

EFFECT OF ANTIANDROGENS ON VALPROIC ACID-INDUCED NEURODEVELOPMENTAL CHANGES IN RATS

HANAN SAYED MOHAMED FARGHALY¹; MARWA HASSAN BAKR²;
HODA GAMAL SAYED³ AND ISRAA EL-SAYED MOHAMED ASHRY¹

¹ Pharmacology Department, Faculty of Medicine, Assiut University, Assiut 71526, Egypt

² Histology and Cell Biology Department, Faculty of Medicine, Assiut University, Assiut 71526; Egypt

³ Pharmacology Department, Faculty of Veterinary Medicine, Assiut University

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ABSTRACT

Autism spectrum disorders are a neurodevelopmental condition characterized by disturbances in social interaction and communication, as well as repetitive and restricted behaviors. No efficient treatment plans have been found so far. The neurodevelopmental effects of antiandrogens on developmental milestones were examined in rats following a single intraperitoneal injection of adult pregnant female rats with valproic acid (VPA) (600 mg/kg) on gestation day 12.5. Control females were injected with physiological saline at the same time. The offspring of set 1 (which received physiological saline) was divided into 2 groups (groups 1&2) and the offspring of set 2 (which received VPA) were divided into 5 groups (groups 3-7). Thus, the seven groups were organized as follows: Group 1, the control group in which pups did not receive any treatment. Group 2, pups were given sesame oil vehicle. Group 3 pups did not receive any further treatment. Group 4 in which the pups were given sesame oil. Group 5 in which the pups received 1 mg/day testosterone propionate subcutaneously. Group 6 in which the pups received flutamide 1 mg/kg subcutaneously. Group 7 in which the pups received finasteride 50 mg/kg subcutaneously. Our results demonstrated that VPA-induced retardation in sensorimotor reflexes, but did not affect physical landmarks while antiandrogens improved developmental milestones. In conclusion, our finding confirms that androgen contributes partially towards VPA-induced neurodevelopmental insults.

Keywords: Autism spectrum disorders, antiandrogens, developmental milestones, testosterone VPA

INTRODUCTION

Autism spectrum disorders (ASDs) are a set of neurodevelopmental disorders

characterized by limited social interaction and communication, repetitive behavior, and narrowed interests. Additionally, it is believed that 69–79% of adults and 60–70% of children with ASDs fulfill the criteria for at least one comorbid mental illness, such as anxiety, attention deficit hyperactivity disorder, or other mood disorders (Stewart *et al.*, 2022). Although the many genetic and environmental factors that contribute to

Corresponding author: Hoda Gamal Sayed
E-mail address: Farghalyhanan@yahoo.com
Present address: Pharmacology Department, Faculty of Veterinary Medicine, Assiut University

ASDs are well acknowledged, clear knowledge of the processes underlying atypical neurodevelopment is inadequate (Sven Bölte *et al.*, 2019).

The major androgen, testosterone, is an anabolic-androgenic steroid hormone that is formed from cholesterol created by progesterone's conversion, with around 50% of the hormone's production coming from androstenedione in the blood (Burger, 2002). Cytoplasmic androgen receptors (ARs) that bind to testosterone stimulate myocellular signaling, which in turn changes the expression of hundreds of genes (Kraemer *et al.*, 2020). With the enzymatic activity of 5-alpha reductase, testosterone is permanently transformed into dihydrotestosterone (DHT) once attached to the ARs (Wilborn *et al.*, 2010). DHT's receptor binding kinetics make it the most powerful hormone, which is widely acknowledged (Ly *et al.*, 2001).

Male fetuses who later in postnatal life were diagnosed with autism, had greater levels of numerous androgens, including testosterone, according to research by Baron Cohen and colleagues (2005). Fetal testosterone levels were also positively connected with autistic features in the general population. The masculinization of the brain is a result of prenatal exposure to high amounts of fetal testosterone, according to several researchers (Auyeung *et al.*, 2010; Baron-Cohen *et al.*, 2005; Baron-Cohen *et al.*, 2015; Chapman *et al.*, 2006; Hines *et al.*, 2015).

