Chronic Testosterone Administration During Pregnancy Causes Autistic-like Traits in Rat Offspring: Possible Relationships with Cerebral Oxytocin, Dopamine, Serotonin and IGF-1 Levels.

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Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that affects boys three times more frequently than girls. Previous studies have suggested that higher fetal testosterone exposure can cause autism-like behaviors in males. The mechanism of effect of fetal testosterone on autism development is not well known. In the present study, we investigated the relationship between prenatal testosterone exposure and ASD in an experimental study with the use of behavioral tests, histopathological examinations and biochemical measurements performed to compare offspring with and without exposure to testosterone. Female rats were randomly distributed into Group 1 (control, n = 6) and Group 2 (Testosterone undecanoate, n = 6). Female rats were caged with a fertile male (three female/one male) for 2–3 days during the oestrus period. Group 1 rats were given 1 ml/kg % 0.9 NaCl saline on the 10th day of pregnancy, while Group 2 rats were given 250 mg/kg testosterone undecanoate. The dams were allowed to raise their litters until weaning on postnatal day 21 (P21). On P21, forty littermates (10 male control, ten female control, ten male Testosterone-exposed, and ten female Testosterone-exposed) were randomly separated and housed. These animals underwent behavioral testing in adulthood on P50. Subsequently, the rats were sacrificed. Biochemical analyses [Brain tissue 5-Hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), Insulin-like growth factor-1 (IGF-1), and oxytocin] and histopathological analyses were performed. Analysis of the behavioral tests (three-chamber social test, open field test) revealed significant differences among the groups, with the testosterone-exposed groups demonstrating autistic traits at a greater degree. Histologically, hippocampal CA1 and CA3 regions displayed significant alterations such as gliosis and neuronal cell death in the testosterone-exposed groups compared to controls. Brain levels of tissue 5-HIAA, HVA, IGF-1 increased while oxytocin level decreased in the testosterone-exposed groups. These results suggest a possible link between chronic prenatal testosterone exposure and neurodevelopmental disorders such as ASD. Testosterone exposure and autism-like traits can be linked to dopamine, serotonin, IGF-1 increase, and oxytocin decrease.

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that affects communication and behavior. It is characterized by early-onset deficits in social and communication skills, restricted interests and repetitive body movements (Posar et al., 2015). Autism prevalence has been increasing in recent years (Maenner et al., 2020). The etiology of ASD is unknown, but it is thought that genetic and environmental factors come together in the development of ASD (Taylor et al., 2020). Current data demonstrates that boys are affected around three times more frequently than girls (Becker, 2012; Ferri et al., 2018). However, the mechanisms underlying male vulnerability are not well-known and remain understudied.

A higher incidence of ASD in males can in fact be among the features that could help to enlighten its etiology. For instance, intrauterine fetal testosterone exposure has gained some recognition as arguably the most popular hypothesis concerning the etiology of autism. Previous studies have suggested that higher fetal testosterone exposure can cause autism-like behaviors in males (Ferri et al., 2018). However,
the mechanism between prenatal high dose testosterone exposure and autistic traits is not well documented. Recent studies showed that testosterone induces molecular changes in dopamine in the nigrostriatal pathway, thus increasing the effect of dopamine in the body (Purves-Tyson et al., 2014). It is well known that increased dopamine causes behavioral anomalies (Klein et al., 2019). Serotonin is another neurotransmitter that is shown to increase in autistic children (Anderson et al., 1987). Dysregulation of serotonin has been linked to behavioral anomalies (Hassan et al., 2019), and the latest studies have shown that there is a possible relationship between increased intrauterine testosterone levels and serotonin levels. Oxytocin is a famous molecule that appears to be involved in the neurobiology of autism development as per the results of recent studies. Oxytocin has been shown to modify synaptic plasticity and modulate social behaviors (Insel, 2010). In addition, empathy induction among individuals has been associated with increased oxytocin levels (Procysyn et al., 2020). IGF-1 (Insulin-like growth factor-1), which reduces neuroinflammation by affecting cytokines and glial activation, is another hormone that can be increased in autism (Riikonen, 2017).

