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Elevated level of prenatal testosterone and vitamin D_3 deficiency during pregnancy, in the presence of prenatal maternal stress, and their association with the development of attention deficit hyperactivity disorder (ADHD)-like symptoms in toddlers

Podwyższone stężenie prenatalnego testosteronu i niedobór witaminy D_3 podczas ciąży, w obecności prenatalnego stresu matki i ich związek z rozwojem objawów zespołu nadpobudliwości psychoruchowej z deficytem uwagi (ADHD) u małych dzieci

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Abstract

Aim of the study: To investigate the hypothesis that the presence of prenatal maternal stress, increased level of prenatal testosterone, and low level of vitamin D_3 in pregnancy is associated with the development of ADHD-like symptoms in toddlers (< 2 years old).

Material and methods: The study group comprised 53 pregnant women and 53 infants of these pregnancies. The population cohort of 53 pregnant women were recruited at their 35th to 37th week of pregnancy and investigated prospectively. The participants were selected through targeted selection. Maternal experience of stressful life events was assessed by stress standardised questionnaires, prenatal testosterone was determined in the mothers' saliva by using the immune enzymatic (ELISA) method, and maternal plasma D vitamin was measured using the ECLIA method, during pregnancy. When the age of the offspring was 6 months and then less than 2 years, the mothers completed the child behaviour and temperament checklist.

Results: A small but statistically significant association was found between the common symptom complex of ADHD and the level of testosterone and vitamin D_3, in the presence of prenatal maternal stress. Multiple regression analysis showed that maternal stressful events during pregnancy significantly predicted ADHD behaviours in offspring.

Conclusion: The study supported the hypothesis that prenatal maternal stress, increased level of prenatal testosterone, and low level of vitamin D3 during pregnancy increases the risk of development of ADHD-like symptoms in toddlers (< 2 years old). Also, the obtained results support the hypothesis that the influence of prenatal factors causes ADHD-like symptoms in offspring through a programming effect.

Key words: fetal programming, attention deficit hyperactivity disorder (ADHD), prenatal maternal stress (PNMS), vitamin D, prenatal testosterone.

Introduction

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder (NDD) presenting with inattention, hyperactivity, and impulsivity. It can be classified into 3 subtypes, depending on the intensity of the symptoms: predominantly inattentive, predominantly hyperactive-impulsive, and combined [1]. ADHD has a global prevalence of 5.9% to 7.1% in children and 1.2% to 7.3% in adults [2]. The aetiology of this condition is a multifactorial with high heritability and with underlying environmental risk factors. Many prospective studies have shown that prenatal maternal stress (PNMS) increases risk in the offspring for childhood behaviour and emotional problems [3]. Research exploring an association between PNMS and ADHD includes 4 prospective longitudinal studies [4–7]. Some studies have investigated the association between vitamin D concentrations during pregnancy and neurodevelopmental outcomes, such as behavioural problems and social competence, in offspring. It has been proposed that vitamin D deficiency could be a risk factor for developing ADHD [8, 9]. Some studies support the hypothesis that prenatal testosterone exposure contributes to the development of ADHD in children [10, 11]. However, some researchers have not found associations between prenatal testosterone exposure and ADHD features or externalising behaviour problems in children [12].

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Material and methods

The study group comprised 53 pregnant women and 53 infants of these pregnancies. Blood samples were obtained at the 35th to 37th gestational weeks by routine method. Non-routine method free testosterone was measured in saliva. The participants were selected through targeted selection.

The control group included 26 pregnant women and study group comprised 27 pregnant women. For the control group the following inclusion criteria were applied: absence of fetal stress, no vitamin D_3 intake during pregnancy. And for the study group: existence of fetal stress, and no vitamin D_3 intake during pregnancy. 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, hypothyreosis, first pregnancy after the age of 40 years, gestosis, fetal developmental defects, and psychiatric disorders. Exclusion criteria were tested at the first structural interview by an obstetric gynaecologist. In addition to this we took into consideration the sociodemographic factors that probably influence the development of behavioural disorders: maternal age, marital status, maternal educational level, professional activity, parity, alcohol consumption, tobacco use/passive smoking during pregnancy, and wanted/unwanted and planned/unplanned pregnancy.

