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Elevated level of testosterone in amniotic fluid during prenatal stress and its association with development of Attention-Deficit/Hyperactivity Disorder (ADHD) like symptoms in toddlers

Zwiększone stężenie testosteronu w płynie owodniowym podczas stresu prenatalnego i jego związek z rozwojem objawów podobnych do zespołu nadpobudliwości psychoruchowej z deficytem uwagi (ADHD) u małych dzieci

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Abstract

Aim of the study: To investigate the "prenatal testosterone hypothesis", according to which, high level of testosterone associated with the development of ADHD like symptoms in younger children, and to investigate whether maternal stress during pregnancy increases the risk of developing ADHD like symptoms in early childhood.

Design: This was a prospective study with three measurement periods: the first one during pregnancy (12 to 25 weeks of gestation); the second period involved the assessment of infants of this pregnancy at the age of 6 months and the third one the assessment of the same infants at the age of 18 months. The research plan is approved by "David Tvildiani Medical University" Research Ethics Committee. The study does not contain serious predictable risks as the expected benefit overweighs them.

Material and methods: The study group is presented by 40 pregnant women and 40 infants of these pregnancies. Pregnant women were asked about their perceived stress levels, critical life events of the past year and pregnancy-specific stress levels using standardized questionnaires. Child's temperament and behavioral regulation were assessed using the questionnaires. Amniotic fluid was collected undergoing amniocentesis. Total testosterone in amniotic fluid was measured by radioimmunoassay.

Results: Although statistically significant relationship was identified between hyperactivity, also impulsiveness symptoms and prenatal stress, no statistically significant relationship was identified between testosterone level and hyperactivity, also impulsivity symptoms, as well as between testosterone level and prenatal stress level.

Conclusion: The performed study did not support the "hypothesis about prenatal testosterone", because there was a significant negative correlation between the frequency of hyperactivity symptoms and testosterone level.

Key words: ADHD, amniocentesis, fetal programming, prenatal stress, prenatal testosterone.

Introduction

David Barker's keen observations have been popularized as the "Barker hypothesis" or "Fetal Origins of Adult Disease" (FOAD). This theory relies on the fact that there are specific developmental periods whereby an organism is "plastic" or "sensitive" to its environment. This phenomenon, known as "programming", refers to the fact that stimuli, when applied during early development, generate permanent changes that persist throughout one's lifespan. Programming is not just limited to the in-utero environment, but extends into childhood, where different organs and systems continue to adapt to various cues [1].

Attention deficit hyperactivity disorder (ADHD) is a common neurodevelopmental disorder, with a prevalence of around 7.2% in children [2]. ADHD is characterized by symptoms of inattention, hyperactivity and impulsivity, and is associated with psychosocial impairment, poor academic functioning and psychiatric problems in children and adolescents [3]. The causes of ADHD are not fully understood; however, several environmental (e.g., exposure to maternal stress during pregnancy, certain foods or inhalants) and genetic risk factors have been proposed [4]. Prenatal influences have received increasing attention as potential causes of ADHD, mainly due to the hypothesis that prenatal exposures predispose individuals to disorders such as ADHD through fetal programming. Fetal programming refers to a process where factors in the intrauterine environment are hypothesized to influence the normal development of the fetus. Prenatal exposures, such as maternal stress, might permanently

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influence the structure, physiology and metabolism, causing long lasting changes that might predispose individuals to later disorders. Prenatal stress has been suggested to influence the child's neurodevelopment and later risk of developing ADHD [5].

Sex hormones play many roles in the development and function of the human body and brain. Organizational effects of hormones are believed to play an important role in the structural organization of the brain and body with subsequent effects on sex-typed behavior. Although much is known about the effects of testosterone in adolescence and adulthood, little is known in humans about how testosterone in fetal development influences later neural sensitivity to valence facial cues and approach-avoidance behavioral tendencies. Thus, organizational theory of prenatal testosterone effects on behavior suggests that prenatal testosterone might influence early-emerging disruptive behavior disorders (DBDs) that are more common in males [6].

