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SPIRONOLACTONE ALTERS THE LEVELS OF NEURONAL FUNCTION MARKERS IN AUTISTIC RATS

Roohi Mirza[1], Poonam Sharma[1], G T. Kulkarni [2] and Bhupesh Sharma[1*]

[1]*Department of Pharmacology, Amity Institute of Pharmacy, Amity University Uttar Pradesh, Noida, India*
[2]*Gokaraju Rangaraju College of Pharmacy, Hyderabad*

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that is characterized by repetitive behavior and impairment in social behavior. ASD is a complex disorder with complex etiology that involves multiple genes, environmental factors, and epigenetic mechanism. Many clinical and pre-clinical study have demonstrated the association of propionic acid (PPA) with ASD. This study evaluates the potential effect of spironolactone in PPA induced ASD phenotype. PPA (250 mg/kg, po) was administered to Albino Wistar rats from post-natal day (PND) 21st to 23rd to induce ASD like neurobehavioral and neurobiochemical alterations. Animals were experimented for behavioral (elevated plus maze, three chambered social interaction apparatus, and Y-maze apparatus), biochemical parameters (BDNF, synapsin II), and blood-brain barrier impairment. Post-natal PPA exposure resulted impairment in social behavior, anxiety, and repetitive behavior in animals. Furthermore, PPA exposure caused reduction in the levels of BDNF and synapsin II in rats' brain. Spironolactone (25 mg/kg and 50 mg/kg) administration was observed to ameliorate post-natal PPA exposed behavioral and biochemical impairments in animals.

Keywords: *Propionic acid, autism, spironolactone, social behavior, repetitive behavior, BDNF, Synapsin*

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopment disorder that characterizes repetitive behavior and impairment in the social interaction[1]. Autistic individuals exhibit co-morbid characteristics namely anxiety, aggressiveness, sleeplessness, gastrointestinal disturbances, and motor impairments[2]. Men are more susceptible to ASD then women.

Propionic acid (PPA) is a saturated fatty acid produced by anerobic fermentation of dietary fibers, sugars, and dairy products via gut microbiota. PPA can regulate cell development and metabolism. Furthermore, it can exert several immunosuppressive properties[3]. Substantial amount of PPA can cross blood-brain barrier (BBB) to cause developmental delay and impairment in the neurotransmitter release[4]. PPA causes cognitive impairment, inflammation, oxidative stress, and immune dysfunction among others as observed in ASD[5-11]. Exposure of PPA results in behavioral abnormalities that are observed in ASD. PPA administration was reported to cause stereotypy and impair attention, locomotion, social behavior, and information processing abilities in experimental animals[12]. Post-natal PPA exposure reduces the levels of cerebral phosphorylated – cAMP response element binding protein (pCREB) and brain derived neurotropic factor (BDNF) as well as increases the levels of cerebral thiobarbituric acid reactive substances (TBARS), interleukin (IL-6), and tumor necrosis factor alpha (TNF-α) in experimental animals [6&13]. Therefore, post-natal PPA exposure is a robust model for mimicking several behavioral and biochemical phenotypes associated with ASD[5&6&11&13&14].

Spironolactone is a mineralocorticoid receptor antagonist that has been reported to provide neuroprotection againt various brain

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*Corresponding author: Bhupesh Sharma, E-mail: bhupeshsharmaresearch@gmail.com

disorders such as ischemia reperfusion injury, traumatic brain injury, Alzheimer's disease to cite a few[15-17]. Spironolactone has reportedly improved primary deficits such as social behavior and hyperactivity in schizophrenic patients as well as experimental mice model[18&19]. Behavioral deficits such as anxiety, depression, and repetitive behavior was found to be mitigated during clinical and pre-clinical studies[20-22]. Furthermore, spironolactone causes inhibition of neuroinflammation, oxidative stress, and neurodegeneration thereby promoting neurogenesis and neuroprotection[15&23-26]. However, the role of spironolactone is yet to be elucidated in experimental model of ASD.

We have hypothesized that spironolactone may play an important role in attenuating the behavioral phenotypes that are associated with ASD. In this study, we have explored the effects of spironolactone against impaired behavioral, biochemical, and BBB parameters that are associated with ASD.

