Research Paper
Shielding Effect of Ryanodine Receptor Modulator in Rat Model of Autism

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ABSTRACT

Introduction: A neurodevelopmental disorder, autism is typically identified with three primary behavioral consequences, such as social impairment, communication problems, and limited or stereotypical behavior. Because of its co-morbidity and lack of therapeutic options, autism is a global economic burden. A short chain of fatty acid, propionic acid is formed biologically by the gut microbiome. Propionic acid levels that are too high can cause leaky intestines, which can lead to autism-like symptoms.

Methods: To induce autism, male Albino Wistar rats were given propionic acid (250 mg/kg/po on the 21st, 22nd, and 23rd postnatal days). Rats also received a ryanodine receptor antagonist (Ruthenium red: 3 mg/kg/po; postnatal 21st to 50th day) to see what influence it had on propionic acid-induced autism. Anxiety, social behavior, and repeated behaviors were all assessed, as well as oxidative stress, inflammatory indicators, neuro signaling proteins, and blood-brain barrier permeability.

Results: Ruthenium red was found to counter the propionic acid-induced increases in anxiety, repetitive behavior prefrontal cortex levels of IL-6, TNF-α, TBARS, Evans blue leakage, and water content along with decreases in social behavior, IL-10, and GSH followed by hippocampus CREB and BDNF levels.

Conclusion: Ryanodine receptor antagonists presented a neuroprotective effect in propionic acid-induced conditions like autism by modulatory effects on social and repetitive behavior, oxidative stress, neuroinflammation, and neuroprotein changes. Ryanodine receptors can be further explored in depth to manage autism as a condition.

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Highlights

• Ruthenium red can reduce the propionic acid-induced anxiety of rats with autism.

• Ruthenium red can improve the propionic acid-induced changes in repetitive behavior of rats with autism.

• Ruthenium red can reduce the propionic acid-induced social behavior dysfunction in rats with autism.

Plain Language Summary

Autism is a complex heterogeneous neurodevelopmental disorder mainly diagnosed with social behavior dysfunction, communication problems, and repetitive behavior. Due to high comorbidity and multiple unknown factors involvement, its exact etiology remains unclear, and so no successful treatment is available. Among the environmentally produced models of autism in rats, the most common is created by propionic acid (PPA). With short-chain type fatty acid, PPA is one of the mediators for the cycle of cell metabolism. This study attempted to study the effect of a ryanodine receptor antagonist (Ruthenium red) on PPA-induced Anxiety, social behavior dysfunction, and repeated behaviors in rats with autism. The results showed the modulatory effects of Ruthenium red PPA-induced conditions including social and repetitive behavior, oxidative stress, neuroinflammation, and neuroprotein changes in rats with autism.

1. Introduction

Autism is a complex heterogeneous neurodevelopmental disorder mainly diagnosed with social behavior dysfunction, communication problems, and repetitive behavior. It is reported that 1 out of 59 children suffers from autism. Autism is four times more frequent in men than in women, and many girls with autism spectrum disorder (ASD) have less apparent symptoms than boys. Autism is a disorder that lasts for life (Maenner et al., 2020). Due to high comorbidity and multiple unknown factors involvement, its exact etiology remains unclear, and so no successful treatment is available.

Among the environmentally produced models of autism in rats, the most common is created by propionic or valproic acid, either prenatally or postnatally. With short-chain type fatty acid, propionic acid (PPA) is one of the mediators for the cycle of cell metabolism. PPA is also a byproduct of enterobacteria in human intestines and a popular food preservative (Choi et al., 2020). Increased PPA and propionic acidemia is associated with autism (Al-Owain et al., 2013; Cotrina et al., 2020) its toxic derivatives, and ammonia. The disease causes multiorgan damage, especially in heart, pancreas, and brain; seizures and intellectual disability are often described. Some PA children also show autism spectrum disorders (ASD. While PPA has a beneficial function, its accumulation has been documented to cause neurotoxicity (Choi et al., 2020; El-Ansary et al., 2012). Enormous amounts of fatty acid with short chains in the rat intestinal tract, as

well as peripheral PPA injection, have all been strongly correlated to autism-related behavior problems. PPA, in neurotoxic amounts, may contribute to autism-like symptoms in animals, according to researchers (Choi et al., 2020; El-Ansary et al., 2012; MacFabe et al., 2011). PPA treatment resulted in increased neuro-inflammation, oxidative stress, poor energy metabolism, aberrant neurotransmission, and a pro-apoptotic effect in rats (El-Ansary et al., 2012). In addition to our prior findings, several other researchers concurred that PPA treatment in rats during the early postnatal day may generate behavioral abnormalities similar to ASD (Choi et al., 2020; El-Ansary et al., 2012; MacFabe et al., 2011; Mirza & Sharma, 2018).

IIncreased cytosolic calcium concentration stimulates neurotransmitter release at synaptic junctions, leads to synaptic function, and regulates behavior pattern modifications in neural function and cell proliferation. (Berridge et al., 1998; Ludwig et al., 2016). Calcium homeostasis is generally regulated by various kinds of calcium channels and their receptors. Ryanodine receptor is one of the calcium Ruthenium red that is known as a selective antagonist of intracellular ryanodine receptor (RyR). Antagonism of the RyR has shown to be useful in the treatment of numerous brain illnesses, including Huntington's disease and Alzheimer's disease (Baker et al., 2013; Calì et al., 2012; Del Prete et al., 2014; Suzuki et al., 2012). It has been documented that the antagonism of the ryanodine receptor exerts a neuro-protective effect along with decreased infarct volume and lower intracellular calcium in the brain (Phillips et al., 2013). Previous

research has also found that ruthenium red has beneficial effects on brain inflammation (Cisneros-Mejorado et al., 2018; Poole et al., 2013) and oxidative stress (Schilling & Eder, 2009). The antagonistic role of ryanodine receptors in ASD has not been explored yet. This manuscript explored the novel job of ryanodine receptors antagonist; ruthenium red in PPA-administered autistic phenotypes in rodents.

2. Materials and Methods

Experimental rat

Pregnant albino Wistar females and their offspring were housed and kept in polypropylene cages at Amity University (Reg No. 1327/PO/ReBi/S/10/CPCSEA). All the conditions and parameter sticky followed as per IAEC/CPCSEA, at a temperature of $25\pm2°C$ with a relative humidity of $50\pm5\%$. Typical laboratory pellet chow food and water have always been available to rats (Ashirwad Industries, Punjab, India). Animals were exposed to the natural light/dark cycle (dark starting at 19:00 hrs. and ending at 07:00 hrs.).

