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Original Article

Progesterone attenuate autism-like-phenotype through modulation of cerebral inflammation and oxidative stress

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ABSTRACT

Objectives: Autism spectrum disorder (ASD) is a neurodevelopmental disorder that characterises repetitive behaviour and impairment in social communication as well as interaction. The complex aetiology of ASD involves multiple genes, epigenetic mechanisms and environmental factors. Propionic acid (PPA) is observed to be well associated with behavioural and biochemical phenotypes akin to ASD. This study evaluates the potential effect of progesterone in PPA-induced ASD phenotype.

Materials and Methods: PPA (250 mg/kg, *po*) was administered to induce ASD-like behavioural and neurobiochemical alterations in Albino Wistar rats from post-natal day 21st to 23rd. Rats were observed for locomotor activity (open filed apparatus), exploratory behaviour (hole board apparatus- no. of rearing, latency to first poke and hole poking), stereotypy behaviour (self-grooming) and biochemical parameters (interleukin-6, tumour necrosis factor-alpha, glutathione and thiobarbituric acid reactive substance).

Results: Post-natal PPA administration resulted in hyperlocomotion, repetitive behaviour and a decrease in exploratory activity. Furthermore, an increase in inflammation and oxidative stress markers was observed in the brain regions of PPA-administered rats. Progesterone (4 mg/kg and 8 mg/kg) administration was observed to attenuate postnatal PPA-induced impairments in experimental animals.

Conclusion: Progesterone (4 mg/kg and 8 mg/kg) administration may protect against behavioural and biochemical alterations that are associated with ASD.

Keywords: Propionic acid, Autism, Progesterone, Exploratory behaviour, Repetitive behaviour

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopment disorder that characterises impairment in two major behaviours: Repetitive behaviour and deficit in social communication as well as interaction.[1] Other comorbid conditions observed in an autistic individual include anxiety, aggressiveness, sleeplessness, gastrointestinal disturbances and motor impairments.[2] Studies suggest that men are more susceptible to the ASD condition than women.

Propionic acid (PPA) is a naturally occurring saturated fatty acid that is produced through anaerobic fermentation of dietary substrates by gut microbiota. PPA modulates cellular processes and metabolism and exhibits immunosuppressive properties.[3] PPA can permeate the blood-brain barrier and cause developmental delays as well as disruption in neurotransmitter release.[3,4] PPA has been implicated in inflammation, oxidative stress, immune dysfunction and

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cognitive impairment, which is also observed in ASD.[5-11] PPA exposure induces behavioural abnormalities akin to ASD. Administration of PPA induces stereotypy and causes impairment in attention, social behaviour, locomotion and information processing abilities in rodents.[5,6,12,13] Postnatal PPA exposure causes a decline in the levels of cerebral phosphorylated – cAMP response element binding protein and brain-derived neurotrophic factor (BDNF). Furthermore, PPA elevates the levels of oxidative (thiobarbituric acid reactive substance [TBARS]) and inflammation stress markers (interleukin 6 [IL-6] and tumour necrosis factor-alpha [TNF-α]) in rodents.[14,15] Consequently, postnatal exposure to PPA serves as a reliable model for replicating behavioural and biochemical phenotypes that are associated with and observed in ASD.[5,6,11,13,16]

Progesterone is an endogenous steroid hormone that is essential for pregnancy, embryogenesis and the menstrual cycle of humans and other species. The potential role of progesterone has been implicated in several neurological such as traumatic brain injury, hypoxia/ischemic brain injury, Parkinson's disease and subarachnoid haemorrhage.[17-20] Progesterone corrects behavioural abnormalities that are observed in ASD in various animal experimental models for diseases. This includes social behaviour, stereotypy, anxiety, depression and hyperactivity.[21-25] In addition to this, progesterone promotes neuroprotection, neurotransmission, myelination and neurogenesis through inhibition of neuroinflammation, oxidative stress and neurodegeneration.[26,27] However, the role of progesterone is yet to be elucidated in the experimental model of ASD. We have hypothesised that progesterone might play a key role in ameliorating behavioural as well as biochemical alterations that are observed in ASD. In this study, we have investigated the effects of progesterone against impaired behavioural (stereotypy, hyperlocomotion and exploration) and biochemical (cerebral inflammation and oxidative stress) parameters that are associated with ASD.