I assessment – in pregnant women (35th–37th week of pregnancy) the existence of specific stress characterised for pregnancy and stress degree were assessed through stress standardised valid questionnaires such as PSS [13], TICS [14], and PDQ [15]. At the same time sociodemographic features were studied. Also, after this visit, blood samples were obtained to measure vitamin D_3 {25-hydroxyvitamin D [25(OH) D]}. The study was conducted using the ECLIA method. On the second day of the planned visit the pregnant women had a saliva specimen collected (hydrocellulose smear) in a special container to measure free testosterone. The study was performed using immune enzymatic (ELISA) method.

II assessment – telephone interviews (due to the COVID-19 pandemic) with the mothers of the infants. The age of the infants was 6 months. Based on the telephone interview, we evaluated infants' temperament and behavioural regulation through a valid IBQ questionnaire [16].

III assessment – the assessment of the same infants at the age of 18 months via telephone interview (again due to the COVID-19 pandemic) by valid questionnaire (Recognizing ADHD in Toddlers [RAT]), developed by us, assessing whether ADHD-like symptoms were present at the specific age.

The mothers' average age was M $\pm$SD: 30.53$\pm$6.45 years. All the participants were married. For both groups of mothers, the pregnancy was wanted. The frequency of passive smoking, and tobacco and alcohol consumption was equal in both groups: 3.8% were consuming, while 96.3% were not. The gestational age when the laboratory samples were collected (vitamin D, testosterone in saliva) was M $\pm$SD: 36.13 $\pm$0.833 weeks. The testosterone level was increased only in 26% of the study group. For 20.8% of participants COVID-19 was an independent stress factor. The children's mean age when they were assessed was M $\pm$SD: 17.87 $\pm$0.520 months. Demographic, clinical, and behavioural parameters of our samples are described in Table I.

Statistical analyses

Data were processed via descriptive and conclusive statistics and other methods, using SPSS Statistics 23.0. The statistical significance of the difference of mean values of interval variables between groups were determined using independent samples t-tests. The statistical significance of the difference of the variations of interval variables were determined using Levin tests. The difference of the distribution of interval variables was studied using Mann-Whitney U test. Intra-group comparisons were evaluated via χ^2 tests and Fisher's exact test. To determine the depth of correlation we used Cramer's V. To study linear relationships between interval variables in the general population and between groups we used Pearson's correlation coefficient, and to study monotonic relationship between interval variables we used Spearman Rank coefficient. To determine linear relationships between variables we used a linear regression model, and to check their value we used the well-known F-test and t-test.

Bioethical standards

The research plan was approved by David Tvildiani Medical University research Ethics Committee (Ethical Committee agreement number 27.10.2019 N 1). Informed consent was obtained from patients' parents for description and publication. The study does not contain serious predictable risks because the expected benefit overweighs them.

Results

Mean data in groups were compared using t-test for independent selection. The difference between the following variables was statistically significant: average D_3 vitamin level in control group mothers M $\pm$SD: 30.71 $\pm$6.33 ng/ml was compared with study group mothers M $\pm$SD: 24.22 $\pm$9.14 ng/ml. Average testosterone level in saliva (normal range: 5–32 pg/ml) was increased in the study group M $\pm$SD: 25.50 $\pm$7.01 pg/ml compared with control group mothers M $\pm$SD: 15.32 $\pm$5.02 pg/ml. Statistically reliable significance occurred according to assessment of the questionnaire of infants' temperament, between study and control groups. In particular, control group infants were more frequently under positive emotions, smiled/laughed more frequently while playing, washing, and dressing, compared with study group. Study group infants expressed negative emotions/irritation more frequently during discomfort due to limitations, and they cried and complained more frequently during motor limitation (dressing, undressing, changing of body position, putting supine, waiting before eating food); also, they retreated more frequently and showed a tendency of fear when seeing sudden and new irritators or when feeling them. In particular, they cried and snivelled when mechanisms made noise, when they saw a strange person, were in a foreign en-