Chemicals, drugs, and reagents

Chemicals and reagents of analytical and laboratory grade were employed in this study. Propionic acid was taken from Lab Sales Corporation India. Evans blue, egtazic acid was purchased from SRL Pvt. Ltd, India. Lowry's reagent, N-naphthylethylenediamine, and 5,5`-dithiobis (2-nitrobenzoic acid) (Ellman's reagent) were obtained from Sigma-Aldrich, India. H_2O_2 and C_5H_5N were provided by Rankem Ltd. India.

ASD induced by PPA

To develop autism-like phenotypes, rats were given 250 mg/kg propionic acid orally on three consecutive postnatal days, the 21st, 22nd, and 23rd. PPA was prepared by mixing with 0.2M phosphate buffer saline. (Bukhari et al., 2020; El-Ansary et al., 2012; Mirza & Sharma, 2018).

Protocol design

A total 48 rats (n=8) divided into 6 groups, were utilized for the protocol of the study. The animals were chosen based on previous research, an experimental ASD-like condition was successfully modeled using albino Wistar rats (Mirza & Sharma, 2018). The timeline, groups, and parameters assessed for the present study are seen in Figure 1.

Group I—Control group- Male rats received 0.2M phosphate buffer saline (5 ml/kg, i.p) on post-natal 21st day, 22nd day and 23rd day; Group II and III — Ruthenium Red (R Red) per se: Rats were given R Red D1 (3 mg/kg, i.p.) and R Red D2 (6 mg/kg, i.p.) from the 24th post-natal day to the end of the study; Group IV—Propionic acid (PPA) group: Rats received three consecutive dose of propionic acid (250 mg/kg, p.o) on post-natal 21st day, 22nd day and 23rd day; Group V and VI —PPA+ R Red D1 and D2 group: Rats received propionic acid on post-natal 21st day, 22nd day and 23rd day followed R Red D1 (3 mg/kg, i.p.) and R Red D2 (6 mg/kg, i.p.) from post-natal 24th day to till of the study (Figure 1).

Anxiety

We assessed anxiety on postnatal day 39th. Anxiety-related behavior changes have been reported in PPA-induced autism. (Kumar & Sharma, 2016b; Mirza & Sharma, 2018). The EPM equipment was built of wood and had four arms that were 90 degrees apart. A 40-centimeter-high wall surrounds two closed arms and two open arms measuring 50×10 cm with a 50-centimeter-high wall above its base. All experimental rats were held in a pre-test arena for 5 minutes apiece to allow them to explore the maze. The animals were quickly transported and released in the maze's center, pointing to the open arm. For 5 minutes, each arm's entries and time taken time were carefully recorded. The tension between both two maze components, namely the open arms, which are aversive, bright, and exposed, and the closed arms, which are covered, dark, and protected, forms the basis for this test. The count of close and open arm entries, as well as time taken in close and open arm, were all measured to determine the % entries and time taken in open arm with the below Equation 1.

1.

$$\%Time\ spent\ "in\ "\ open\ arm=$$

$$\frac{Time\ spent\ "in\ "\ open\ arm}{time\ spent\ "in\ "\ open\ arm+Time\ spent\ "in\ "\ closed\ arm}\times100$$

$$\%Open\ arm\ entries=$$

$$\frac{Number\ of\ entries\ "in\ "\ open\ arm}{Closed\ arm\ entries+open\ arm\ entries}\times100$$

Social interaction assessment

On a postnatal day 40th, social interaction was evaluated by three-chamber sociability and novelty test apparatus (30 cm long and 70 cm wide divided into three identical chambers) for the rat. The test arena was di-

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vided into three equal chambers, each with an entrance point, and measured 76×30×35 cm. Animals were granted free access to all three chambers, and each trial started with the animal being placed in the apparatus's middle chamber. Animals that were to be placed beneath a wire cage spent 30 minutes under the wire cage for acclimation 24 hours before the commencement of the sociability phase. To stimulate exploration of the side chambers, all rats were assimilated to the equipment for 5 minutes before the start of the test trial. The rats were subjected to a 10-minute sociability test after the habituation phase was over. During the sociability phase, a stranger rat was placed under a wired cage on either the right side or left side chamber, while a vacant cage was held on the opposite side. The arrangement of wired cages was randomized to eliminate side preferences, and the stranger animal chamber and the empty cage were dubbed stranger chamber and empty chamber, respectively. Time taken in empty and stranger chambers by rats was noted during the sociability phase trial. Following the sociability phase was completed, the social preference phase began after the last animal trial. During the social preference phase, each animal was given 10 minutes to examine the entire arena. During this phase, the rat that had previously been considered a stranger was now made familiar, and now another novel rat stayed in the paradigm alongside the familiar rat. The two chambers are now known as the familiar and novel chambers. The test rat's time spent in novel and familiar chambers was recorded. The Equation 2 below was used to calculate the sociability index (SI) and social preference index (SPI) (Kumar et al., 2015; Kumar & Sharma, 2016a, b).

$$2. \quad SI = \frac{Total\ time\ in\ stranger\ chamber}{Total\ time\ in\ empty\ chamber}$$

$$SPI = \frac{Total\ Time\ in\ novel\ chamber}{Total\ time\ in\ familiar\ Chamber}$$

Repetitive behavior assessment

Repetitive behavior is measured through the y maze by observing the percentage of spontaneous alternations (Kumar et al., 2015; Mirza & Sharma, 2018). A decrease in spontaneous alternations reported an indication of increased stereotype or repetitive behavior. The rats were exposed to the starting arm during an 8-minute trial with free exploration in the three arms of the maze. The number of spontaneous alternations made was calculated using the succession of arm entries. As the rat entered each of the three independent arms, the alternation was counted. The number of % spontaneous alternations was determined as follows (Equation 3):

$$3. \quad \%Spontaneous\ alternation = \frac{Total\ alternations}{Total\ Arm\ entries\text{-}2} \times 100$$

Preparation for biochemical assessments

On postnatal day 50th, a high dose of thiopental sodium (90 mg/kg; i.p) was used to isolate the PFC tissue by anaesthetization. The homogenate supernatant was collected for various biochemical estimates as defined in the procedures below. Absorption was taken with a spectrophotometer (PerkinElmer). The key brain parts engaged in ASD etiology and neurochemical abnormalities include the prefrontal cortex, hippocampus, and cerebellum (Chauhan et al., 2011; Kumar & Sharma, 2016 b). As a result, we chose the cerebellum and prefrontal cortex brain parts for this investigation.

