	199.3 (32.6)	215.5 (41.9)	217.3 (54.9)	0.71
ARS-Total-Baseline	34.8 (6.6)	44.1 (7.2)	41.7 (5.7)	0.09
ARS-Total-Endpoint	18.3 (12.8)	29.5 (11.3)	27.6 (5.3)	0.12
ARS-IA-Baseline	19.8 (1.7)	23.7 (3.4)	22.3 (3.9)	0.11
ARS-IA-Endpoint	10.5 (5.8)	17.3 (6.9)	14.5 (3.1)	0.06
ARS-HIP-Baseline	15.0 (7.4)	19.4 (3.5)	19.5 (4.6)	0.42
ARS-HIP-Endpoint	7.8 (7.6)	12.2 (5.2)	13.1 (3.3)	0.34

*Kruskal-Wallis test, IA: Inattentive, HIP: Hyperactive/Impulsive, C: Combined, DA: Dopamine, NE: Norepinephrine, ARS: Du Paul ADHD Rating Scale, IQR: Median

lapping cases), leading to a final sample of 50 patients.

The control group consisted of students from a primary school located within the epidemiological catchment area of the clinic and were matched for class and age to the patients with ADHD. Sociodemographic variables and WISC-R results of the study sample and control group are listed in Table 1. Mothers of participants in the control group had statistically significantly higher education (Z=-2.3, p=0.02), and families in the control group were found to have statistically significantly higher income (Z=-2.0, p<0.05) compared to those of patients with ADHD.

Partial correlations controlling for the ages of patients with ADHD and for ADHD subtypes revealed that baseline NE levels correlated significantly with DA (r=0.47, p=0.001), while they were not related to symptom severity as measured by ARS and CGI. In contrast, there was a statistically significant negative correlation with baseline DA levels and total scores in ARS (r=-0.30, p=0.04). Baseline ARS scores and subscale scores completed by the senior researcher correlated significantly with CGI scores determined by physicians in the outpatient department, verifying the clinical symptom severity of patients (r=0.56, p=0.001; r=0.41, p=0.004; and r=0.39, p=0.01 for ARS-total, inattention, and hyperactivity/impulsivity scores, respectively).

Table 2 shows the total methylphenidate dose received and changes in NE and DA levels along with psychometric evaluations according to ADHD subgroups after 8 weeks of stimulant treatment. Plasma NE levels in all ADHD subgroups were reduced with 8 weeks of stimulant treatment, while changes in DA levels were more complex. Plasma DA levels were reduced with

treatment in the ADHD-inattentive subgroup while they were elevated in the hyperactive/impulsive and combined subgroups. However, there were no statistical differences between ADHD subgroups for these variables. Endpoint NE levels were also found to be correlated with endpoint DA levels after controlling for ages and ADHD subtypes of patients (r=0.42, p=0.003). Endpoint catecholamine levels were not significantly correlated with symptom severity as measured by ARS.

DISCUSSION

This prospective case-control study evaluated plasma NE and DA levels of pre-pubertal male children diagnosed with ADHD and those of age- and gender-matched controls. We hypothesized that patients with ADHD and controls would differ for plasma NE levels and that NE levels would also correlate with symptom severity and treatment response. The analyses for DA were planned as exploratory. In contrast to our a priori hypotheses, we found no difference between groups in terms of plasma catecholamine levels. Plasma NE levels were also not related to symptom severity or treatment response. In contrast, we observed that baseline DA levels were negatively correlated with ARS total scores. Plasma DA and NE levels were found to be correlated at both baseline and the endpoint. No statistically significant relationship could be found between NE or DA levels at the endpoint and any of the psychometric evaluations.

The negative results could be explained by our focus on plasma. In some studies for measuring catecholamine metabolites, urine samples (the second morning urine) were taken and analyzed by HPLC using electrochemical detection. Other studies have examined the baseline cerebrospinal fluid (CSF) of boys with ADHD in relation to stimulant drugs. The recent consensus is that urinary levels of NE and DA may be more informative in patients with ADHD and that concurrent evaluation of multiple neurotransmitter systems (i.e., neuropeptide Y and NE) may be more informative[16-18].

Plasma catecholamine levels are also notoriously unstable and change in response to exercise, diet, and stress (especially for NE)[9-11,14,15]. We have tried to control effects of diet via BMI evaluations and stress with parental reports, but these measures were rather crude and may not have adequately covered daily diet or stressful experiences of participating children[17,18]. Indeed, it is widely known that parents are better reporters for externalizing problems in their children, while children are more reliable reporters for internalizing problems and subjective evaluations such as stress levels[28]. The lack of a reliable and valid scale of subjectively experienced stress for children and adolescents in Turkish led us to use a parent report for approximating stress levels. It may also be argued that evaluating NE/DA levels via canule insertions, repeating evaluations on consecutive days, and using average levels in analyses may be more informative[9-11,14,15]. Evaluation of polymorphisms in monoamine oxidase and dopamine beta hydroxylase genes in our sample may have changed our results.

Limitations of the study included dependence on parental reports for calculating methylphenidate doses. Pill counts may have reflected drug use better and may lead to different results in correlation analyses, although at the cost of alienating parents. The negative results may also be a reflection of the small sample size in subtypes of ADHD. It may also be beneficial to evaluate blood serum levels of methylphenidate to reinforce the results of the study. Another limitation is that we evaluated the ADHD symptoms using a single clinical measure (ARS). Baseline and endpoint evaluations reported by parents and teachers may have led to more nuanced evaluation of symptoms and treatment effects in the sample.

To conclude, we have not found statistically significant differences between plasma levels of NE and DA in a pre-pubertal male sample with ADHD and controls. Both baseline and endpoint NE and DA levels were significantly correlated. While there was a signal that baseline DA levels may correlate with ADHD symptoms as evaluated

via ARS, this was not true for endpoint analyses. Because this negative correlation disappeared after treatment, this finding about baseline DA levels may also be evaluated as an early treatment neuromarker. Further studies are needed to reexamine this unexpected finding and to see if this result can be verified. The results may have been affected by sampling, reporting, and measurement bias, and the measures used to control for the effects of stress and diet were rather crude. Further studies may benefit from concurrent measurements of plasma and urinary levels of catecholamines and their metabolites as well as evaluating enzymes (levels/polymorphisms) involved in their metabolism and co-transmitters.

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