MATERIAL AND METHODS

Animals

In the current study, Albino Wistar rats (adult male) were employed and housed in animal house of Amity University (Reg No. 1327/PO/ReBi/S/10/CPCSEA). This experimental study was approved by the Institutional animal ethics committee of Amity University, Uttar Pradesh, India.

Chemical and reagents

Spironolactone was obtained from Supramax pharmaceutical, Dehradun, India. Propionic acid was purchased from Lab Sale Corporation, New Delhi, India.

Drugs and administration

The untreated pregnant female rats' male offspring received propionic acid (250 mg/kg) dissolved in 0.2 M phosphate buffer saline for three consecutive days from post-natal day (PND) 21 to 23.

Experimental animals were administered with treatments (drug/vehicle) from PND 24 to PND 50. Spironolactone (25 mg/kg, ip; 50 mg/kg, ip) was dissolved in Tween 20 (5% in distilled water as vehicle) and was administered 1 hr prior to behavioural assessments. These doses were selected as per the previously published research reports[17, 27].

Experimental design

In current study 7 groups were employed with 8 animals each (n = 8; male). The choice of animals was based on the previously published research reports[6,13].

The groups were divided as follows:

- **Group I (Control group):** Male offspring were exposed to behavior and biochemical assessments from PND 44 to PND 50.
- **Group II (PBS):** 0.2 M PBS was administered to male offspring from PND 21 to 23.
- **Group III (Tween 20):** Tween 20 was administered to male offspring from PND 24 to PND 50.
- **Group IV (Spironolactone perse):** Spironolactone (50 mg/kg) was administered to male offspring from PND 24 to PND 50.
- **Group V (PPA):** PPA (250 mg/kg, po) was administered to male offspring and behavioral assessments were conducted from PND 44 to PND 50.
- **Group VI, VII (Spironolactone):** Spironolactone (25 mg/kg, ip; 50 mg/kg, ip) was administered to male offspring (PND 24 to PND 50) who received post-natal PPA from PND 21 to PND 23.

Behavioral assessments

All behaviors were conducted during the light phase i.e., between 09:00 Hrs. and 18:00 Hrs. from PND 44 to PND 50.

Assessment of social interaction

The impairment in the social interaction is the main characteristic of ASD[28]. Social interaction is assessed using three chambered social interaction apparatus. In this apparatus the animals are assessed on four features: sociability, social index, social preference, and social preference index. The tests consisted of three sessions. During session 1, animals were allowed to explore the apparatus for the duration of 5 minutes. This was followed by session two, wherein a stranger rat was kept inside the grid cage (either left or right side) and the other side was kept empty. The grid cage with the stranger rat was designated as stranger chamber and individually each rat was placed to explore these three chambers for next 10 minutes, giving us the sociability phase. Session 2 was followed by

session three, wherein the stranger animal and the chamber in which the rat was residing during session 2 were now during session 3, termed as a familiar rat and the familiar chamber respectively. At the beginning of session 3, an unfamiliar rat from another litter was placed inside the grid cage of previously kept empty chamber, now designated as the novel chamber and again the testing rats were individually placed to explore these three chambers for the next 10 minutes. This phase was referred to as social preference phase.

The ratio of the duration for which the test animal stayed in stranger chamber to the empty chamber was expressed as sociability index (SI) and the ratio of the duration for which the animal explored the novel chamber to the familiar chamber was expressed as social preference index (SPI).

Assessment of repetitive behavior

Repetitive behavior is another one of the major co-morbid symptoms of ASD[7,28,29]. Repetitive behavior in rats were measured using Y-maze apparatus. Individual rats were placed in the start arm and the series in which the animals enter the other arms were recorded for 8 minutes to calculate the spontaneous alterations. Rat entering the three arms in succession was recorded as one alteration. A decrease in the % spontaneous alteration is regarded as a marker for increase in repetitive behavior. The % Spontaneous alteration is calculated using the formula:

$$\% \text{ Spontaneous alternation} = \frac{\text{Total alternations}}{(\text{Total Arm entries} - 2)} \times 100$$