This experimental study exposed pregnant rats to testosterone and assessed the results on the offspring via behavioral parameters, such as locomotor activity and anxiety-like behavior, using the open field test and social interaction using the three-chamber test. Additionally, we aimed to evaluate the possible association between prenatal testosterone exposure and other biochemical parameters, including 5-Hydroxyindoleacetic acid (5-HIAA) (serotonin's primary metabolite), homovanillic acid (HVA) (associated with dopamine levels in the brain), oxytocin, and IGF-1. We also evaluated the effect of prenatal testosterone exposure on the cornu ammonis 1 (CA1) and cornu ammonis 3 (CA3) regions of the hippocampus via neuronal morphology, total neuron count, and GFAP immunoexpression.

**Materials And Methods**

**Animals**

The experimental procedures employed in the present study were approved by the Animal Ethics Committee (199545685/2). The rats used in the experiment were obtained from the Experimental Animal Laboratory of Bilim University. All experiments were carried out with respect to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (US) (2011).

Twelve female and four male Wistar adult rats (220 ± 10 g) were included in the study. The rats were housed in plastic cages and maintained under standard conditions with 12-hour light/dark cycles at room temperature (22 ± 2°C) and a humidity of 45–60%.

**Study design**

Female rats were randomly distributed into Group 1 (control, n = 6) and Group 2 (recipients of testosterone undecanoate, n = 6). All animals were monitored daily for behavior and health conditions throughout the study. Female rats were caged with a fertile male (three female/one male) for 2–3 days during the
oestrus period. Mating was checked by looking at the white vaginal plaque in rats. Then, male rats were removed from the cages.

Group 1 rats were given 1 ml/kg % 0.9 NaCl saline on the 10th day of pregnancy. Group 2 rats were given 250 mg/kg testosterone undecanoate (Nebido Ampul, Bayer, 250 mg/ml) on the 10th day of pregnancy. On the day of birth, to ensure standardized maternal care, litter size was reduced to 9 pups per dam. The dams were allowed to raise their own litters until weaning on postnatal day 21 (P21). On P21, forty litters (10 male control, ten female control, ten male Testosterone-exposed, and ten female Testosterone-exposed) were randomly separated and housed in same-sex and same study group cages with ad libitum access to standard rodent chow and tap water. These animals underwent behavioral testing in adulthood on P50. All behavioral experiments were conducted between 10.00 AM and 3.00 PM. A summary of the study design is presented in Fig. 1.

Behavioral tests

Three-chamber sociability and social novelty test

The sociability test was performed as previously described with minor modifications (Ellegood and Crawley, 2015; Erbas et al., 2018; Moy et al., 2004). Briefly, a Plexiglas cage (40 cm x 90 cm x 40 cm) was divided into three equal regions (40 cm x 30 cm x 40 cm). On the first day, rats were allowed to habituate in the test cage for 5 min (pre-test session). Twenty-four hours later, to test sociability, a stranger rat was placed inside a small plastic cage with mesh-like holes in one side chamber and an empty cage in the third chamber. Then, the test rat was placed in the center chamber, and the time spent in each region by the test rat was recorded for 10 min (sociability test). The test rat was considered to be in the chamber when its head and two front paws entered the chamber. The field floor was then cleaned between each test with 70% alcohol and dried with a paper towel to remove any traces of olfactory stimuli from the previous rat.

Open field test

The open-field test was conducted in an open-air box with dimensions of 50 cm x 50 cm x 40 cm (Erbas et al., 2018). At the beginning of the test, rats were gently placed in the center of the box and freely explored the arena for 5 minutes. Then, each rat was observed for 5 min to evaluate spontaneous activity level. The total number of ambulation (number of floor divisions crossed with the four paws) were recorded. The field floor was then cleaned between each animal with a 70% alcohol-water solution and dried with a paper towel to remove olfactory cues.