Oxidative stress assessments

As oxidative stress markers, gutathione (GSH) and lipid peroxidation (TBAR) were assessed in the prefrontal cortex in this investigation.

Glutathione (GSH)

The quantitative estimation of the reduced form of GSH was assessed using a spectrophotometer (PerkinElmer) at 412 nm (Kumar et al., 2015). In a test tube, PFC supernatant and trichloroacetic acid (10 percent w/v) were combined at 1:1 proportion and the test tubes were centrifuged (10 min;1000 g at 4°C) allowing the supernatant (0.5 mL) to mix with 0.3 M Na_2HPO_4 (2 mL). The spectrophotometric absorbance was taken at 412 nm after adding 25 mL of freshly produced Ellman's reagent (Ellman's reagent dissolved in 1 percent w/v sodium citrate). The reference curve was drawn using 10-100 M of the glutathione (reduced form). To express GSH, micromoles/mg protein units were used.

Lipid peroxidation

Lipid peroxidation was assessed as close to our earlier investigations (Kumar et al., 2015). The brain part (PFC) supernatant sample (0.2 mL) was taken in a test tube. In addition, 8.1 percent $CH_3(CH_2)11SO_4Na$ (0.2 mL), 30 percent CH_3COOH at pH 3.5 (1.5 ml), and 0.8 percent $C_4H_4N_2O_2S$ (1.5 mL) were added, followed by volume built up to 4 mL with water and incubation at 95°C for 1 hour. The liquid was cooled and then distil water (1 mL) was added, followed by the n-butanol-pyridine combination (15:1 v/v; 5 mL). The centrifuge tubes were handled at 4000 g for 10 min. $CH_2(CH(OCH_3)_2)_2$(1-10 nM) was used for plotting of standard calibration curve. The results were demonstrated using nanomoles per milligram of protein.

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Neuroinflammation and neurotrophin

Elisa method for tissue necrosis factor-α (TNF-α), iterleukin-6 (IL-6), interleukin-10 (IL-10), cAMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF)

These were assessed by the enzyme-linked immune-sorbent assay-sandwich method in the PFC and hippocampus area of the brain. The rat ELISA kits (RayBio®) were used to measure the levels of TNF-, IL-6, IL-10, CREB, and BDNF. The procedure mentioned in the product information leaflet was completely followed and samples were run in triplicate for optical density measurement, average optical density was considered for the final calculation of concentration. Briefly, IL-6, IL-10, TNF-α, BDNF, and CREB quantitation was made in PFC supernatant at 450 nm on 96-well percolated to specific antibody plates. Supernatant and standard were taken out into the well. The immobilized antibody was bound with TNF-α, IL-6, IL-10, CREB, and BDNF present in the sample, respectively. The tubes were washed, and the plates were coated with biotinylated anti-Rat TNF-α, IL-6, IL-10, CREB, and BDNF antibodies. The well was washed to eliminate unbound biotinylated antibodies. The wells were filled with HRP-conjugated streptavidin. After washing, the wells were treated with a TMB substratum solution. TNF-α, IL-10, IL-6, CREB, and BDNF were all linked in a blue color creation in the sample. When the stop solution was pipette into the wells, the color changed to yellow. Results were expressed in pg/mL and ng/mL (Mirza and Sharma, 2018).

Permeability of the blood-brain barrier (BBB) and water content

As stated earlier, the permeability of BBB and water content were estimated using the levels of Evans blue in the cerebellum (Manaenko et al., 2011; Kumar et al., 2015b). In a brief, the rats were administered 4% dye/Evans blue (4 mL/kg; intra-peritoneal) and left to circulate for 2 hours. Before collecting the samples, the rats (anesthetized) were trans-cordially perfused with the saline to excrete out the remaining dye in the vessels. Weighed cerebellum for quantitative spectroscopic calculation. In short, the cerebellum was precisely measured. The dye was extracted from the cerebellum using a homogenizer at pH 7.4 in 3.5 mL of 0.1 mol/L phosphate buffer saline. Then 6 mL of 60% trichloroacetic acid was added for protein precipitation. After cooling for half an hour, the processed cerebellum was vortexed for two minutes. The cerebellar tissue was processed for 40 minutes through a centrifuge at 4000 rpm to obtain pellets. Using a spec-

trophotometer, the dye levels were determined at 610 nm. The results were estimated through an Evans blue standardized curve of µg of Evans blue/g of cerebellar tissue. For 48 hours wet-weighted cerebellum tissue was put in the oven at 105°C weighed out after 48 hours of dry cerebellum. The wet-dry method was used to calculate the volume of water in the cerebellum (Manaenko et al., 2011). The water content was estimated as follows (Equation 4):

$$4. \ \%Water\ content = \frac{wet\ weight - dry\ weight}{wet\ wet} \times 100\%$$

Statistical analysis

For statistical analysis, Sigma statistical software, version 3.5 was employed. The results were presented in the form of a Mean±SD. All variables' statistics were examined with a two-way analysis of variance (ANOVA) and a Bonferroni post-test. The results were scrutinized as statistically significant at $P < 0.05$.

3. Results

Anxiety

PPA recorded a substantial decrease in open arm entries ($F_{(1, 42)} = 693.858$, $P < 0.05$) and time spent ($F_{(1, 42)} = 218.038$, $P < 0.05$) relative to control animals. Treatment with R Red substantially decreased PPA administered drop in open arms entries ($F_{(2, 42)} = 123.074$, $P < 0.05$) and time spent ($F_{(2, 42)} = 159.8$, $P < 0.05$) (Figure 2).

Social behavior

Sociability, sociability index, social preference, and social preference Index

PPA has decreased time went through in stranger chamber ($F_{(1, 42)} = 106.277$, $P < 0.05$) and expanded time went through in empty chamber ($F_{(1, 42)} = 228.979$, $P < 0.05$), this reveals that PPA-treated rats are less sociable. The use of R Red in PPA-exposed animals highly reduced the time went through in the stranger chamber ($F_{(2, 42)} = 31.543$, $P < 0.05$) followed by an increase in time went through in the empty chamber ($F_{(2, 42)} = 68.632$, $P < 0.05$). PPA-treated rats have shown a lower sociability index ($F_{(1, 42)} = 502.727$, $P < 0.05$) when compared to control rats, which was markedly attenuated by R Red ($F_{(2, 42)} = 131.026$, $P < 0.05$) (Figure 3).