Assessment of anxiety

Anxiety is a common co-morbid trait that is expressed with ASD [30]. The anxiety behavior of the animals was assessed using elevated plus-maze apparatus. Animals were set down in a pre-test arena to explore the maze for 5 minutes. Post this, rats were individually placed at the center facing towards the open arm and were assessed for % open arm entries and % time spent in open arm using the formula.

$$\% \text{ Open arm entries} = \frac{\text{Number of entries in open arm}}{\text{Closed arm entries} + \text{Open arm entries}} \times 100$$

$$\% \text{ Time spent in open arm} = \frac{\text{Time spent in open arm}}{\text{Time in open arm} + \text{Time in closed arm}} \times 100$$

Biochemical assessments

Tissue preparation for biochemistry

The assessment of behavioral parameters was followed by biochemical assessments. The experimental animals were euthanized using thiopental sodium (90 mg/kg, *ip*) to remove the brain. The biochemical assays were performed in different brain regions (cerebellum, hippocampus, frontal cortex, and remaining brain) therefore, the required brain regions were dissected from individual rat's brain. These regions were homogenized with cocktail protease inhibitor in cooled RIPA buffer (Sigma-Aldrich (R0278)) (50mM Tris HCl, 10% nonidet-40, 1.5 M NaCl, 2.5% deoxycholic acid,10mM EDTA, pH 7.4) using homogenizer (Polytron (PT 1600 E)). The homogenized brain regions were separately centrifuged at 3000 rpm for 15 minutes at 4°C and the obtained supernatant was stored at -80°C and was utilized for biochemical assessments[6,28]. The remaining brain was utilized for the assessment of BBB permeability by evaluating % brain water content.

Assessment of brain protein

Brain protein were assessed using Lowry's method at 750 nm using bovine serum albumin as a standard. The recorded values were reported as mg/ml of supernatant[7].

Assessment of BDNF and synapsin II

The biochemical estimation of BDNF and synapsin II in selected brain regions were carried out using a microplate reader at 450 nm. All kits were based on sandwich in-vitro ELISA principle. The concentrations for BDNF and Synapsin-II were expressed as pg.ml^{-1}[7&31].

Assessment of BBB permeability

Increase in % brain water content indicates BBB permeability. Brain water content (%) was estimated using wet and dry method[7]. The remaining brain was immediately weighed and then was dried for next 24 hrs at 100°C. Post 24 hour, the dried tissue was again weighed and the % brain water content was calculated as:

$$\% \text{ Brain water content} = \frac{\text{Wet weight-Dry weight}}{\text{Wet weight}} \times 100$$

Statistical analysis

The analysis of data was performed using sigma stat 12.5 (Systat Softwares, Inc.). All results are represented as mean ± S.D. The data for sociability and social behaviour were analysed using three-way ANOVA followed by Bonferroni's post-hoc test, where PPA, drug treatment and time spent in each chamber (PPA/without PPA × drug treatment × time spent in stranger/empty chamber & PPA/without PPA × drug treatment × time spent in novel/familiar chamber) were considered as independent factors. Data for rest of the behavioral and biochemical assays were assessed using two-way ANOVA followed by Bonferroni's post hoc test. Data were statistically significant at $p< 0.05$.

RESULTS AND DISCUSSION

Results

Effect of PPA and spironolactone on social behavior

Sociability and sociability index

During sociability phase, control rats showed normal behavior by spending more duration in the stranger chamber than the empty chamber as compared to the PPA administered animals with low sociability index. However, administration of spironolactone (25 mg/kg, ip; 50 mg/kg, ip) in these animals, significantly increased their stay duration in the stranger chamber increasing their sociability index **(Fig. 1A-1B)**.