Table I. Demographic, clinical, and behavioural parameters of samples

	Control group	Study group
Mothers' educational level	30.8% secondary education, 69.2% higher	33.3% secondary education, 66.7% higher
The frequency of mother's professional activity during pregnancy	34.6% worked, 65.4% unemployed	55.6% worked, 44.4% unemployed
Economic status of families	100% average condition	96.3% average, 3.7% high
Planned/unplanned pregnancy	61.5% planned, 38.5% unplanned	44.4% planned, 55.6% unplanned
Parity	I – 34.6%, II – 30.8%, III and more – 34.6%	I – 22.2%, II – 37.0%, III and more – 40.7%
The amount of D_3 vitamin	M ±SD: 30.71 ±6.33 ng/ml Min = 20.23 ng/ml Max = 45.36 ng/ml	M ±SD: 24.22 ±9.14 ng/ml Min = 12.94 ng/ml Max = 52.63 ng/ml
The amount of free testosterone in saliva	M ±SD: 15.32 ±5.02 pg/ml Min = 5.6 pg/ml Max = 23.6 pg/ml	M ±SD: 25.50 ±7.01 pg/ml Min=12.2 pg/ml Max = 35.2 pg/ml
Type of delivery	53.8% caesarean section 46.2% physiological	44.4% caesarean section 55.6% physiological
Week of birth	M ±SD: 39±.0.80 week	M ±SD: 39 ±1.18 week
Sex distribution of children	11 girls, 15 boys	16 girls, 11 boys
Child's birth weight	M ±SD: 3473 ±349.351 g	M ±SD: 3424 ±371.9 g
Child's birth length	M ±SD: 50.65 ±1.47 cm	M ±SD: 51.04 ±1.48 cm
Duration of breastfeeding	M ±SD: 5.85 ±5.15 months, not implemented 26.9%	M ±SD: 5.81 ±4.30 months, not implemented in 14.8%
Stress severity	100% mild degree	18.5% mild degree, 55.6% moderate degree, 25.9% severe degree
The frequency of hyperactivity symptoms	11.5%	74.1%
The frequency of impulsivity symptoms	11.5%	77.8%
ADHD total score (hyperactivity, as well impulsivity symptoms)	7.7%	63%
At the presence of prenatal stress, the risk of the development of the syndrome is increasing by	15.4%	88.9%

vironment, or tasted new food, and they had higher gross motor activity level, compared to the control group. The infants of the study group required more time and calming strategies so that they could replace negative influence, compared to control group infants, although a statistically significant difference was not detected.

A statistically significant difference was detected also between the degrees of specific stress characterised for pregnancy. In study group mothers stress was more often manifested related to delivery and newborn-related stress, stress related to body changes and physical symptoms, and related to emotional relationship with the infant, compared with study group mothers.

Also, there was a statistically significant difference in stress degree, between moderate and severe stress, in the study group mothers. A statistically significant difference was revealed in the determination of chronic stress. We concluded that the study group mothers were under chronic stress; in particular, they are more under stress while doing daily home tasks such as professional life, family and housewife functions; they were more excited toward possible events, frequently had bad expectations about, for instance, natural disasters, job loss, injury, illness; they were more frequently under social stress caused by permanent conflicts that were not properly managed; they frequently had problems at work – stress due to obligations at work becasuse they had little self-motivation; and they had more traumatic, intrusive memories from the past, which frequently cause chronic stress.

In the study group infants, hyperactivity and impulsivity symptoms were manifested more often than in the control group. The same tendency was revealed using a nonparametric Mann-Whitney U Test comparing not only 2 groups' averages but also the distribution between them. Thus, the given test is more complex than the above-mentioned t-test.

Pearson's χ^2 and Fisher's tests did not reveal significant associations between socio-demographic variables and developed symptom complex, although a statistically significant relationship was identified between the degree of vitamin D_3 deficit, testosterone level, degree of stress, and developed symptom complex (hyperactivity, impulsivity), and these links were very strong Pearson's R $r = 0.676$, $p < 0.001$; Spearman correlation $r = 0.636$, $p < 0.001$. We revealed a statistically significant relationship also between vitamin D_3 deficit and the level of testosterone by Fisher's exact test $x^2 = 6.050$, $p = 0.04$ ($p < 0.05$), while by χ^2 test only a 10% value was obtained $x^2 = 5.456$, df = 2, $p = 0.073$ ($p < 0.1$), Cramer's V = 0.32, between vitamin D_3 deficit and stress level, between D_3 vitamin deficit and impulsivity symptoms, between vitamin D_3 deficit and ADHD common symptom complex, between testosterone level and stress level, between level and frequency of hyperactivity symptoms, between testosterone level and ADHD common symptom complex (ADHD total score), between stress degree and the frequency of hyperactivity symptoms, between stress level and the frequency of impulsivity symptoms, and between stress level and ADHD common symptom complex.