PPA administration has decreased time went through the novel chamber ($F_{(1, 42)} = 1477.037$, $P < 0.05$) and expand time went through the familiar side of the chamber ($F_{(1, 42)} = 502.086$, $P < 0.05$), in contrast to control rats,

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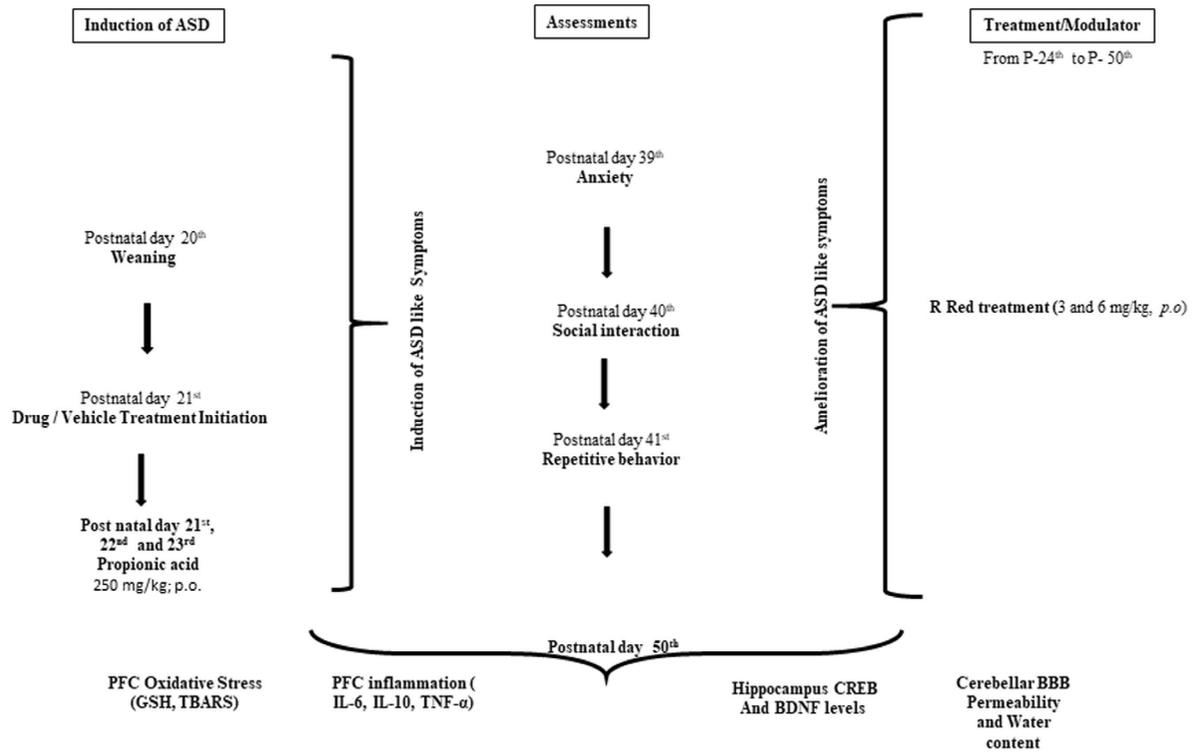
Figure 1. Diagram of the experimental technique

Abbreviations: E: Embryonic day; PFC: Prefrontal cortex; P: Postnatal day; PPA: Propionic acid; R Red: Ruthenium Red; D1: Dose 1; D2: Dose 2.
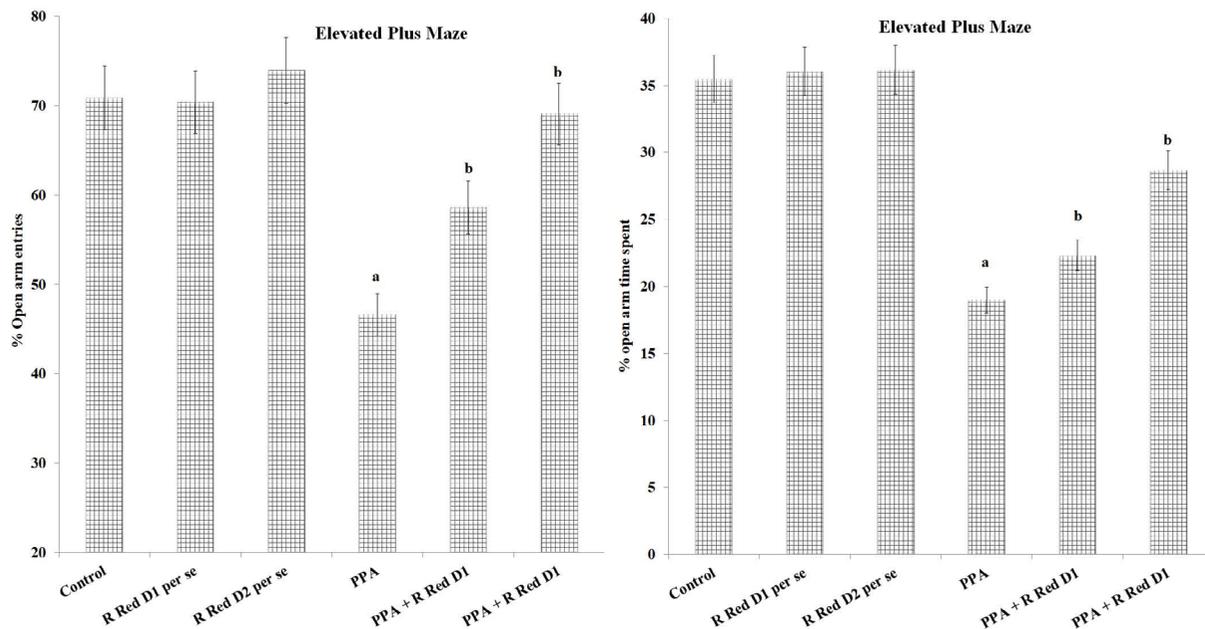
Figure 2. %open arm entry and %open art time spent on elevated plus maze

The statistics is presented with Mean±SD, two-way ANOVA with Bonferroni's post-test. [a]$P<0.05$ vs control rats, [b]$P<0.05$ vs PPA treated rats.