Prenatal exposure to VPA is well known to induce developmental neurotoxicity in the central nervous system (Ogawa *et al.*, 2007; Ornoy *et al.*, 2015). An approximately three-fold risk ratio between exposure to VPA during pregnancy and substantial dysmorphic feature abnormalities as well as reduced intrauterine development has been demonstrated in prospective and retrospective research studies (Wood *et al.*, 2015). Clinical research has shown that consuming VPA during pregnancy is linked to neurocognitive disabilities in children and congenital abnormalities, such as an

increased risk of neural tube defects, including spina bifida and exencephaly (Cummings *et al.*, 2011; Kulaga *et al.*, 2011). VPA exposure during pregnancy has been linked to ASDs-like symptoms in both human and animal models (Nicolini and Fahnestock, 2018). It is believed that VPA was responsible for juveniles' delayed development of crucial motor reflexes and cognitive disabilities. In addition, Purkinje neurons' dendritic arborization and cell density were also reduced following exposure to VPA (Wang *et al.*, 2018).

Behavioral deficits are consistent with autism induced by maternal challenge with VPA in human offspring are also found in prenatally VPA-exposed rodents. A model of autism that possesses both construct validity (similarity in underlying causes) and face validity (resemblance to human symptoms) has been developed in animals exposed to prenatal VPA (Nicolini and Fahnestock, 2018). Rodier *et al.* (1996) developed the VPA model by giving rat embryos a single dose of 350 mg/kg VPA. Since then, mice exposed to VPA during pregnancy have been employed extensively to study the molecular mechanisms underlying autistic behavior and, in addition, to evaluate prospective treatments for autism. These findings show that a single prenatal exposure to VPA causes permanent behavioral deficits in mice, which are the primary symptoms of autism (Nicolini and Fahnestock, 2018).

Schneider and Przewlocki (2005) examined the behavior of rats that had been given a single injection of VPA on day 12.5 of pregnancy. Their research showed that male rats treated with VPA had a variety of behavioral deficits that were similar to those seen in autistic people, including: Social deficits which are indicated by (1) fewer social explorations (2) increased latency to social behaviors; (3) decreased sensitivity to pain at the spinal and supraspinal levels and increased sensitivity to non-painful stimuli, suggesting sensory system deficits; and (4) increased locomotor and repetitive/

stereotypic activities combined with lower exploratory activity. Also, these scientists noted that rats exposed to VPA had delayed maturation (late eye opening), decreased body weight, delayed motor development, and delayed nest-seeking response mediated by the olfactory system, which supports early olfactory discriminating deficiencies.

Recently Sever *et al.* (2022) examined the effects of finasteride given to rats with an autism model caused by propionic acid. The results showed that finasteride (5 mg/kg/day given via oral gavage for 15 days) treatment significantly improved behavioral disorders such as cognitive impairment, communication deficits, and restricted interests and movements. It's possible that finasteride's effect on ASDs is partially explained by its antiandrogenic properties, which prevent the creation of DHT, an active androgen, by inhibiting the 5-reductase enzyme.

The goal of this study was to assess the data supporting the link between hyperandrogenism and autistic developmental milestone as well as to assess the efficacy of anti-androgen medications on valproic acid induced decline in developmental milestones.

MATERIALS AND METHODS

Animals

A total of fifteen male and female (8-10 weeks old) Wistar rats were obtained from the animal house facility, Faculty of Medicine, Assiut University, Assiut, Egypt (Approval number: 17300700). Rats were housed in standard specific pathogen-free facilities, under a 12 h/12 h light/dark cycle and acclimated for 1 week. Food and water were available *ad libitum*. Ten female rats were mated with five males using one male rat and two female rats per breeding cage in clean capacious stainless steel cages under standard laboratory conditions, including aerated rooms, humidity with suitable

temperature (25 ± 5 °C) maintained at normal daylight cycle.