MATERIALS AND METHODS

Animals

The present study employed male Albino Wistar rats (male) that were housed in the animal house of Amity University (Reg No. 1327/PO/ReBi/S/10/CPCSEA). The animal house was set at a temperature of 25 ± 2°C with relative humidity of 50 ± 5%. These rats were provided with water and a standard laboratory pellet chow diet (Ashirwad Industries, Punjab, India). The rats were exposed to 12 h natural light and 12 h dark cycle. This experimental study was approved by the Institutional Animal Ethics Committee (IAEC) of Amity University Uttar Pradesh, India, under the approved protocol 'CPCSEA/IAEC/AIP/2020/03/18.'

Chemical and reagents

Progesterone was procured from Central Drug House, Pvt. Ltd., New Delhi, India. PPA was purchased from Lab Sale Corporation, New Delhi, India. The IL-6 and TNF-α enzyme-linked immunosorbent assay kits were procured from Ray Biotech, Inc., Norcross, GA.

Drugs and administration

The male offspring from the untreated pregnant female rats received PPA (250 mg/kg) diluted in phosphate buffer saline (PBS) for injection in a 9 g/L sodium chloride solution from postnatal day (PND) 21 to 23.

Animals in the treatment groups were administered with drug/vehicle from PND 24 to PND 50. Progesterone (4 mg/kg, sc; 8 mg/kg, sc) was dissolved in sesame oil and was administered an hour before behavioural assessments. These doses were selected for administration on the basis of the previously published research reports.[28,29]

Experimental design

In the present study, animals were randomly divided into seven groups. Each group had eight animals (n = 8; male), and these animals were selected on the basis of previously published research reports.[6,13]

These groups include:

- Group I (Control group): Male offspring (No treatment/vehicle) underwent behaviour assessments from PND 44 to PND 50.
- Group II (PBS): Male offspring were administered with 0.2 M PBS from PND 21 to 23.
- Group III (Sesame oil): Male offspring were administered with sesame oil from PND 24 to PND 50.
- Group IV (Progesterone per se [8 mg/kg]): Male offspring were administered progesterone (8 mg/kg; sc) from PND 24 to PND 50.
- Group V (PPA): Male offspring were administered with PPA (250 mg/kg, *po*), and the behavioural assessments were performed from PND 44 to PND 50.
- Groups VI and VII (Progesterone [4 mg/kg, sc; 8 mg/kg]): Male offspring that received PPA were administered with progesterone (4 mg/kg; 8 mg/kg; *sc*) from PND 24 to PND 50.

Behavioural assessments

All behavioural parameters were performed in experimental animals during the light phase (09:00–18:00 h) from PND 44 to PND 50. The experiments were conducted during the daytime to obtain a clear understanding of the behavioural experiment conducted, as rats are nocturnal animals, and they exhibit peak activity during the night.

Assessment of locomotor activity

Rats were assessed for locomotor activity using an open-field apparatus. This apparatus consists of an open box with a floor divided into 25 squares. The individual rats were placed separately in a fixed corner facing toward the wall, and the number of squares crossed during a 5-min session (floor units which rats crossed with four feet) were regarded as a measure of locomotor activity.[30]

Assessment of self-grooming activity

Self-grooming is an innate grooming behaviour that rats perform on themselves. Excessive self-grooming denotes stereotypic behaviour. For the assessment of self-grooming, individual rats were placed in a clean and empty cage without any bedding and were allowed to habituate for 10 min. Post habituation, the cumulative time sent by the test rat in grooming all body regions was recorded for 10 min. Grooming behaviour elicited by the rat included head washing, genital/tail grooming, body grooming, scratching and paw as well as leg licking.[30]

Assessment of exploratory activity

The exploratory activity in the rats was examined with the help of a hole board apparatus. The hole board apparatus comprises a wooden platform (40 cm × 40 cm) which was raised to a height of 15 cm from the floor of a grey platform with dimensions 40 cm × 40 cm × 40 cm × 40 cm × 40 cm × 40 cm. The platform was divided equally into 16 square compartments. Each square compartment had a circular hole of a diameter of 3 cm at the centre. A rat was placed at the centre of the platform for 5 min to explore. After this habituation, the exploratory activity of the rat was assessed as the latency of the first poke, number of rearing and hole pocking for 3 min.[7]