We aimed to investigate that testosterone in humans acts as a fetal programming mechanism on the reward system and influences behavioral approach tendencies later in life. As a mechanism influencing atypical development, fetal testosterone (FT) might be important across a range of neuropsychiatric conditions that asymmetrically affect the sexes, the reward system, emotion processing, and approach behavior.

Material and methods

A sample of 50 pregnant women was formed by target selection. Seven of them refused to participate in the research after the second interview. One infant died within the first few hours after he was born, one infant was adopted and one infant was excluded from the study group due to congenital pathology. Thus, the study group is presented by 40 pregnant women and 40 infants of these pregnancies.

Based on that the invasive, non-routine method research in pregnant women selected by us (the use of amniotic fluid obtained through amniocentesis as a biomarker) is restricted all over the world due to ethical reasons. The number of study participants corresponds to the number of the participants of similar studies in the world. For this 1 ml amniotic fluid was required which was taken during routine study of genetic pathologies.

The data were collected from May, 2019 through May, 2021 on basis of "Pineo Medical Ecosystem, Perinatology'' department and laboratory "Ltd Georgia Pacific Partnership". Material-technical support and result analysis were conducted in "Academician K. Nemsadze Pediatric Clinic Globalmed".

For the exposed group (exp. group; $n = 30$) there were the following inclusion criteria: existence of prenatal maternal stress and for non-exposed group (non-exp. group; $n = 10$), absence of prenatal maternal stress. Informed consent of pregnant woman was obtained.

The following exclusion criteria were used for both groups: medical risk-factors, such as acute and chronic diseases, especially gestational diabetes, familial case of fat metabolism disorder, arterial hypertension, hyperthyroidism, first pregnancy after the age of 40 years, gestosis, existence of fetal development defects, psychiatric disorders.

Exclusion criteria were checked during the first structural interview by obstetrics-gynecologist and geneticist.

In addition to this we took into consideration the social-demographic factors that probably influence the development of behavioral disorders: maternal age, nationality, marital status, maternal educational level (medium, high), professional activity, sequence of pregnancy, alcohol consumption and tobacco use/passive smoking during pregnancy, wanted/unwanted pregnancy, planned/unplanned pregnancy.

I assessment. The existence of specific stress characterized for pregnancy and stress quality was assessed through stress standardized questionnaires such as PSS [7], TICS [8], PDQ [9]. At the same time social- demographic features were studied. Also, prenatal testosterone from amniotic fluid of 40 pregnant women was obtained through amniocentesis (12 to 25 weeks). Free testosterone was measured through radioimmunoassay.

II assessment. Telephone interview (due to COVID-19 pandemic) with the mothers of infants. The age of infants was 6 months. Based on the telephone interview we evaluated infants' temperament and behavioral regulation through valid questionnaire IBQ [10].

III assessment. The assessment of the same infants at the age of 18 months via telephone interview again due to COVID-19 pandemics by valid questionnaire developed by us (RAT-Recognizing ADHD in toddlers), assessing whether the attention deficit hyperactivity syndrome like symptoms were present at the specific age.

Statistical analysis

The data were processed via different methods of descriptive and conclusive statistics using SPSS 20.0. The relationship between categorical variables was checked using Pearson's χ^2 statistical method. A correlation analysis was performed using Pearson's correlation coefficient. Means within the group were compared via *t* criterion for independent selection. Intergroup comparisons for independent selection were evaluated via non-parametric Mann Whitney *U* test ad Kruskal-Wallis test. Intragroup comparisons were evaluated via Ranks *Z* test. The comparison between means for more than two independent selections was measured via one factor ANOVA. Intergroup comparisons were measured via Bonferroni test. For the determination of the relationship between variables regressive analysis was used.

Ethical standards

The research plan is approved by "David Tvildiani Medical University" Research Ethics Committee (N1 27.10.2019).