Hippocampus histopathology

The CA1 and CA3 regions of the hippocampus were chosen as the target areas to be examined for hippocampus evaluation. Briefly, following behavioral tests, animals were euthanized, and their brains were removed and fixed in 10% formaldehyde (0.1 M phosphate buffer saline, PBS) for three days. Then, they were moved into 30% sucrose and stored at 4°C until infiltration was complete. The brains were cut
coronally on a sliding microtome at 40 µm and mounted on gelatinized glass slides. Cresyl violet staining to quantify the number of surviving neurons were performed in six sections per studied group by an image analysis system (Image-Pro Express 1.4.5, Media Cybernetics, Inc. USA).

For GFAP immunohistochemistry, brain sections were incubated with H₂O₂ (10%) for 30 min to eliminate endogenous peroxidase activity and blocked with 10% normal goat serum (Invitrogen) for one hour at room temperature. Subsequently, sections were incubated in primary antibodies against GFAP (Abcam, Inc., MA, US; 1/1000) for 24 h at 4°C. Antibody detection was performed with the Histostain-Plus Bulk kit (Invitrogen) against rabbit IgG, and 3,3' diaminobenzidine (DAB) was used to visualize the final product. All sections were washed in PBS and photographed with an Olympus C-5050 digital camera mounted on the Olympus BX51 microscope. To calculate the GFAP immunostaining index, GFAP-positive cells were counted at 40X magnification in randomized Sect. (3–4) for each rat. All histopathological examinations were performed by the same investigator, who was blinded to the study groups.

**Tissue biochemical analysis**

After decapitation, brains were rapidly removed and stored at -20°C until biochemical analyses. For tissue analysis, whole cerebral tissues were homogenized with a glass homogenizer in 5X volume of phosphate-buffered saline (pH, 7.4) and centrifuged at 5000 g for 15 min. The supernatant was then collected, and total protein concentration in the brain homogenates was determined according to Bradford's method using bovine serum albumin as standard (Bradford, 1976).

The brain levels of IGF-1 (Shanghai LZ Biotech Co., Ltd.), oxytocin (Shanghai LZ Biotech Co., Ltd.), 5-HIAA (Shanghai LZ Biotech Co., Ltd.), HVA (Cusabio) in the tissue supernatants were measured using commercially available rat enzyme-linked immunosorbent assay (ELISA) kits. All samples from each animal were measured in duplicate according to the manufacturer's guidelines.

**Statistical analysis**

Statistical evaluation was performed using SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Shapiro-Wilk's W and Levene's tests were used to check normality of distribution and homogeneity of variance, respectively. The results are presented as the mean ± standard error of the mean (SEM). Resultant p-values of < 0.05 were accepted to demonstrate statistical significance.

**Results**

**Three-Chamber and Open Field Test**

In the three-chamber sociability and social novelty test, both male and female testosterone-exposed rats spent less time with the stranger rat than the male and female rats of the control group, indicating somewhat less social affiliation and more autism-like phenotype (p < 0.05 for males; p < 0.001 for females). Both male and female testosterone-exposed rats spent significantly less time in the center of
the open field area, which suggests increased anxiety and less locomotor activity (p < 0.05 for males; p < 0.05 for females). Results are shown in Fig. 2.