Biochemical assessments

Tissue preparation for biochemistry

Biochemical assessments were carried out after the completion of behavioural studies. The rats were euthanised through administration of thiopental sodium (90 mg/kg, *ip*) and their brains were removed after decapitation. The removed brains were washed with ice-cold phosphate buffer solution (pH 7.4) to remove hair and other debris. The brain was sectioned carefully to isolate the cerebellum, frontal cortex and hippocampus regions. The brain samples were homogenised in RIPA buffer (1:9 w/v) using polytron (PT 1600 E) homogeniser. Each 1 mL of RIPA buffer contained 10 µL of cocktail protease inhibitor. The homogenised brain samples were centrifuged at 3000 rpm for 15 min (4°C) using a centrifuge machine (Remi, C-24 PLUS, India) to collect the supernatant.[31] This supernatant was stored at −80°C for biochemical analysis in the cerebellum, frontal cortex hippocampus and remaining brain regions.[6]

Assessment of brain protein

Brain protein levels were calculated using Lowry's method. Bovine serum albumin was used as a standard. The supernatant was mixed with Lowry's reagent and after keeping this mixture undisturbed for 15 min, Folin-Ciocalteu reagent was added. This tube was vortexed and then incubated for 30 min at room temperature. The absorbance of the tube was recorded at 750 nm, and the values were expressed as mg/mL of supernatant.[7]

Assay of thiobarbituric acid reactive substance and glutathione (GSH)

TBARS assesses lipid peroxidation and is used as an oxidative stress marker. The TBARS levels were estimated using a microplate reader (with slight modifications) at 532 nm.[31] For the estimation of TBARS, isolated supernatant (100 µL) was mixed with equal volumes of sodium dodecyl sulphate (8.1%), 250 µL of 1:1 mix of 30% acetic acid (pH 3.5) and 0.8% thiobarbituric acid. This mixture was incubated for 60 minutes at 95°C. After incubation, these samples were centrifuged (4000 g) for 10 min, and the butanol fraction was taken for further assessment. The results were expressed as nM/mg of protein.[32,33]

GSH is a crucial antioxidant and plays an important role in cellular defence against oxidative stress. The GSH levels in the brain were recorded at 412 nm using a microplate reader. For the estimation of GSH levels, brain supernatant was mixed with 10% w/v trichloroacetic acid (1:1 ratio), and this mixture was centrifuged at 1000 g for 10 min at 4°C. Following centrifugation, the resulting supernatant was combined with 0.25 mL of 0.001 M DTNB in 2 mL of 0.3 M disodium hydrogen phosphate. A standard curve was built using fixed doses of GSH between 10 and 100 µM; the values were represented as µM/mg of protein.[6,32]

Assessment of IL-6 and TNF-α

The biochemical estimation of TNF-α and IL-6 was carried out in the selected brain regions (frontal cortex, cerebellum, and hippocampus) using their respective ELISA kits as per manufacturers' instructions (RayBio®, USA). All kits were based on the sandwich *in vitro* ELISA principle, and the optical density of the samples was measured using a microplate reader at 450 nm. The concentrations for TNF-α and IL-6 were expressed as pg/mL.[31,33]

Statistical analysis

The data were represented as mean ± Standard deviation. The data were analysed using a two-way analysis of variance

followed by Bonferroni's post hoc test using Sigma stat 12.5 (Systat Softwares, Inc.). Data were statistically significant at $P < 0.05$.

RESULTS

Effect of progesterone on locomotion in postnatal PPA exposed rats

PPA-administered rats, compared to control groups, showed an increase in the number of square crossings that indicate hyper-locomotion. However, the administration of progesterone (4 mg/kg; 8 mg/kg; sc) to PPA-administered rats showed a significant reduction in the number of square crossings in open field apparatus in comparison to the PPA-administered group [Figure 1a].

Effect of progesterone on self-grooming in postnatal PPA exposed rats

PPA-administered rats showed enhanced self-grooming activities such as head washing, genital/tail grooming, body grooming, scratching, and paw as well as leg licking activities in comparison to the control group; however, treatment with progesterone (4 mg/kg; 8 mg/kg; sc) significantly attenuated the PPA-induced increase in the self-grooming activities in the postnatal PPA-exposed rats [Figure 1b].