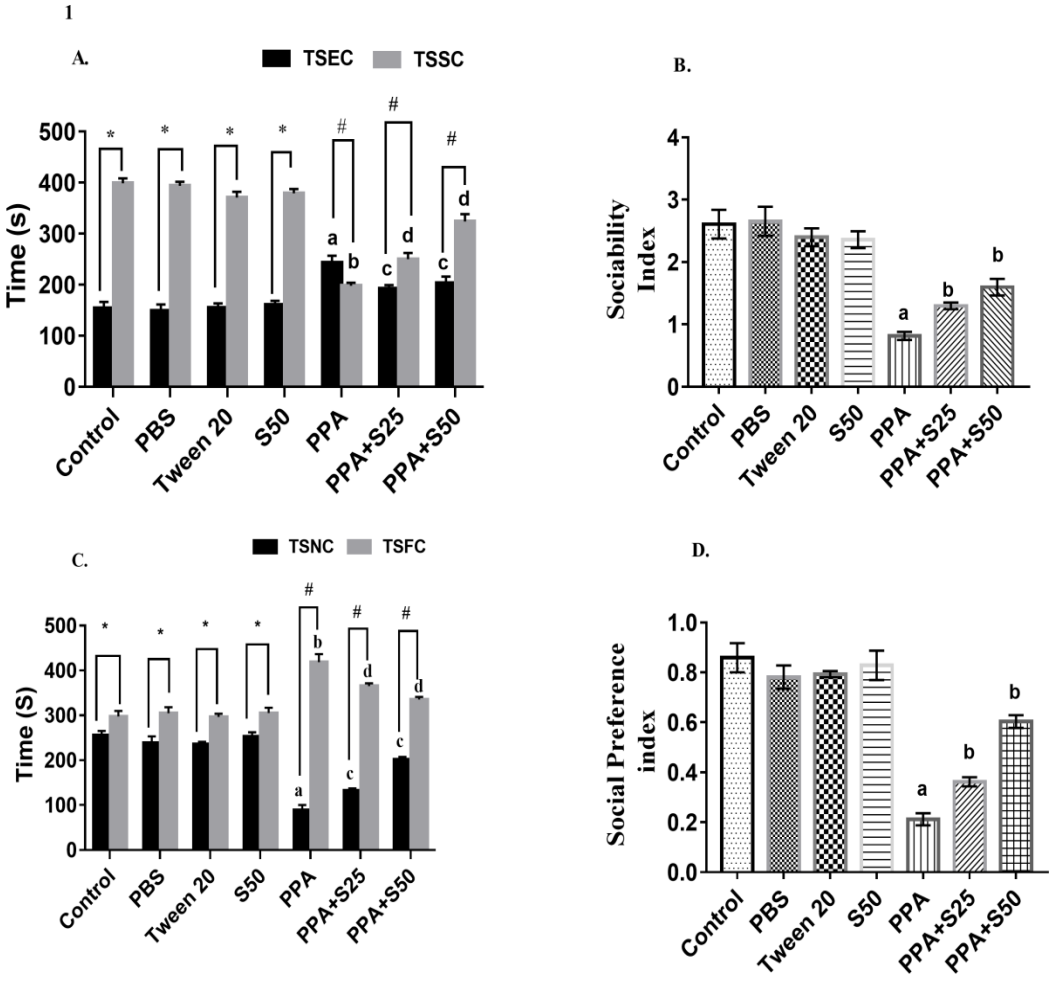

Fig. 1: Spironolactone administration and its effect on social behaviour.

Social preference and social preference index

PPA rats in comparison to the control animals showed a decline in the social preference index by staying for longer duration in the familiar chamber rather than novel chamber with unfamiliar rat. However, administration of spironolactone (25 mg/kg, ip; 50 mg/kg, ip) in the PPA exposed animals ameliorated the PPA induced decline in the social preference index of these animals by increasing the duration of stay in the novel chamber as compared to the familiar one **(Fig. 1C-1D).**

Results are expressed as mean ± S.D (n = 8; male); three-way ANOVA followed by Bonferroni's post hoc test (a & c).

Sociability: (F (1,84) = 375.31), a p< 0.001 vs TSEC control group, (F (1, 84) = 1909.007), b p< 0.001 vs TSSC control group; (F (2, 84) = 41.153), c p< 0.001 vs TSEC PPA group, (F (2, 84) = 184.603), d p< 0.05 vs TSSC PPA group,

Social preference: (F (1,84) = 1912.467), a p<0.001 vs TSNC control group, (F (1,84) = 466.735), b p< 0.001 vs TSFC control group; (F (2,84) = 148.144), c p< 0.001 vs TSNC PPA group, (F (2, 84) = 55.954), d p< 0.05 vs TSFC PPA group,

*p<0.001 vs TSEC/TSSC and TSNC/TSFC within control groups; # p< 0.001 vs TSEC/TSSC and TSNC/TSFC within PPA administered groups.