Abbreviations: PPA: Propionic acid; R Red: Ruthenium Red; D1: Dose 1; D2: Dose 2.

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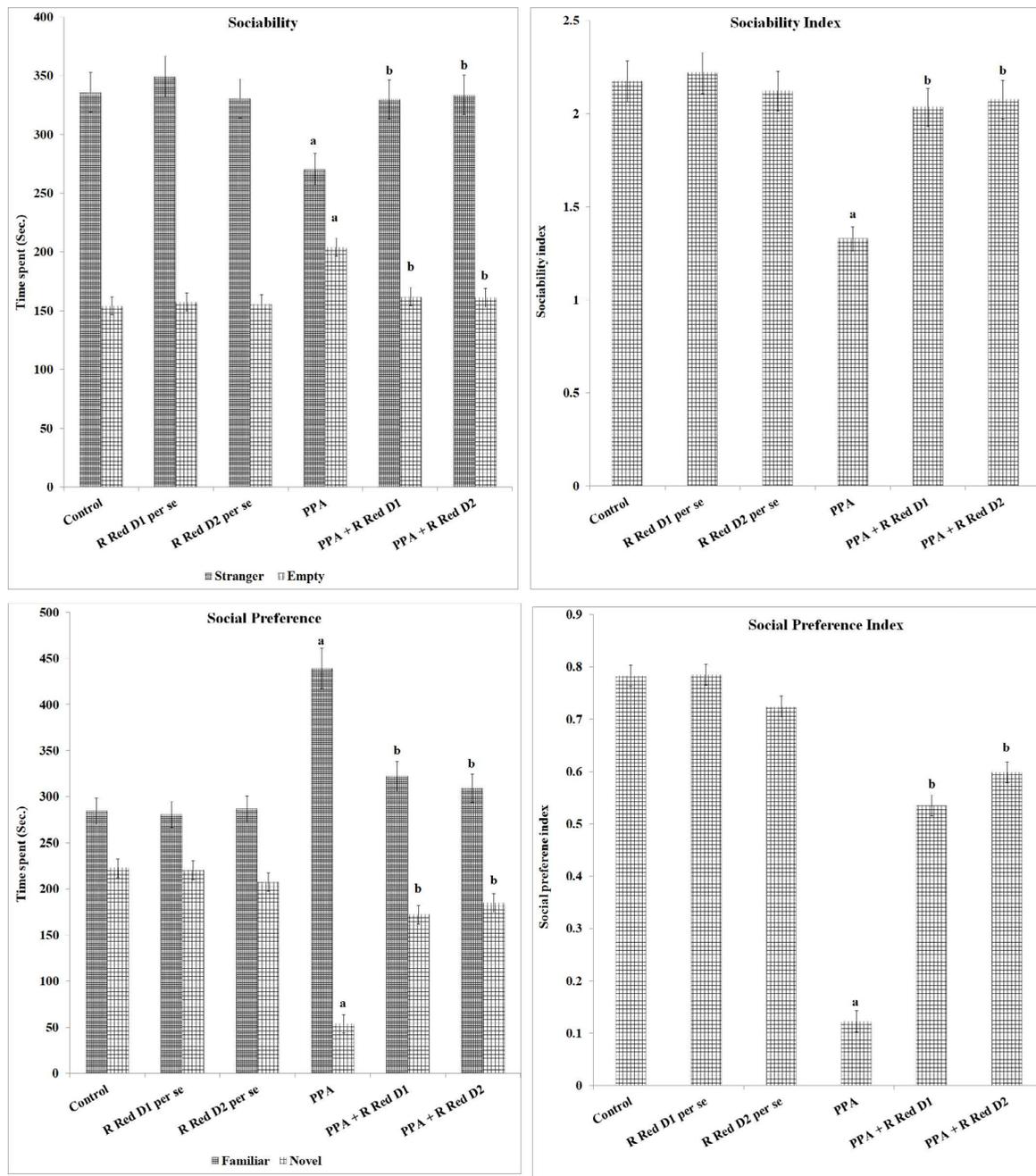
Figure 3. Sociability, sociability index social preference and social preference index on three-chamber sociability and Novelty test apparatus

The statistics is presented with Mean±SD, two-way ANOVA followed by Bonferroni's post-test [a]P<0.05 vs control rats, [b]P<0.05 vs PPA treated rats.

Abbreviations: PPA: Propionic acid; R Red: Ruthenium Red; D1: Dose 1; D2: Dose 2.

it reveals that PPA-treated rats have a decreased social preference. The use of R Red has significantly mitigated PPA associated reduction of time went through the novel chamber ($F_{(2, 42)}$=421.444, P<0.05) and increased time went through the familiar chamber ($F_{(2, 42)}$=60.493, P<0.05) (Figure 3). PPA-treated rats have shown a lower social preference index ($F_{(1, 42)}$=2585.419, P<0.05) in

contrast to control rats, which was significantly mitigated by R Red ($F_{(2, 42)}$=444.524, P<0.05).

Repetitive behavior

PPA exposure has considerably reduced the % spontaneous alteration in rats ($F_{(1, 42)}$=623.665, P<0.05). This

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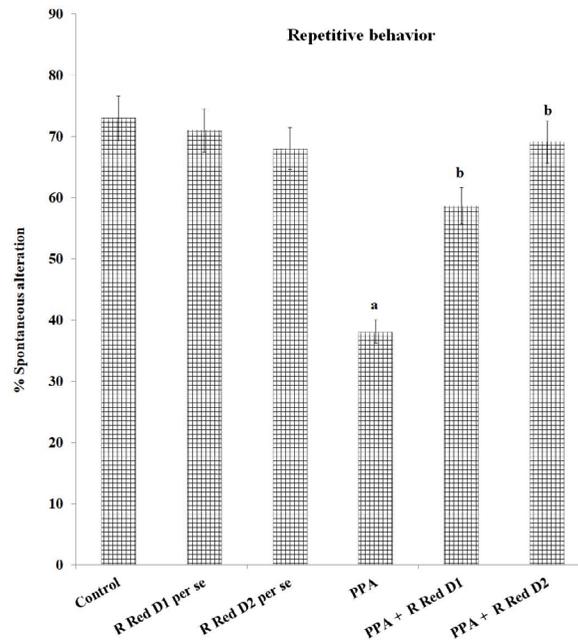
Figure 4. Repetitive behavior measured as % spontaneous alteration on Y maze

The statistics is presented with Mean±SD, two-way ANOVA with Bonferroni's post-test.