Effect of progesterone on exploratory activity in postnatal PPA exposed rats

PPA rats showed an increase in the exploratory behaviour as observed through an increase in the latency of the first poke as well as a decrease in the number of hole poking and rearing in comparison to control rats. However, treatment with progesterone (4 mg/kg; 8 mg/kg; *sc*) significantly attenuated the PPA-induced increase in the exploratory activity in the PPA-administered rats [Figure 2a-c].

Effect of progesterone on brain oxidative stress in postnatal PPA-exposed rats

PPA-administered rats showed a significant increase in oxidative stress (increased TBARS and decreased GSH) in different brain regions compared to control animals; however, treatment with progesterone (4 mg/kg; 8 mg/kg; sc) significantly attenuated the PPA-induced increase in the brain TBARS levels and decrease in the brains' GSH levels in the postnatal PPA-exposed rats [Figures 3a and b].

Effect of progesterone on brain inflammation markers in postnatal PPA exposed rats

PPA-administered rats, in comparison to control rats, showed significant increases in the levels of IL-6 and TNF-α in the cerebellum, frontal cortex, and hippocampus regions of the brain; however, treatment with progesterone (4 mg/kg; 8 mg/kg; sc) in postnatal PPA-exposed animals significantly decreased the elevation of inflammatory status in the cerebellum, frontal cortex, and hippocampus regions of the brain [Figure 3c and d].

DISCUSSION

Autistic animals have shown hyperlocomotion during the experimental studies.[3] In the present study, rats administered with PPA have shown an increase in the number of square crossings during an open field test, signifying an increase

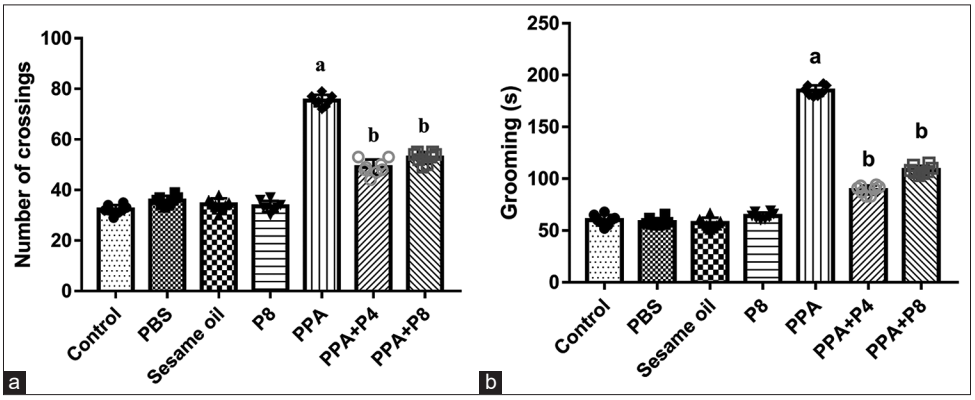

Figure 1: Effect of progesterone administration on locomotion and repetitive behaviour. Results are expressed as mean ± S.D ($n = 8$; male); two-way ANOVA followed by Bonferroni's *post hoc* test. **(a)** Number of square crossings: (F [1,42] = 1511.663), a: $P < 0.001$ versus control group; (F [2,42] = 152.296), b: $P < 0.05$ versus PPA group. **(b)** Grooming: (F [1, 42] = 3493.017), a: $P < 0.001$ versus control group; (F [2, 42] = 796.243), b: $P < 0.05$ versus PPA group. PBS: Phosphate buffer saline, P8: Progesterone (8 mg/kg), PPA: Propionic acid, P4: Progesterone (4 mg/kg), ANOVA: Analysis of variance, S.D: Standard deviation.