Results

Descriptive results

All the study participants were Georgian ethnicity. Maternal age varied from 19 to 43 years (M ±SD): 32.03 ±6.792. Professional activity during pregnancy in both groups was as follows: 60% was unemployed, 40% was employed. In 70% of the control group and 60% of the study group, the pregnancy was unplanned, but in both cases, pregnancy was 100% want-

Table I. Demographic and clinical parameters

Parameter	Control group	Study group
Marital status	Married – 90%	Married – 97%
	Divorced – 10%	Divorced – 3%
Educational level	High education – 70%	Secondary – 37%
	Secondary – 30%	High education – 63%
Tobacco and alcohol consumption/ passive smoking during pregnancy	100% did not consume alcohol and tobacco, and did not note passive smoking	3% consumed tobacco and alcohol and was a passive smoker
Family economic status	Middle – 100%	Middle – 83%
		Low – 17%
Planned/unplanned pregnancy	Planned – 30%	Planned – 40%
	Unplanned – 70%	Unplanned – 60%
Testosterone level	High – 30%	High – 46.7%
	Normal – 60%	Normal – 53.3%
	Low – 10%	Low level was not identified
Prenatal stress level	Low stress was identified	Moderate – 86.7%
		High – 13.3%
Child sex	6 females	17 females
	4 males	13 males
Child hight at birth	M ±SD: 49.9 ±0.568 cm	M ±SD: 50.7 ±2.781 cm
Delivery type	Cesarean section – 40%	Cesarean section – 47%
	Physiological delivery – 60%	Physiological delivery – 53%
Birth week	M ±SD: 39.50 ±0.707 weeks	M ±SD: 38.03 ±2.025 weeks
Breastfeeding duration	6 months – 40%	6 months – 10%
Children's mean age when evaluation was performed	M ±SD: 18.30 ±0.823 months	M ±SD: 18.47 ±0.937 months

ed. Gestational age when amniocentesis was performed was (M ±SD): 19.08 ±2.596 week of pregnancy. Prenatal stress level was determined with PSS score [10]. Parity was: first pregnancy – 18%, second one – 30%, the third one – 20%, 4th and more – 32%. Premature children comprised 13%, i.e. that is 4 children in exp. group. Demographic and clinical parameters of sample are described in Table I.

Analytical results

According to independent sample tests, statistically significant difference was revealed between the quality of specific stress characterized for pregnancy, in exp. group stress vs. the mothers of non-exp. group ($p < 0.001$).

Also, there was a statistically significant difference between stress levels, in exp. group mothers had highly pronounced moderate and severe stress levels ($p < 0.001$). Statistically significant difference was revealed also at the determining chronic stress. The exp. group mothers were showed more chronic stress, in particular, they were more under stress while performing daily activities and stress determined by permanent conflicts that were not managed properly and they had higher social recognition deficit ($p < 0.001$), which established by TICS.

According to questionnaire assessing the temperament non-exp. group infants are more frequently under positive emotions than the exp. group. Exp. group infants required more time and relaxing strategies so that they shifted attention from negative influence. Also, they more frequently expressed negative emotions due to limitations and showed the tendency to feel fear when they saw new irritants. Moreover, they had higher rough motor activity level. They often kicked their arms and legs, and became nervous in various situations compared with non-exp. group ($p < 0.001$). The same tendency was revealed using nonparametric statistical method.

In exp. children compared to non-exp. group hyperactivity and impulsiveness symptoms were more revealed ($p < 0.001$). The same tendency was revealed using nonparametric statistical method.

As regards to the level of testosterone in exp. group, it was increased (M ±SD): 0.77 ±0.38 nmol/l compared to the non-exp. group although parametric $t(25.483) = -1.760$, $p = 0.090$), as well as nonparametric ($U = 112.500$, $p = 0.241$) statistical methods between groups significant statistical difference was not revealed.

We did not identify significant associations between socio-demographic variables and children behavioral characteristics. Although statistically significant relationship was identified between hyperactivity symptoms and prenatal stress ($\chi^2 = 26.437$, $df = 6$, $p < 0.001$). Also, statistically significant relationship was established between impulsiveness symptoms and prenatal stress ($\chi^2 = 19.500$, $df = 14$, $p = 0.001$). As regards to the testosterone level, statistically significant difference was not revealed between exp. and non-exp. groups ($p = 0.169$). No statistically significant relationship was identified between testosterone level and hyperactivity symptoms ($p = 0.777$), or between testosterone level and prenatal stress level ($p = 0.278$). Using these tests, no significant positive rela-

tionship was revealed between testosterone level and 5 factors assessing infant temperament.