### Tissue Biochemical Analysis

The concentration of IGF-I, 5-HIAA and HVA measured from brain tissues were significantly higher in both male (p < 0.001; p < 0.05; p < 0.001, respectively) and female (p < 0.001; p < 0.001; p < 0.05, respectively) testosterone-exposed rats compared to control male and female rats. The concentration of oxytocin measured from brain tissues was significantly lower in both male (p < 0.001) and female (p < 0.05) testosterone-exposed rats compared to the male and female rats of the control group (Table-1).

|                      | Control group male rats | Control group female rats | Testosterone exposed male rats | Testosterone exposed female rats |
|----------------------|-------------------------|---------------------------|-------------------------------|-------------------------------|
| **IGF-1 (U/mg protein)** | 7.2 ± 0.3               | 6.3 ± 0.6                 | 24.8 ± 5.5 **                 | 19.5 ± 3.8 **                 |
| **Oxytocin (pg/mg protein)** | 65.1 ± 8.8              | 70.9 ± 7.4                | 22.6 ± 7.2 **                 | 48.9 ± 10.6 *                 |
| **5-HIAA (µmol/mg protein)** | 13.2 ± 2.3              | 8.8 ± 1.6                 | 33.1 ± 6.7 *                  | 24.5 ± 6.2 **                 |
| **HVA (ng/mg protein)** | 0.73 ± 0.09             | 0.68 ± 0.04               | 1.2 ± 0.1 **                  | 0.9 ± 0.3 *                   |

Results were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA. * p < 0.05, ** p < 0.001 different from control groups.

Abbreviations: 5-HIAA: 5-Hydroxyindoleacetic acid; HVA: Homovanillic acid; IGF-1: Insulin-like growth factor-1; SEM: standard error of the mean.

### Histopathological evaluation of the groups

Decreased neuronal count, and dysmorphological changes in both the CA1 and CA3 regions of the hippocampus, which indicates neuronal body degeneration, were present in both testosterone-exposed male (p < 0.001) and female (p < 0.001) rats. In contrast, the cresyl violet staining results of control group rats demonstrated normal characteristics (Figure-3 for males and figure-4 for females). In both the CA1 and CA3 regions of the hippocampus, GFAP immunoreactivity of hippocampal sections showed increased glial activity in testosterone-exposed male (p < 0.05) and female (p < 0.05) rats compared to the respective control groups. (Figure-5 for males and figure-6 for females; Table-2).
Table 2
Total neuron count and GFAP immunostaining in the hippocampal sections of study groups.

|                      | Control group male rats | Control group female rats | Testosterone exposed male rats | Testosterone exposed female rats |
|----------------------|--------------------------|----------------------------|--------------------------------|---------------------------------|
| **CA1 Total neuron count** | 67.9 ± 5.5               | 66.5 ± 2.9                 | 35.4 ± 5.1 **                  | 51.7 ± 2.5 *                    |
| **CA3 Total neuron count** | 45.4 ± 4.8               | 59.1 ± 4.3                 | 28.7 ± 4.2 **                  | 30.8 ± 3.7                      |
| **GFAP immunostaining**  |                          |                            |                                |                                |
| **CA1**               | 21.6 ± 1.4               | 20.7 ± 1.5                 | 33.2 ± 2.7 *                  | 29.2 ± 1.9 *                    |
| **GFAP immunostaining** | 19.1 ± 0.9               | 18.5 ± 1.2                 | 27.1 ± 3.3 *                  | 32.3 ± 1.2 *                    |

Results were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA. * p < 0.05, ** p < 0.001 different from control groups.

Abbreviations: CA: Cornu Ammonis; GFAP: Glial fibrillary acidic protein.