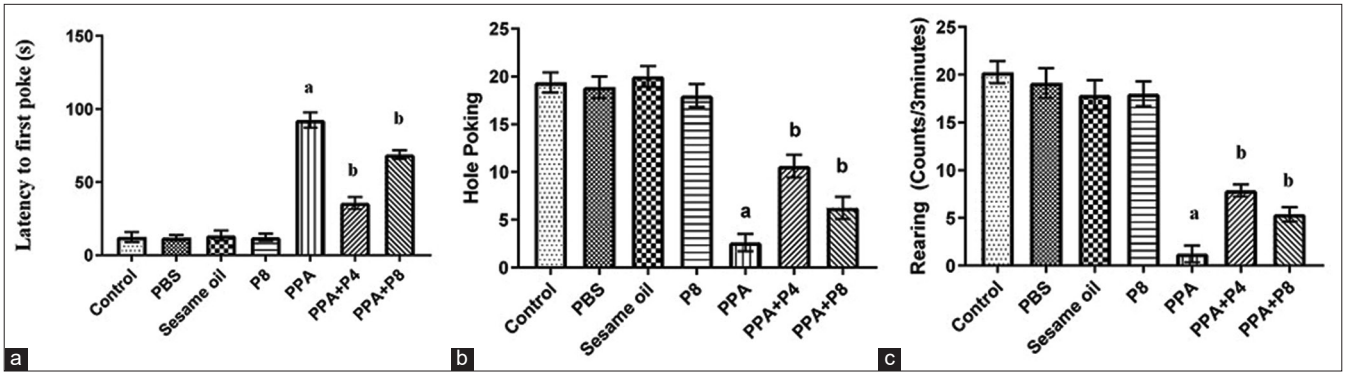

Figure 2: Effect of progesterone administration on exploratory activity. Results are expressed as mean ± S.D (n = 8; male); two-way ANOVA followed by Bonferroni's *post hoc* test. **(a)** Latency to first poke: (F [1, 42] = 2898.639), a: $P < 0.001$ versus control group; (F [2, 42] = 272.418) and b: $P < 0.05$ versus PPA group. **(b)** Hole poking: (F [1, 42] = 1448.832), a: $P < 0.001$ versus control group; (F [2, 42] = 74.818), b: $P < 0.05$ versus PPA group. **(c)** Rearing: (F [1, 42] = 2108.065), a: $P < 0.001$ versus control group; (F [2, 42] = 86.364), b: $P < 0.05$ versus PPA group. PBS: Phosphate buffer saline, P8: Progesterone (8 mg/kg), PPA: Propionic acid, P4: Progesterone (4 mg/kg), ANOVA: Analysis of variance, S.D: Standard deviation.

in locomotor activity as compared to the normal control animals. Evidence suggests hyperlocomotion in rodents with elevated levels of cerebral TNF-α in comparison to rodents with normal cerebral TNF-α levels.[34] Furthermore, hyperlocomotion was also recorded in GSH-knockout mice during the experiment of the open field apparatus.[35] In the present study, we have observed a significant elevation and reduction in the TNF-α and GSH levels, respectively, in the studied brain regions of the PPA-administered rats. Therefore, the observed hyper-locomotory effect in these PPA rats might have been due to an increase and decrease in brain TNF-α and GSH levels, respectively.

PPA-administered rats in the present study have shown an increase and decrease in repetitive and exploratory behaviour, respectively. Clinical observation suggests the association of restricted and repetitive behaviour with increased cerebral IL-6 levels in autistic children.[36] Furthermore, oxidative stress in the frontal cortex and hippocampal region regions of the brain was observed to impact exploratory behaviour in the experimental animals.[37] Consequently, a rise in the cerebral inflammatory status and oxidative stress markers in PPA-administered rats in the present study might have been responsible for an increase and decrease in the repetitive and exploratory behaviour, respectively. In addition, cerebral inflammation and oxidative stress are reported to cause behavioural impairment in the PPA-induced ASD animal model.[6-8,31-33,38] PPA induces the excessive formation of reactive oxygen species in both the frontal cortex and hippocampus. Oxidative stress activates the nuclear factor kappa-light-chain-enhancer of activated B-cells, which further increases the secretion of inflammatory markers, forming a vicious circle of inflammatory markers secretion and free radicals formation.[39,40] Furthermore, a decrease in the GSH levels was recorded in the postmortem brain of individuals suffering from autism, suggesting the crucial role of GSH depletion in autistic phenotype.[41]

Progesterone potentiates BDNF mRNA and protein levels in the hippocampus and cerebral cortex regions of the brain[42], which further initiates the activation of prosurvival cell signalling pathways.[43] In ADHD mice, an increase in the expression of BDNF is reported to protect against hyperactivity and hyper-locomotion.[44] Furthermore, administration of progesterone inhibits the release of proinflammatory cytokines such as IL-6, TNF-α and interferon-γ that are known to cause hyperlocomotion in ketamine-induced cognitive dysfunction and hyperlocomotion in rats.[33,45] Furthermore, another study has demonstrated that progesterone plays a beneficial role in synapse formation in the hippocampal region of the brain.[46] These results are akin to the results obtained in the present study, where subcutaneous administration of progesterone (4 mg/kg and 8 mg/kg) in postnatal PPA-exposed experimental animals increases the expressions of antioxidant marker GSH as well as decreases the expression of brain inflammatory markers such as IL-6, TNF-α in the cerebellum, hippocampus and frontal cortex regions of the brain. Thus, in this study, the progesterone-mediated decrease in hyperlocomotion could have been due to an increase in the GSH and a decrease in the TBARS expression in the brain.