[a]$P<0.05$ vs control rats, [b]$P<0.05$ vs PPA treated rats.

PPA - Propionic acid; R Red – Ruthenium Red; D1- dose 1; D2- dose 2.

shows the repetitive behavior of PPA-exposed rats. Treatment with R Red suggestively mitigates PPA associated decreased % spontaneous alteration ($F_{(2, 42)}=274.682$, $P<0.05$) (Figure 4), which implies a decrease in PPA-induced repetitive behavior.

PFC Neuro-inflammation, oxidative stress, and hippocampus CREB and BDNF

PPA has increased PFC inflammation [IL-6 levels ($F_{(1, 18)}=973.189$, $P<0.05$), TNF-α levels ($F_{(1, 18)}=1105.281$, $P<0.05$) and decreased IL-10 levels ($F_{(1, 18)}=219.928$,
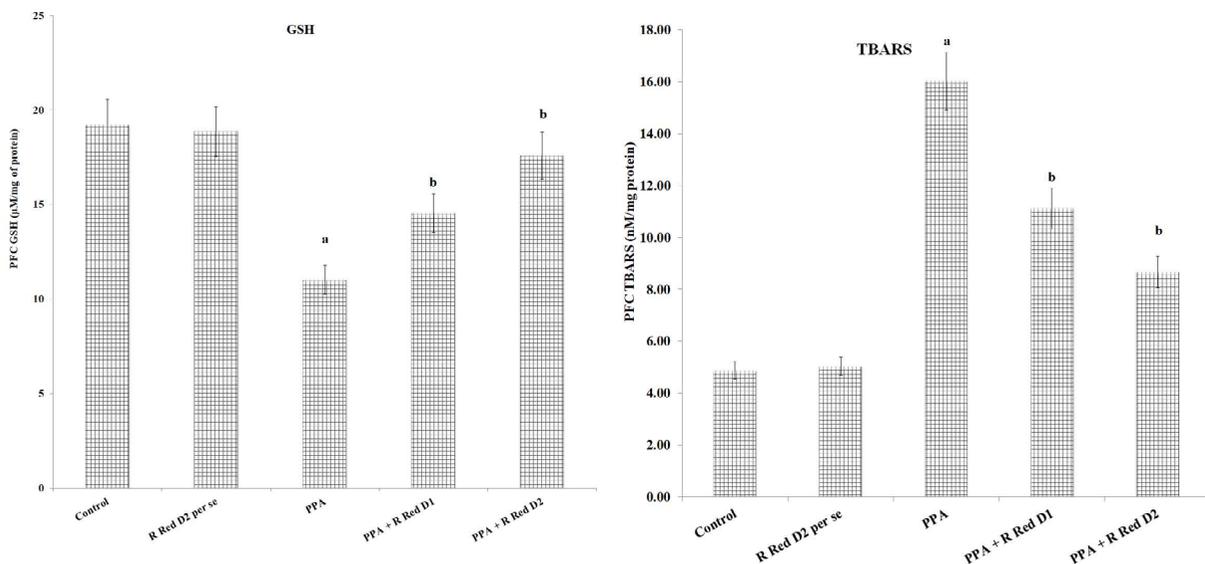
Figure 5. Prefrontal cortex oxidative stress and effect of various agents

The data is presented with Mean±SD, two-way ANOVA followed by Bonferroni's post-test. [a]$P<0.05$ vs control rats, [b]$P<0.05$ vs PPA treated rats.

Abbreviations: PPA: Propionic acid; R Red: Ruthenium Red; D1: Dose 1; D2: Dose 2.

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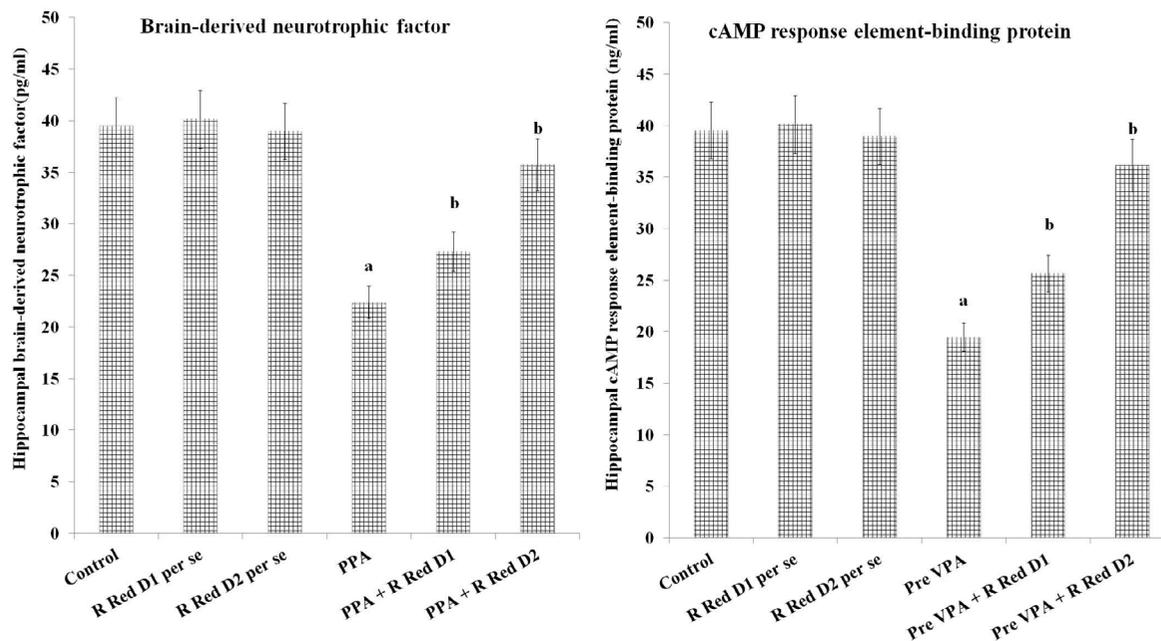
Figure 6. Hippocampal brain-derived neurotrophic factor (BDNF) and cAMP response element-binding protein (CREB) and effect of various agents

The data is presented with Mean±SD, two-way ANOVA followed by Bonferroni's post-test.