Groups and Treatment:

After mating, vaginal smears were examined every morning for the detection of spermatozoa using a light microscope (Ishola *et al.*, 2020). The day on which spermatozoa were found in vaginal smear, was considered the first day of gestation (GD1) (Schneider and Przewlocki, 2005). Then, each pregnant rat was housed separately and divided into 2 sets; control and VPA-treated rats. Valproic acid was dissolved in 0.9% saline at a concentration of 250 mg/ml. Pregnant females received a single intraperitoneal injection of 600 mg/kg sodium valproate on GD12.5, and control females were injected with the same amount of physiological saline during the same period (Hou *et al.*, 2018). The offspring of set 1 (which received physiological saline) were divided into 2 groups of ten pups each and the offspring of set 2 (which received VPA) were divided into 5 groups of ten pups each. Finally, seven groups (10 neonatal rats each, Figure 1) were organized as follows:

Group 1 (control): Pups from set 1 and did not receive any treatment.

Group 2 (Sesame oil- treated control): Pups from set 1 and were given sesame oil vehicle only from postnatal day (PND) 1 until PND10.

Group 3 (VPA-induced model of autistic disorder): Pups from set 2 (pups of VPA-treated mothers) and did not receive any further treatment.

Group 4 (VPA+sesame oil): Pups from set 2 (pups of VPA-treated mothers) that were given sesame oil from PND1 until PND10.

Group 5 (VPA+testosterone): Pups from set 2 (pups of VPA-treated mothers) received 1 mg/day testosterone propionate subcutaneously; dissolved in sesame oil at a concentration

of 1 mg/ml every 2 days from PND1 until PND10.

Group 6 (VPA+Flutamide): Pups from set 2 (pups of VPA- treated mothers) received flutamide 1 mg/kg subcutaneously; dissolved in sesame oil at a concentration of 1 mg/ml, every two days from PND1 until PND10 (Abi *et al.*, 2017).

Group 7 (VPA+Finasteride): Pups from set 2 (pups of VPA- treated mothers) received one injection of finasteride 50 mg/kg subcutaneously at day 2 of birth (dissolved in sesame oil at a concentration of 3mg/ml (Li *et al.*, 2018).

Developmental Milestones:

Development of selected neonatal reflexes and growth milestones were evaluated (Lim *et al.*, 2008; Osório *et al.*, 2009), beginning on PND 1, we monitored newborn rats every day for the development of the following sensorimotor reflexes and physical landmarks:

A- Sensorimotor Reflexes:

1) Surface Righting Reflex:

Beginning on PND 5, to see if the pup could turn over onto its belly, or the prone position, it was gently handled and laid on a cushioned horizontal surface in the supine position (Figure 2, Panel A&B), and the first appearance of the surface righting reflex was defined as the ability of the pups to turn over to the prone position and stand with all four paws in contact with the board within 10 sec (Figure 2, Panel C). All pups were checked and recorded every day until they all reached the standard (Jiang *et al.*, 2022).

2) Air Righting Reflex:

Pups were naturally dropped into comfortable bedding from the supine position (30 cm high) (Figure 3). If a rat landed in a typical prone position, it was seen as displaying a positive response (belly

facing downwards against the bedding). The test began on P10 (Jianget. al., 2022).

3) Forelimb Grasping:

The task was performed between PND 4-14. A metal bar was suspended 30 cm above a soft surface (Figure 4). The animal was held and its forepaws were permitted to contact the bar. Complete acquisition of the grasping reflex was assumed when the animal was able to grasp the bar with both forepaws. A maximum of 30 seconds of the animal's ability to hold onto the bar with just its forepaws (the wire suspension time) was also recorded (Osório *et al.*, 2009).

4) Negative Geotaxis Reflex:

The task was performed on PND 5. The pups were placed facing downwards on a slope with a 45° incline, held there for 3 sec and then released (Figure 5, Panel A&B). It was determined whether the pups could rotate 180 degrees and face upwards within 30 seconds. The first day the animals appeared to successfully accomplish the task was recorded (Feather-Schussler and Ferguson, 2016; Jiang *et al.*, 2022).

Reflexes were considered acquired only after they were observed for 2 consecutive days.

B- Physical Landmarks:

Physical developmental milestones were examined by assessing the following:

1) Pinnae Detachment:

Defined as the opening of the ear channel (beginning on PND 2) (Feng *et al.*, 2006).