The present study has observed the protective effects of progesterone administration (4 mg/kg and 8 mg/kg) against increased repetitive behaviour as well as impaired exploratory activity in the PPA-treated rats. These results are in accordance with the previously published reports where the cocaine sensitisation rat model has demonstrated the beneficial effects of progesterone administration against

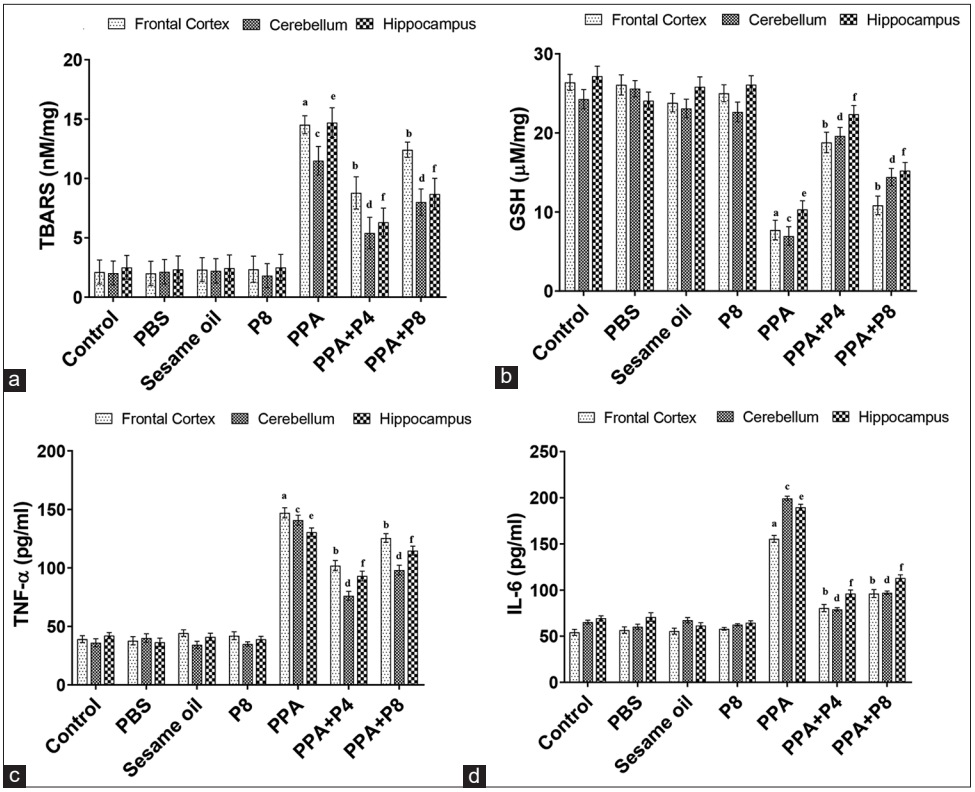

Figure 3: Effect of progesterone administration on cerebral oxidative stress and inflammatory markers Results are expressed as mean ± S.D (n = 8; male); two-way ANOVA followed by Bonferroni's post hoc test. (**3a**) TBARS: Frontal cortex (F (1, 42) = 1130.157), a: p<0.001 versus control group; (F (2, 42) = 36.501), b: p<0.05 versus PPA group. Cerebellum (F (1, 42) = 443.856), c: p<0.001 versus control group; (F (2, 42) = 30.389), d: p<0.05 versus PPA group. Hippocampus (F (1, 42) = 513.7), e: p<0.001 versus control group; (F (2, 42) = 59.402), f: p<0.05 versus PPA group. (**3b**) GSH: Frontal cortex (F (1, 42) = 1606.387), a: p<0.001 versus control group; (F (2, 42) = 115.005), b: p<0.05 versus PPA group. Cerebellum (F (1, 42) = 919.987), c: p<0.001 versus control group; (F (2, 42) = 177.589), d: p<0.05 versus PPA group. Hippocampus (F (1, 42) = 1127.581), e: p<0.001 versus control group; (F (2, 42) = 129.703), f: p<0.05 versus PPA group. (**3c**) TNF-α: Frontal cortex (F (1, 42) = 946.783), a: p<0.001 versus control group; (F (2, 42) = 642.398), b: p<0.05 versus PPA group. Cerebellum (F (1, 42) = 5502.655), c p<0.001 versus control group; (F (2, 42) = 386.948), d: p<0.05 versus PPA group. Hippocampus (F (1, 42) = 6046.561), e: p<0.001 versus control group; (F (2, 42) = 126.985), f: p<0.05 versus PPA group. (**3d**) IL-6: Frontal cortex (F (1, 42) = 3724.988), a: p<0.001 versus control group; (F (2, 42) = 727.146), b: p<0.05 versus PPA group. Cerebellum (F (1, 42) = 15333.05), c: p<0.001 versus control group; (F (2, 42) = 5393.849), d: p<0.05 versus PPA group. Hippocampus (F (1, 42) = 6811.425), e: p<0.001 versus control group; (F (2, 42) = 1166.197), f: p<0.05 versus PPA group. PBS: Phosphate buffer saline, P8: Progesterone (8 mg/kg), PPA: Propionic acid, P4: Progesterone (4 mg/kg), ANOVA: Analysis of variance, S.D: Standard deviation, TNF-α**:** Tumour necrosis factor-alpha, IL-6: Interleukin, GSH: Glutathione, TBARS: Thiobarbituric acid reactive substance. [Note: For the frontal cortex, "a" is for the statistical comparison of the PPA group with the control group; "b" is for the statistical comparison of treatment groups with the PPA group for all the parameters, i.e., TBARS, GSH, TNF-α, and IL-6. For the cerebellum region, "c" is for the statistical comparison of the PPA group with the control group; "d" is for the statistical comparison of treatment groups with the PPA group for all the parameters, i.e., TBARS, GSH, TNF-α, and IL-6. For the Hippocampus region, "e" is for the statistical comparison of the PPA group with the control group; "f" is for the statistical comparison of treatment groups with the PPA group for all the parameters, i.e., TBARS, GSH, TNF-α, and IL-6.]