Sociability index: (F (1,42) = 1216.972), a p< 0.001 vs SI control group, (F (2,42) = 72.364), b p<0.05 vs SI PPA group

Social preference index: (F (1,42) = 1389.502), a p<0.001 vs SPI control group, (F (2,42) = 90.056), b p<0.05 vs SPI PPA group

TSEC- Time spend in empty chamber; TSSC- Time spend in stranger chamber; TSNC- Time spend in novel chamber; TSFC- Time spend in familiar chamber;

PBS- Phosphate buffer saline; S50- Spironolactone (50 mg/kg); PPA- Propionic acid; S25- Spironolactone (25 mg/kg).

Effect of PPA and spironolactone on repetitive behavior

Post-natal PPA exposed rats in comparison to control rats showed decrease in the % spontaneous alterations however, treatment with spironolactone (25 mg/kg, ip; 50 mg/kg, ip) significantly attenuated the post-natal PPA administration mediated increase in the % spontaneous alterations in the post-natal PPA exposed rats **(Fig. 2A).**

Effect of PPA and spironolactone on anxiety

Post-natal PPA exposed rats in comparison to control rats showed more anxious behavior by spending less percentage of total time and a smaller number of entries in the open arm in compared to closed arm. However, administration of spironolactone (25 mg/kg, ip; 50 mg/kg, ip) in post-natal PPA exposed animals significantly decreased the levels of anxiety in animals and animals were observed to spent more percentage of time and more entries in the open arm as compared to control arms **(Fig. 2B)**.

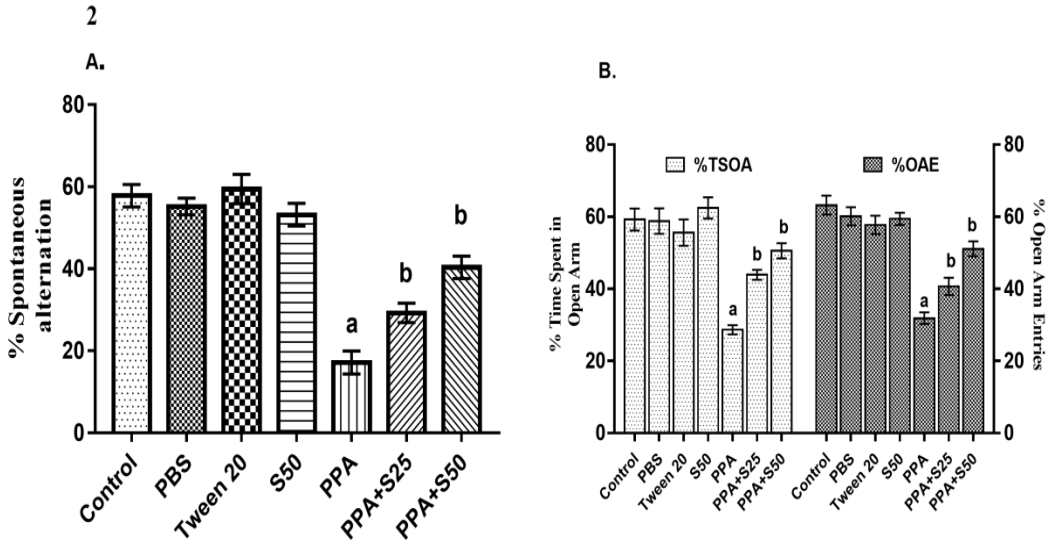

Fig. 2: Spironolactone administration and its effect on repetitive behavior and anxiety.

Results are expressed as mean ± S.D (n = 8; male); two-way ANOVA followed by Bonferroni's post hoc test.

3A. % Spontaneous alteration: (F (1, 42) = 1230.769), a p< 0.001 versus control group; (F (2, 42) = 118.911), b p< 0.05 versus PPA group.

3B. Anxiety: Time spend in open arm - (F (1, 42) = 1078.253), a p< 0.001 versus control group; (F (2, 42) = 69.469), b p< 0.05 versus PPA group.

Open arm entries - (F (1, 42) = 1169.377), a p<0.001 versus control group; (F (2, 42) = 115.381), b p<0.05 versus PPA group

PBS- Phosphate buffer saline; S50- Spironolactone (50 mg/kg); PPA- Propionic acid; S25- Spironolactone (25 mg/kg).