2) Eye Opening:

Defined as the opening of both eyes (between PND 7-17) (Heyser, 2004).

Statistical analysis

The data were represented as the group means \pm standard errors of the mean (SEM). The significance of differences between groups was analyzed using one-way analysis of variance (ANOVA) followed by the post hoc Dunnett's test for multiple comparisons.

RESULTS

1. Sensorimotor Reflexes:

1.1. Surface Righting Reflex:

As shown in (Figure 6), there was a significant delay in surface righting reflex appearance in pups of VPA-treated mothers (6.83 ± 0.48 days) when compared to pups of the control group (3.67 ± 0.33 days, $p < 0.0008$) and sesame oil-treated pups of saline-treated mothers (3.7 ± 0.29 days, $p < 0.0006$). Compared to the pups of VPA-treated mothers (6.83 ± 0.48 days), there was no significant difference in surface righting reflex appearance in sesame oil-treated pups of VPA-treated mothers (6 ± 0.87 days, $p < 0.71$) and testosterone-treated pups of VPA-treated mothers (5.6 ± 0.75 days, $p < 0.43$).

Compared to pups of VPA-treated mothers (6.83 ± 0.48 days), there was a significant early appearance of surface righting reflex in flutamide-treated pups of VPA-treated mothers (4 ± 0.33 days, $p < 0.0014$) and finasteride-treated pups of VPA-treated mothers (3.7 ± 0.29 days, $p < 0.0006$) (Figure 6).

1.2. Air Righting Reflex:

There was a significant delay in air righting reflex appearance in pups of VPA-treated mothers (6.3 ± 0.76 days) when compared to the pups of the control group (3.83 ± 0.31 days, $p > 0.002$) and sesame oil-treated pups of saline-treated mothers (3.71 ± 0.29 days, $p < 0.0009$). However, when compared to the pups of VPA-treated mothers (6.3 ± 0.76 days) there was no significant difference in air righting reflex appearance in sesame oil-treated pups of VPA-treated mothers (7.86 ± 0.5 days, $p < 0.09$) and testosterone propionate treated pups of VPA-treated mothers (8 ± 0.55 days, $p < 0.09$) (Figure 6).

Moreover, compared to pups of VPA-treated mothers (6.3 ± 0.76 days), there was a significant early appearance of air righting reflex in flutamide-treated pups of VPA-treated mothers (3.63 ± 0.18 days, $p < 0.0004$) and finasteride-treated pups of VPA-treated

mothers (3.29 ± 0.42 days, $p < 0.0001$) (Figure 6).

1.3. Forelimb Grasping Reflex:

There was a significant delay in the appearance of forelimb grasping reflex in pups of VPA-treated mothers (13.67 ± 0.9 days) when compared to the pups of the control group (7.3 ± 0.49 days, $p < 0.0001$) and sesame oil-treated pups of saline-treated mothers (7.1 ± 0.4 days, $p < 0.0001$). However, there was no significant difference in forelimb grasping reflex appearance in the pups of VPA-treated mothers (13.67 ± 0.92 days) when compared to sesame oil-treated pups of VPA-treated mothers (12.7 ± 0.42 days, $p < 0.65$) and testosterone propionate treated pups of VPA-treated mothers (11.8 ± 0.8 days, $p < 0.13$) (Figure 6).

Compared to pups of VPA-treated mothers (13.67 ± 0.92 days), there was a significant early appearance of forelimb grasping reflex in flutamide-treated pups of VPA-treated mothers (5.25 ± 0.41 days, $p < 0.0001$) and finasteride-treated pups of VPA-treated mothers (3.88 ± 0.35 days, $p < 0.0001$) (Figure 6).