stereotypic behaviour in animals.[22] Furthermore, oxidative stress in the frontal cortex and hippocampal regions of the brain is observed to impair exploratory activities in rodents during pre-clinical studies.[37] Progesterone (4 mg/kg and

8 mg/kg) administration to the PPA-treated rats in this study caused a marked decrease in the levels of oxidative stress markers (TBARS) in the frontal cortex as well as hippocampal regions of the brain. Therefore, progesterone-mediated beneficial effects against PPA-induced decrease in the exploratory behaviour as well as an increase in the repetitive behaviour might have been due to its ameliorating effects against elevated inflammation and oxidative stress markers in animals. Furthermore, the present study utilises sesame oil as a vehicle for progesterone administration. Sesame oil is a delivery vehicle for all fat-soluble compounds, such as steroidal hormones and toxins. The administration of sesame oil to male offspring from PND 24 to PND 50 has shown no significant effects against any of the parameters assessed, which clearly suggests that the beneficial effects observed in the present study were observed through the administration of progesterone and not its vehicle.

CONCLUSION

Therefore, we can conclude that the administration of progesterone (4 mg/kg and 8 mg/kg) has significantly enhanced the exploratory behaviour as well as reduced the hyperlocomotion and stereotypy behaviour in postnatal PPA-treated rats in the present study. This beneficial effect of progesterone was observed through the activation of anti-inflammatory as well as anti-oxidative stress markers and the inhibition of inflammatory as well as oxidative stress markers in the frontal cortex, hippocampus and cerebellum regions of the brain.

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Ethical approval

The experiments in the current study were conducted as per the approved study protocol (CPCSEA/IAEC/AIP/2019/01/22), dated: January 19, 2019; by IAEC of Amity University Uttar Pradesh, India (CPCSEA Reg. No. 1327/PO/ReBi/S/10/CPCSEA). Animals were housed and taken care off as per the guidelines set by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

Declaration of patient consent

Patient's consent was not required as there are no patients in this study.

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Conflicts of interests

There are no conflicts of interest.

Use of artificial intelligence (AI)-assisted technology for manuscript preparation

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

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