Discussion

In this study, firstly, we found that chronic prenatal high-dose testosterone exposure caused autistic-like traits in rats, regardless of sex. Previous studies had shown that fetal testosterone exposure could lead to autistic traits in children (Auyeung et al., 2009; Auyeung et al., 2010; Hassan et al., 2019; James and Grech, 2020). Furthermore, Xu et al. found that testosterone levels were increased in the mothers of autistic children (Xu et al., 2013). Even though many studies have shown the effect of prenatal testosterone on autistic traits, the mechanism of effect of testosterone with respect to the development of autistic traits is unclear. We found that prenatal testosterone exposure leads to an increase in brain tissue 5-HVA levels. There are studies in the literature showing the effect of dopamine on autistic-like behaviors. Tyson et al. showed that testosterone could cause molecular changes in dopamine structure within the nigrostriatal pathway, thus increasing the effect of dopamine (Purves-Tyson et al., 2014). Putnam et al. showed that testosterone injections on castrated rats increased dopamine levels in the medial preoptic area which is an essential site for male sexual behavior (Putnam et al., 2001). Theije et al. showed that, in addition to an increase of dopamine levels in the amygdala, rats exposed to allergenic food demonstrated reduced social behavior and increased repetitive behaviors (de Theije et al., 2014). Our findings correlate with the previous studies and show the association between testosterone increase and autistic traits, possibly through the alteration of dopamine levels.
Another striking result in our study is that prenatal testosterone exposure increases brain levels of 5-HIAA, the primary metabolite of serotonin. Previous studies have concluded that serotonin is one of the neurotransmitters that increases in patients with social disturbance and autistic children (Abdulamir et al., 2018; Naffah-Mazzacoratti et al., 1993; Yang et al., 2015). Dayem et al. found higher serum serotonin levels in autistic children compared to healthy controls, but also noted that there was no correlation with autism severity (Dayem H, 2018). In a similar study, Abdulamir et al. reported relatively elevated levels of serotonin and serotonin reuptake transporter (SERT) in autistic children, and their results demonstrated a correlation between serotonin level and autism severity (Abdulamir et al., 2018). Kranz et al. showed that high-dose testosterone treatment in female-to-male transgender people increased SERT binding in the brain, which may play a role in autistic traits (Kranz et al., 2015). Our findings appear to be well supported by the previous studies, and autism-like traits can be linked to serotonin increase as a result of prenatal testosterone exposure.

The present study showed that prenatal testosterone exposure decreased oxytocin level in the rat brain. Oxytocin is a neurotransmitter that plays an essential role in social and affiliative behaviors. Research related to this role has uncovered that oxytocin administration may improve autistic traits in rats, and therefore, oxytocin has been considered a promising therapeutic agent for therapy in autistic trait behavior (Sala et al., 2011; Teng et al., 2016). Teng et al. showed the efficiency of subchronic oxytocin treatment to increase sociability and ameliorate repetitive stereotypic movements in rats (Teng et al., 2016). Procyshyn et al. showed that experimental empathy induction increases oxytocin levels and decreases testosterone levels in people, indicating an inverse relationship between oxytocin and testosterone (Procyshyn et al., 2020). In accordance with these data, our results have shown that high-dose prenatal testosterone exposure caused decreased oxytocin levels in both male and female gender, which may, in turn, indicate a relationship with autistic behavior.

Another significant finding in our study is increased neuronal loss and astrogliosis on the CA1 and CA3 regions of the hippocampal sections among rats exposed to high-dose testosterone, which indicates neurodegeneration in both male and female offspring. This conclusion may be related to our biochemical findings as a result of several possible mechanisms. For instance, it is likely that prenatal testosterone exposure may cause neuronal inflammation via IGF-1 increase, leading to cytokine inhibition and glial activation, thereby resulting in decreased neuroinflammation (Riikonen, 2017). Another mechanism may be that testosterone exposure inhibits oxytocin, thus causing neurodegeneration and gliosis (Sunnetci et al., 2020). Besides this, increased metabolites of serotonin and dopamine can also cause neurodegeneration, consistent with the literature (Klein et al., 2019; Yang et al., 2015).

We believe that the results of this study, taken together, indicate that prenatal testosterone exposure may cause autistic traits via increased levels of dopamine, testosterone, and IGF-1, and decreased levels of oxytocin. The present findings might help to understand the mechanism of prenatal high-dose testosterone exposure in the development of autistic traits. Further studies should be done to confirm our findings and reveal mechanistic associations.
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