Effect of PPA and spironolactone on neuronal function marker

Post-natal PPA exposed rats in comparison to control rats showed significant decrease in the neuronal function markers such as synapsin II and BDNF in different areas of brain however, treatment with spironolactone (25 mg/kg, ip; 50 mg/kg, ip) significantly attenuated the post-natal PPA administration mediated decrease in the brain neuronal function markers in rats' brain **(Fig. 3A-3B)**

Results are expressed as mean ± S.D (n = 8; male); two-way ANOVA followed by Bonferroni's post hoc test.

3A. BDNF: Frontal cortex (F (1, 42) = 2412.921), a p< 0.001 versus control group; (F (2, 42) = 74.462), b p< 0.05 versus PPA group. Cerebellum (F (1, 42) = 1127.162), a p< 0.001 versus control group; (F (2, 42) = 43.77), b p<0.05 versus PPA group. Hippocampus (F (1, 42) = 1748.797), a p< 0.001 versus control group; (F (2, 42) = 77.547), b p< 0.05 versus PPA group.

3B. Synapsin II: Frontal cortex (F (1, 42) = 6492.311), a p<0.001 versus control group; (F (2, 42) = 175.928), b p< 0.05 versus PPA group. Cerebellum (F (1, 42) = 3842.567), a p< 0.001 versus control group; (F (2, 42) = 108.909), b p<0.05 versus PPA group. Hippocampus (F (1, 42) = 3055.306), a p<0.001 versus control group; (F (2, 42) = 230.051), b p<0.05 versus PPA group.

PBS- Phosphate buffer saline; S50- Spironolactone (50 mg/kg); PPA- Propionic acid; S25- Spironolactone (25 mg/kg).

Effect of PPA and spironolactone on BBB permeability

Post-natal PPA exposed rats shows higher % brain water content as compared to the control rats showing BBB permeability however, treatment with spironolactone (25 mg/kg, ip; 50 mg/kg, ip) in post-natal PPA exposed animals, significantly reduced the % brain water content in the rats **(Fig. 4)**.

Results are expressed as mean ± S.D (n = 8; male); two-way ANOVA followed by Bonferroni's post hoc test.

Brain water content: (F (1, 42) = 740.976), a p< 0.001 versus control group; (F (2, 42) = 111.176), b p< 0.05 versus PPA group.

PBS- Phosphate buffer saline; S50- Spironolactone (50 mg/kg); PPA- Propionic acid; S25- Spironolactone (25 mg/kg).

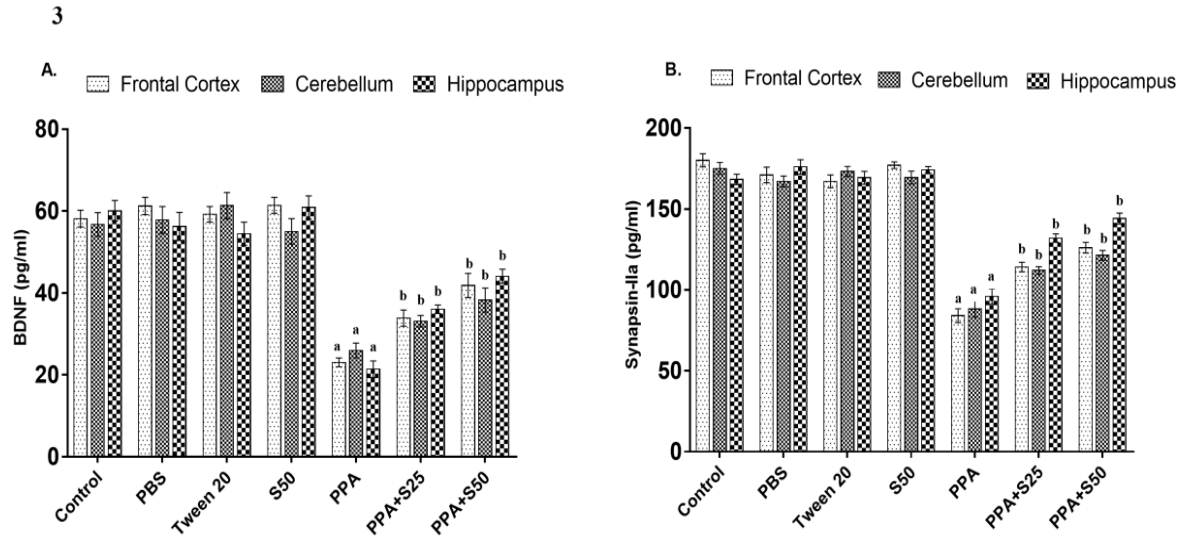

Fig. 3: Spironolactone administration and its effect on neuronal function marker.