1.4. Negative Geotaxis Reflex:

There was a significant delay in the appearance of negative geotaxis reflex in pups of VPA-treated mothers (13 ± 0.55 days) when compared to the pups of the control group (6.2 ± 0.3 days, $p < 0.0001$) and sesame oil-treated pups of saline-treated mothers (6.1 ± 0.5 days, $p < 0.0001$). However, there was no significant difference in negative geotaxis reflex appearance in the pups of VPA-treated mothers (13 ± 0.55 days) when compared to sesame oil-treated pups of VPA-treated mothers (11.57 ± 0.43 days, $p < 0.19$) and testosterone propionate treated pups of VPA-treated mothers (11 ± 0.84 days, $p < 0.06$) (Figure 6).

When compared to pups of VPA-treated mothers (13 ± 0.55 days), there was a significant early appearance of negative geotaxis reflex in flutamide-treated pups of VPA-treated mothers (6.13 ± 0.29 days, $p <$

0.0001) and finasteride-treated pups of VPA-treated mothers (5.43 ± 0.48 days, $p < 0.0001$) (Figure 6).

2. Physical Landmarks:

Major physical landmarks of rodent development were evaluated in the rat pups. Physical landmarks include ear detachment and eye opening.

2.1. Ear Detachment:

There was no significant difference in ear detachment in pups of VPA-treated mothers (3.3 ± 0.21 days) when compared to the pups of the control group (3.2 ± 0.17 days, $p < 0.97$) and sesame oil-treated pups of saline-treated mothers (3.14 ± 0.14 days, $p < 0.93$). Also, there was no significant difference in the pups of VPA-treated mothers (3.3 ± 0.21 days) when compared to sesame oil-treated pups of VPA-treated mothers (3.57 ± 0.2 days, $p < 0.83$) and testosterone propionate treated pups of VPA-treated mothers (3.6 ± 0.24 days, $p < 0.82$) (Figure 6). Compared to pups of VPA-treated mothers (3.3 ± 0.21 days), there was no significant difference in flutamide-treated pups of VPA-treated mothers (3.13 ± 0.13 days, $p < 0.88$)

and finasteride-treated pups of VPA-treated mothers (3.14 ± 0.14 days, $p < 0.93$) (Figure 6).

2.2. Eye Opening:

There was no significant difference in eye opening in pups of VPA-treated mothers (14.4 ± 0.6 days) when compared to the pups of the control group (13.2 ± 0.2 days, $p < 0.1$) and sesame oil-treated pups of saline-treated mothers (13.57 ± 0.29 days, $p < 0.37$). Moreover, there was no significant difference in the pups of VPA-treated mothers (14.4 ± 0.6 days) when compared to sesame oil-treated pups of VPA-treated mothers (14.86 ± 0.51 days, $p < 0.86$) and testosterone propionate treated pups of VPA-treated mothers (14 ± 0.32 days, $p < 0.94$) (Figure 6).

Compared to pups of VPA-treated mothers (14.4 ± 0.6 days), there was no significant difference in flutamide-treated pups of VPA-treated mothers (13.25 ± 0.16 days, $p < 0.12$) and finasteride-treated pups of VPA-treated mothers (13.14 ± 0.26 days, $p < 0.08$) (Figure 6).

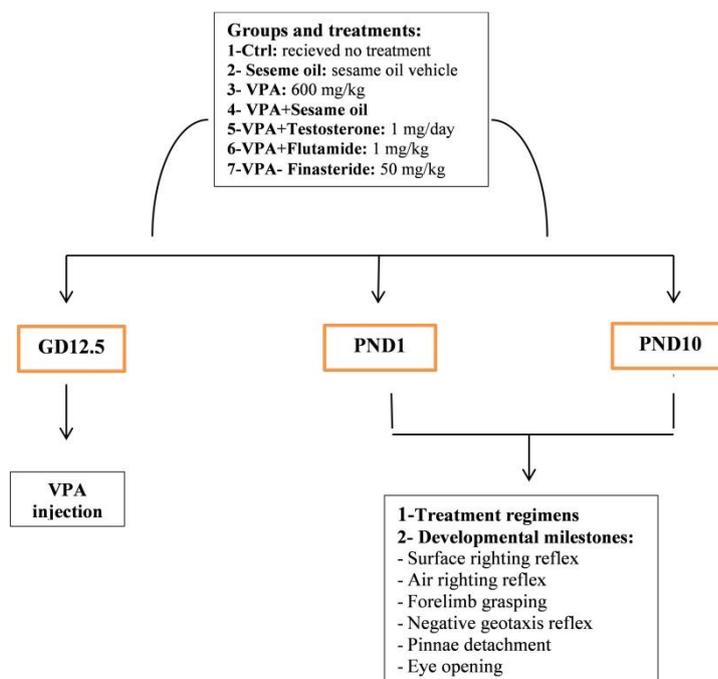


Figure 1: Study design of groups and treatment. (GD: day of gestation, PND: Post-natal day)