Regression

In order to study the impact of maternal stress and vitamin D_3 on the frequency of common symptom complex we used regression analysis. As a dependent variable the frequency of ADHD common symptom complex was used, while as constant variables we used D_3 level and prenatal stress. According to the obtained results, the model is somewhat significant, with a determination coefficient $R^2 = 0.396$. To determine the reliability of the obtained result we used multifactorial dispersion analysis ANOVA method, which confirmed the above-mentioned $F = 16.375$, $p < 0.001$. Although, in this model only the maternal stress variable was statistically significant, at $p < 0.001$, while the level of vitamin D_3 was statistically insignificant. To study the influence of maternal stress and testosterone level on the frequency of hyperactivity symptoms we used regression analysis. Hyperactivity symptom was used as a dependent variable, while as constant variables we used testosterone level and prenatal stress. It proved to be somewhat significant, with determination coefficient $R^2 = 0.407$. To determine the reliability of the obtained result we used multifactorial dispersion analysis ANOVA method, which confirmed the above mentioned $F = 17.169$, $p < 0.001$. However, in this model only maternal stress variable was statistically significant $p < 0.001$, while the testosterone level was statistically insignificant, $p = 0.249$.

Discussion

The results of this study are theoretically and practically applicable in a variety of ways.

The research project subject – ADHD – is a serious global problem of health care. The share of its development in the structure of child morbidity is increasing globally each year. This study is also important because ADHD is a severe neurobiological disorder that starts in childhood, and nearly 50% of cases persist until adulthood. Its development is mostly accompanied by concomitant mental complaints. Based on the disorder, severe functional disorders occur in various areas of daily life (academic/professional functioning, social relationships, everyday problems, and criminal). Along with the familiar symptoms, other complex emotional symptoms emerge that further complicate the daily life of the people with this symptom complex.

Thus, the verification of disease predictors at the fetal programming level will significantly contribute to further preventive measures. However, it is necessary to study not only the incidence/prevalence of the disease (in Georgia we have no precise data), but also to accurately verify the syndrome development predictors, which will be encouraged by our study, as well as other studies. In addition, the study methodology is complex, i.e. the determination of the presence (and severity) of stress in pregnant women by clinical evaluation (interview-valid questionnaires) and testosterone levels in saliva and vitamin D from plasma, and the assessment of the development in the context of symptoms characteristic for ADHD. The results, along with other international studies, will contribute to an in-depth understanding of the problem, as well as establish the scientific basis for the elaboration of practical recommendations for the management of pregnant women and their children.

Based on the results obtained by us, prenatal maternal stress was significantly associated with higher levels of ADHD symptoms in the offspring. The associations were significant for stress during pregnancy overall but not for each pregnancy trimester separately or according to its degree (mild, moderate, severe). The results are in line with my hypothesis and consistent with the findings of most previous studies [17, 18].

Vitamin D status during prenatal brain development may influence risk of ADHD symptoms in childhood. However, there are no prospective studies addressing this hypothesis. Our findings indicate a positive association between prenatal $25(OH)D_3$ concentrations and ADHD-like symptoms at < 2 years of age.

Maternal salivary testosterone, indexing the free concentrations of biologically active testosterone at each gestational period did not differ according to fetal sex, which is consistent with reports based on maternal serum testosterone in pregnancy [19, 20].

The results of the current study support a modest association between individual variations in maternal testosterone levels and toddlers' neurobehavioural development. Higher maternal salivary testosterone at 36 weeks' gestation was significantly associated with hyperactivity total score. The current findings provide preliminary empirical support that maternal testosterone is associated with interference of fetal neuromaturation development in late gestation. The modest level of these detected associations may be attributable to the fact that the measurement of maternal testosterone, whether in serum or saliva, provides only a proxy for actual fetal exposure, which is directly measurable only within clinically indicated procedures that are infrequently administered and confer risk.

Conclusions

The performed study supported the hypothesis that the existence of prenatal stress, increased level of prenatal testosterone, and low level of vitamin D_3 during pregnancy increases the risk of development of attention deficit hyperactivity syndrome-like symptoms in toddlers (< 2 years old). Also, the obtained results once more support the hypothesis that the impact of prenatal factors can cause the development of ADHD-like symptoms in the offspring through fetal programming.

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