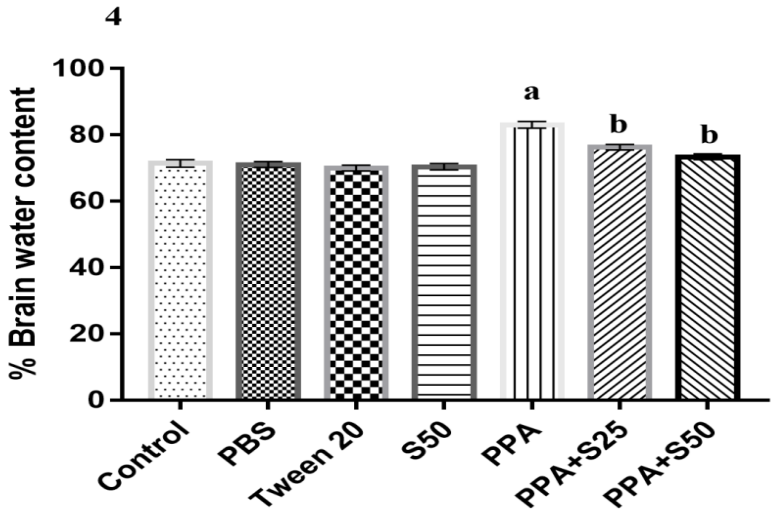

Fig. 4: Spironolactone administration and its effect on brain water content.

Discussion

In the current study, we observed that post-natal exposure to PPA in rats caused behavioral and biochemical abnormalities as observed in ASD which were attenuated by administration of spironolactone. These results are comparable with the previously published research reports[6,11,13]. This is the first study which is reporting the efficacy of spironolactone against decreased synapsin II in the brain of post-natal PPA exposed rats.

Repetitive behavior and decrease in social behavior are the two major symptoms of ASD. In the current study, PPA exposure increased stereotype and decreased social behavior in the experimental rats. These results are in synchronization with the previously published results reports wherein, propionic acid administration alters social behavior and increases repetitive behavior, validating the PPA induced experimental autistic phenotypes in animals[4&11&32&33]. Research reports suggest that decline in the cerebral BDNF expressions is associated with increased stereotypy behavior in experimental animals[34-36]. In addition to this, pre-natal stress animal model has shown reduced social behaviour and sociality upon reduction in the brains' BDNF expression[37]. Furthermore, in study by Dyck and colleagues, (2009), the knockout of synapsin II gene was held responsible for observed decrease in the social behaviour in schizophrenic mice[38]. Therefore, in this study the decrease in the BDNF and synapsin II expressions in PPA exposed rats might be a cause for behavioral alterations.

Anxiety occurs as a co-morbid trait, associated with ASD[39]. This study shows anxiety in post-natal PPA administered animals during behavioral assessment on elevated plus maze apparatus. Cirulli and colleagues, (2004) have observed a decline in the anxiety like behavior in animals with increase in the hippocampal BDNF levels using elevated plus maze apparatus[40]. Furthermore, synapsin II is also involved in neurogenesis[41] and alleviation in the hippocampal neurogenesis exerts anxiety like behavior[42]. This study records alleviation in the cerebral BDNF and synapsin II levels in the PPA treated rats therefore, an increase in the anxiety like behavior in these experimental rats might have been due to the alterations observed in the BDNF and synapsin II levels.