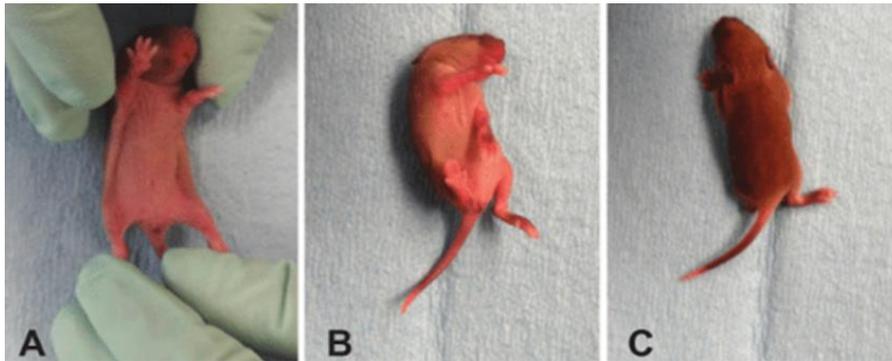


Figure 2: Surface righting reflex (Feather-Schussler and Ferguson, 2016).



Figure 3: Air righting reflex (Yan et al., 2010)

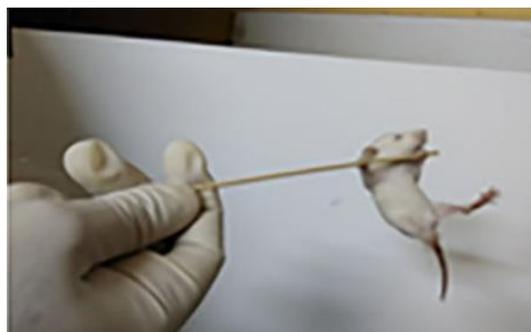


Figure 4: Forelimb grasping (Anshu et. al., 2022)

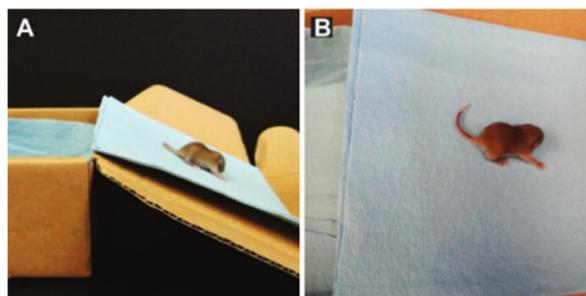


Figure 5: Negative geotaxis reflex (Feather-Schussler and Ferguson, 2016)