[a]P<0.05 vs control rats, [b]P<0.05 vs PPA treated rats.

PPA - Propionic acid; R Red – Ruthenium Red; D1- dose 1; D2- dose 2.

P<0.05)], oxidative stress [decrease GSH levels ($F_{(1, 18)}$=163.265, P<0.05) and increased TBARS levels ($F_{(1, 18)}$=1257.099, P<0.05)] along with decreased hippocampus CREB ($F_{(1, 18)}$=476.678, P<0.05) and BDNF ($F_{(1, 18)}$=199.643, P<0.05). R Red treatment has significantly mitigated PPA induced increased, inflammation [IL-6 levels ($F_{(2, 18)}$=84.89, P<0.05), TNF-α levels ($F_{(2, 18)}$=364.755, P<0.05) and decreased IL-10 levels ($F_{(2, 18)}$=138.116, P<0.05), oxidative stress [decrease GSH levels ($F_{(2, 18)}$=685.007, P<0.05) and increased TBARS levels ($F_{(2, 18)}$=175.039, P<0.05) along with decreased hippocampus CREB ($F_{(2, 18)}$=29.509, P<0.05) and BDNF ($F_{(2, 18)}$=78.896, P<0.05) (Figure 5, Figure 6, and Figure 7).

Permeability of BBB and water content

A considerably higher Evans blue concentration ($F_{(1, 18)}$=971.701, P<0.05) and water content ($F_{(1, 18)}$=181.92, P<0.05) were present in the cerebellum of PPA-exposed rats in contrast to control rats. Treatment with R Red expressively mitigates PPA-induced increased amount of Evans blue ($F_{(2, 18)}$=163.993, P<0.05) and water content in the cerebellar tissue ($F_{(2, 18)}$=94.856, P<0.05) (Figure 8), which suggest amelioration in PPA induced BBB dysfunction.

4. Discussion

In the current investigation, we used PPA to generate autism-like symptoms in rats. PPA-exposed rats resulted in social behavior dysfunction, anxiety, and repetitive behavior. Also, PPA rats showed an increase in PFC TBARS, IL-6, TNF-α, Cerebellum Evans blue concentration, and water content. On the other hand, PPA rats demonstrated a decrease in IL-10, GSH, CREB, and BDNF levels. We also assessed the impact of ruthenium red in PPA-exposed rats. Ruthenium Red was found to mitigate the effect of PPA in rats.

Calcium-stimulated second messenger pathways can trigger the CREB, which downstream to BDNF but calcium excitotoxicity could reverse this pathway. The CREB/BDNF signaling pathway is critical in synaptic plasticity, morphogenesis, and behavioral conduct (Chen et al., 2012; Wang et al., 2014). Phospho-CREB controls the transcription of neuroprotective proteins like BDNF. In the brain, BDNF governs synapse development and synaptic strengthening. Reduction in BDNF level causes abnormal plasticity, learning, and memory formation (Tye & Bolton, 2013; Uutela et al., 2012) Fmr1 knockout (KO. According to research, lowering p-CREB levels can contribute to spatial memory impairment (Bourtchuladze et al., 1994) a cellular model of

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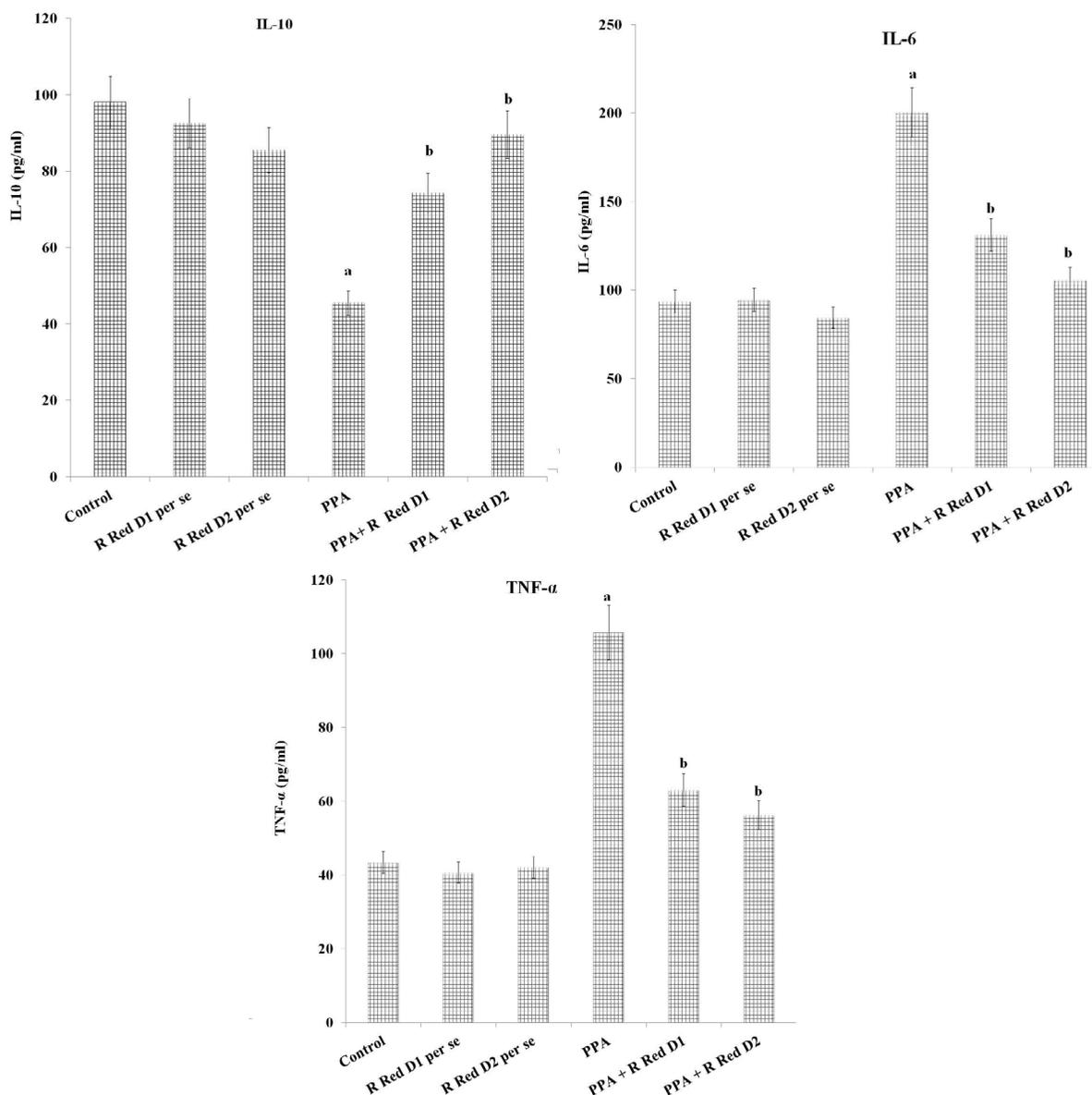
Figure 7. Prefrontal cortex inflammation (IL- 6, IL-10 and TNF-α levels) and effect of various agents