In the current study, the administration of spironolactone (25 mg/kg and 50 mg/kg) in post-natal PPA exposed rats increases social behaviour and reduces stereotypy as well as anxiety like behaviour. Spironolactone was observed to reduce stereotypic behaviour in autistic individual in a pre-clinical study [43]. Rodents with synapsin II knockout gene has been illustrated to show repetitive behaviour in animals[44]. In this study the administration of (25 mg/kg and 50 mg/kg) spironolactone increases the levels of synapsin in post-natal PPA exposed rats. Furthermore, increase in the expressions of BDNF is reported to increase social behavior in animal model of stroke and dementia[45]. Additionally, it is observed that activation of BDNF-TrkB signaling reduces anxiety like behavior in acute social stress mice model[46-47]. Therefore, in this study the administration of

Spironolactone (25 mg/kg and 50 mg/kg) causes an increase in the expression of BDNF in different brain regions of pre-natal VPA exposed rats.

Therefore, spironolactone mediated increase in the social behavior as well as decrease in the repetitive behavior and anxiety might have been due to its beneficial effect against reduced BDNF as well as synapsin II expressions in the brain.

Conclusions

Thus, we can conclude that spironolactone (25 mg/kg, ip and 50 mg/kg, ip) administration in the current study increases the social behavior and decreases the stereotypy as well as anxiety like behavior in post-natal PPA exposed rats possibly via activating the expressions of BDNF as well as synapsin II in brains' cerebellum, hippocampus, and frontal cortex regions. Moreover, detailed studies are required to understand the complete molecular pathways that are activated and is responsible for the effect of spironolactone in experimental ASD.

Data availability

The raw/processed data required to reproduce these findings will be available from authors, if required.

Funding

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Ethical approval

The experiments were conducted as per the study protocol approved by Institutional Animal Ethics committee (IAEC) of Amity University Uttar Pradesh, India (CPCSEA Reg. No. 1327/PO/ReBi/S/10/CPCSEA). Animals were housed and taken care as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

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عقار سبيرونولاكتون يغير مستويات علامات الوظيفة العصبية في الجرذان المصابة بالتوحد

روحي ميرزا[١] – بونام شارما[١] – جي تي كولكارني[٢] – بوبش شارما[١]*

[١] قسم علم الأدوية ، معهد أميتي للصيدلة ، جامعة أميتي أوتار براديش ، نويدا ، الهند

[٢] كلية جوكاراجو رانجاراجو للصيدلة ، حيدر أباد

ا ضطراب طيف التوحد (ASD) هو ا ضطراب في النمو العـ صبي يتميز بالـ سلوك المتكرر و ضعف الـ سلوك الاجتماعي. ASD هو ا ضطراب معقد مع مـ سببات معقدة تنطوي على جينات متعددة وعوامل بيئية وآلية جينية. أظهرت العديد من الدرا سات الـ سريرية وما قبل الـ سريرية ارتباط حمض البروبيونيك (PPA) مع ا ضطراب طيف التوحد. تقيم هذه الدرا سة التأثير المحتمل للـ سبيرونولاكتون في النمط الظاهري لاضـــطراب طيف التوحد الناجم عن حمض البروبيونيك (PPA). تم إعطاء PPA (250 مجم / كجم ، عن طريق الفم) لجرذان الألبينو من اليوم الحادى و العشرين الى اليوم الثالث و العشرين ما بعد الولادة للحث على ا ضطراب طيف التوحد مثل التغيرات الـ سلوكية العـ صبية والكيميائية الحيوية العصـــبية. تم اختبار الحيوانات للســـلوك (متاهة مرتفعة ، وجهاز تفاعل اجتماعي ثلاثي الحجرات ، وجهاز متاهة Y) ، والمعلمات الكيميائية الحيوية (BDNF، synapsin II) ، وضـــعف حاجز الدم في الدماغ. أدى التعرض لحمض البروبيونيك بعد الولادة إلى ضعف في السلوك الاجتماعي والقلق والسلوك المتكرر في الحيوانات. علاوة على ذلك ، تـ سبب التعرض لحمض البروبيونيك في انخفاض مـ ستويات BDNF و synapsin II في دماغ الجرذان. لوحظ أن إعطاء سبيرونولاكتون (٢٥ مجم / كغم و ٥٠ مجم / كجم) يخفف من العاهات الـ سلوكية والكيميائية الحيوية المعر ضة لحمض البروبيونيك بعد الولادة في الحيوانات.