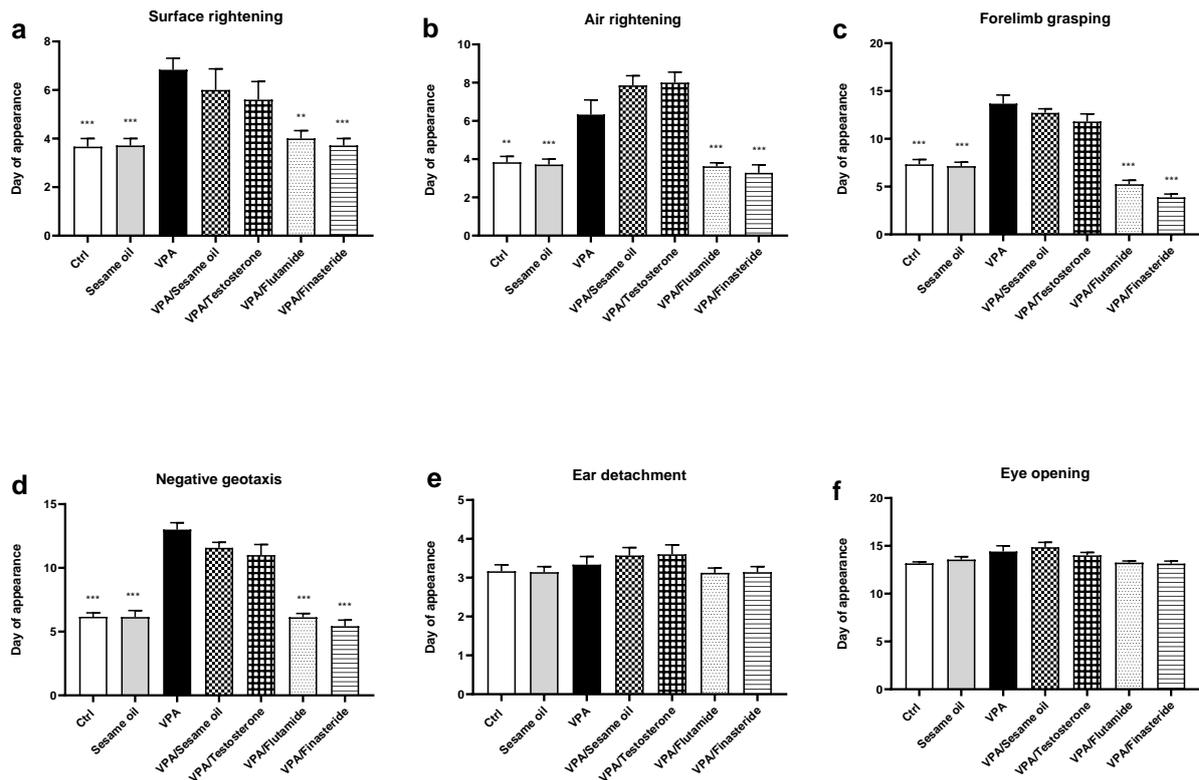


Figure 6: Effect of flutamide and finasteride on developmental milestones of prenatal VPA rat model of autism. (a) Surface righting reflex, (b) air righting reflex, (c) forelimb grasping, (d) negative geotaxis, (e) ear detachment and (f) eye opening. (Ctrl: Control, VPA: Valproic acid). **Values are expressed as mean ± SEM. of 8 rats.**
 * P<0.05, ** p<0.01; *** p<0.001 compared to VPA group.

DISCUSSION

Rodent pups are born with closed eyes and ears, underdeveloped sensory processing, and unpredictable, disorganized movements. Throughout the first 21 postnatal days, these processes continue to grow in a temporally planned way. During this first critical period, it is possible to identify the effects of prenatal insult on the development of brain functions and identify early indicators of later behavior by assessing developmental milestones, such as physical landmarks (eye opening and pinnae detachment) and sensorimotor abilities (air righting reflex, surface righting reflex, forelimb grasping, and negative geotaxis) (Heyser, 2004). It is recognized that the ontogeny of sensorimotor developmental milestones can serve as an indicator of neural circuit or brain area maturity in several species,

including humans (Rice and Barone S Jr, 2000).

The main findings of the current research were that in-utero exposure to VPA caused developmental retardation with a delayed onset of sensorimotor reflexes maturation. In the current study, we demonstrated that pups of VPA-treated mothers had delayed sensorimotor reflexes development, as evidenced by the late emergence of air righting, surface righting, forelimb grasping, and negative geotaxis responses, all of which indicate a delayed sensorimotor system's development in the brain. In the same line, a single intraperitoneal injection of VPA (450 mg/kg on gestational day 12.5) showed delayed ontogeny of negative geotaxis in rats (Anshu *et al.*, 2022). Also, subcutaneous injection of sodium valproate (200 mg/kg on gestational days 12–17) showed a delayed

appearance of surface righting as well as negative geotaxis in mice (Wagner *et al.*, 2006). And single intraperitoneal injection of 600 mg/kg VPA in mice revealed a significant delay in negative geotaxis and surface righting reflexes (Kim *et al.*, 2022).