Regression

The frequency of hyperactivity symptoms was not significantly related to testosterone level and prenatal stress ($R = 0.744$, $R2 = 0.553$). In order to determine the reliability of obtained results we used multifactorial dispersion analysis ANOVA method. This method revealed that the difference between mean values of dependent and constant variables was not statistically significant ($F = 22.918$, $p < 0.001$); for testosterone: ß = –0.185, $t(39) = –1.615$, $p = 0.115$; for prenatal stress: ß = –0.775, $t(39) = 6.762$, $p = 0.000$. Consequently these factors are independent from each other and there is not interaction effect between them.

To study the influence of maternal stress and testosterone level on the frequency of impulsiveness symptoms we used again regression analysis, but according to the obtained results the frequency of impulsivity symptoms was not significantly related to testosterone level and prenatal stress ($R = 0.559$, $R2 = 0.312$). To determine the reliability of obtained result we again used multifactorial ANOVA method. This method revealed that the difference between mean values of dependent and constant variables was not statistically significant ($F = 8.408$, $p < 0.001$); for testosterone: ß = 0.231, $t(39) = –1.623$, $p = 0.113$; for prenatal stress ß = –0.448, $t(39) = 3.148$, $p = 0.003$. Consequently these factors are independent from each other and there is not interaction effect between them.

In both cases – hyperactivity/impulsivity – a significant negative correlation with testosterone level was revealed (impulsivity $p = 0.113$, hyperactivity $p = 0.115$), but a significant positive correlation with prenatal stress was shown (impulsivity $p = 0.003$, hyperactivity $p = 0.000$).

Discussion

Fetal exposure to testosterone is increasingly implicated in the programming of future reproductive and non-reproductive behavior. 14–18 gestational weeks is considered as a peak level of testosterone in fetal plasma serum and we studied testosterone level in the gestational period. In this study we used amniotic fluid, as a biomarker, to study fetal exposition. In Georgia amniocentesis procedure is performed only in one clinic and besides this type of study is being performed for the first time in Georgia. In this study there was no relationship between testosterone of amniotic fluid and attention deficiency hyperactivity syndrome like symptoms in younger children, although there are some studies involving the results of performed studies, similar to our hypothesis, and they do not reveal any significant relationship between hyperactivity and increased activity of androgens [11, 12]. Some authors assume that this relationship could exist in particular age group [13]. Despite the fact that in our current sample there is no relationship between testosterone and behavioral characteristics in young children, it could be conditioned by the quantitative scarcity of samples in this particular age group which is one of the main limitations of this study. Large scale future research in this particular age group should be conducted to shed more light on this issue and study a potential effect of active testosterone on behavioral problem, such as attention deficiency and hyperactivity syndrome. It is also poorly studied how testosterone affects neurocognitive function in the patients with attention deficit hyperactivity syndrome. According to our study attention deficit hyperactivity syndrome like symptoms are almost equal in girls and boys.

In terms of the literature related to maternal prenatal stress and attention deficiency and hyperactivity syndrome, this study is consistent with existing literature and generalizes it showing that even after the control of a large number of studies [14, 15].

Conclusions

The performed study did not support the hypothesis about, prenatal testosterone, as there was a significant negative correlation between the frequency of hyperactivity symptoms and testosterone level. Along with it a significant negative correlation between maternal perceived stress and testosterone level was revealed meaning that the higher the perceived stress level the lower the testosterone level and vice versa. The model is significant at the expense of the perceived stress $p < 0.001$. Along with it a close relationship between prenatal stress level and the frequency of hyperactivity and impulsivity (Pearson's R and Spearman correlation $p < 0.001$), was revealed that once more emphasizes the significance of fetal programming as a basis during the development of neurological-behavioral disorders.

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