Moreover, we found that pups of VPA-treated mothers did not show any delay in physical milestones acquirement including eye opening and pinnae detachment indicating that prenatally VPA exposure did not affect physical milestones maturation. In the same line, single intraperitoneal injection of VPA (450 mg/kg on gestational day 12.5) showed no delay in eye opening in rats (Anshu *et al.*, 2022).

Additionally, we found that testosterone-treated pups of VPA-treated mothers had delayed development of their sensorimotor reflexes (indicated by the late appearance of air righting, surface righting, forelimb grasping, and negative geotaxis responses). While flutamide- (1 mg/kg, intraperitoneally every two days from PND1 until PND10) treated rats showed normal development of sensorimotor reflexes indicated by normal appearance of (air righting, surface righting, forelimb grasping and negative geotaxis responses).

Finasteride (50 mg/kg, subcutaneously once)-treated rats showed early development of sensorimotor reflexes (air righting, surface righting, forelimb grasping and negative geotaxis responses) when compared to VPA group. In the same line finasteride (50 mg/kg/ intraperitoneally for 3 days) maintained grasping and righting reflex in rats (Mladenovic *et al.*, 2014).

CONCLUSION

Prenatal exposure to VPA retarded sensorimotor developmental milestones maturation including surface righting reflex, air righting reflex, forelimb grasping and negative geotaxis reflex. Anti-androgen therapy should be considered an effective

means to significantly help improve the sensorimotor reflexes of patients diagnosed with ASDs.

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تأثير مضادات الأندروجين علي التغيرات السلوكية المحدثة بحمض الفالبرويك في الجرذان

حنان سيد محمد فرغلي ، مروه حسن بكر ، هدى جمال سيد ، إسراء السيد محمد عشري

Email: Farghalyhanan@yahoo.com Assiut University web-site: www.aun.edu.eg

التوحد هو حالة نمو عصبي تتميز بإضطرابات في التفاعل الإجتماعي والتواصل، فضلاً عن السلوكيات المتكررة والمقيدة. ولم يتم العثور على خطط علاج فعالة حتى الآن. في هذه الدراسة تم فحص التأثيرات التطورية العصبية لمضادات الأندروجين على مراحل النمو في الجرذان بعد الحقن داخل الغشاء البريتوني لحمض الفالبرويك (٦٠٠ ملليجرام لكل كجم في اليوم ١٢,٥ من الحمل). وتم حقن الإناث في المجموعة الضابطة بنفس الكمية من المحلول الملحي الفسيولوجي في نفس الفترة الزمنية. تم تقسيم نسل المجموعة ١ (والتي تلقت محلول ملحي فسيولوجي) إلى مجموعتين فرعيتين وتم تقسيم نسل المجموعة ٢ (والتي تلقت حمض الفالبرويك) إلى ٥ مجموعات فرعية. وبهذا، تم عمل سبع مجموعات على النحو التالي: المجموعة الضابطة التي لم يتلق فيها الجرذان أي علاج. المجموعة الثانية: أعطيت الجرذان زيت السمسم. ولم تتلق جرذان المجموعة الثالثة أي علاج آخر (غير حمض الفالبرويك الذي تلقتته الأم اثناء حملها). المجموعة الرابعة حيث تم إعطاءها زيت السمسم. المجموعة الخامسة التي تلقت فيها الجرذان ١ مجم يوميا من بروبيونات التستوستيرون تحت الجلد. المجموعة السادسة حيث تلقت الجرذان الفلوتاميد ١ مجم لكل كجم تحت الجلد. المجموعة السابعة التي تلقت فيها الجرذان فيناسترايد ٥٠ مجم لكل كجم تحت الجلد.

وقد اظهرت هذه الدراسة أن حمض الفالبرويك قد تسبب في التخلف في ردود الفعل الحسية ولكنه لم يؤثر على بعض التغيرات الجسدية المختبره. وبعد إعطاء مضادات الأندروجين أدى ذلك الي تحسن في ردود الفعل الحسية.