The data is presented with Mean±SD, two-way ANOVA followed by Bonferroni's post-test.

[a]$P<0.05$ vs control rats, [b]$P<0.05$ vs PPA treated rats.

Abbreviations: PPA: Propionic acid; R Red: Ruthenium Red; D1: Dose 1; D2: Dose 2, IL-6: Interleukins 6; IL-10: Interleukins10; TNFα: Tumor necrosis factor-α.

memory in Aplysia. Our studies with fear conditioning and with the water maze show that mice with a targeted disruption of the α and δ isoforms of CREB are profoundly deficient in long-term memory. In contrast, short-term memory, lasting between 30 and 60 min, is normal. Consistent with models claiming a role for long-term potentiation (LTP. Double corten, dendritic growth, production of the neuronal microtubule-associated protein, and BDNF have all been linked to CREB activity. Autistic traits such as poor synaptic plasticity and neuron survival could emerge as a result of this (Jagasia et al., 2009). We found a significant drop in CREB and BDNF concentration in the hippocampus tissue of PPA-exposed rats, which was consistent with other studies (Wu et al., 2017). Ryanodine receptors (RyR) regulate calcium-linked calcium release, which in turn regulates activity-dependent calcium inflow. Calcium signals in hippocampus neurons' postsynaptic pathways drive learning and memory, and synaptic plasticity. R Red treatment also alleviated behavioral impairment in PPA rats, as well as raising p-CREB and BDNF levels.
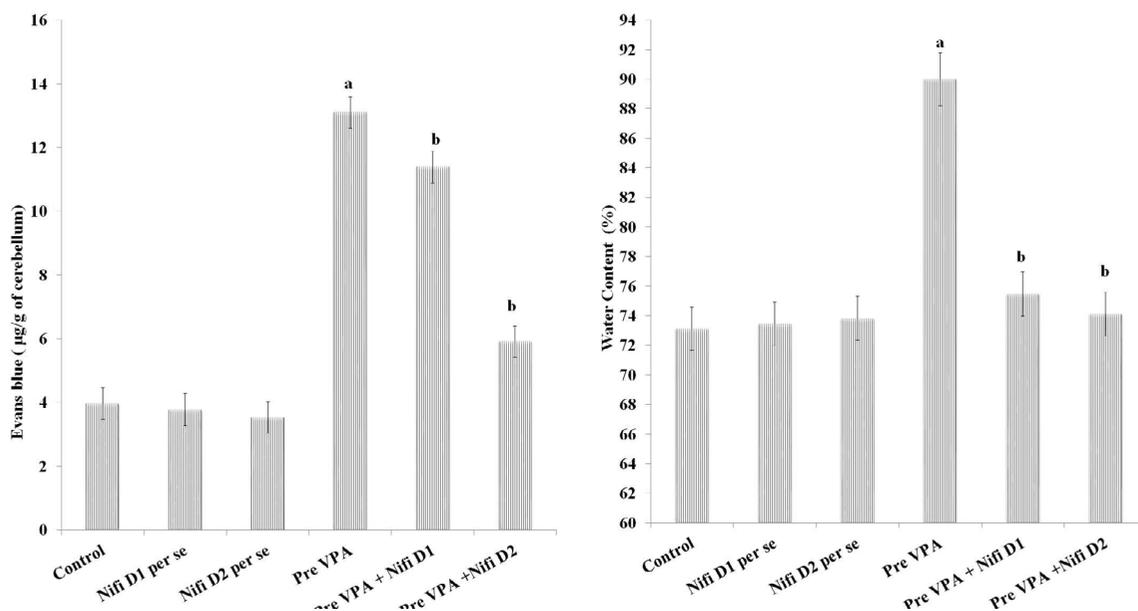
Figure 8. Permeability of BBB assessed by Evans blue concentration and water content in cerebellum and effects of drugs.

The results are representing with Mean±SD, two-way ANOVA followed by Bonferroni's post-test. [a]P<0.05 vs control rats, [b]P<0.05 vs PPA treated rats. The cerebellum of PPA-exposed rats showed more staining than control rats, whereas the cerebellum of R Red-exposed rats showed less staining than PPA rats.

PPA: Propionic acid; R Red: Ruthenium Red; D1: Dose 1; D2: Dose 2; BBB: Blood Brain Barrier.

PPA contains anti-inflammatory and pro-inflammatory cytokine modulatory effects, however IL-6, IL-10, and TNF- are connected to ASD, neuroinflammation, and other disorders (Abdelli et al., 2019). In rats treated with PPA, we discovered higher levels of IL-6, TNF-, and lower levels of IL-10, suggesting neuroinflammation in rats with PPA-induced ASD. PPA treatment boosts glial cell proliferation at the initial of stem cell development, similar to the neuro-inflammation reported in post-partum autism brains (Abdelli et al., 2019). PPA treatment triggered microgliosis, astrogliosis, and the release of cytokines like TNF- and IL-6 (MacFabe et al., 2011). PPA modulates PTEN/AKT pathway followed by results in neuroinflammation and disturbed neurocircuitry in autism (Abdelli et al., 2019). In PPA-induced ASD symptoms, the specific process concerning PPA-induced alterations in IL-6, IL-10, and TNF- remains unknown. In contrast, the relationship between these neuroinflammatory indicators and ASD behaviors has already been extensively researched in a variety of autism studies (Choi et al., 2020; El-Ansary et al., 2012; MacFabe et al., 2011). According to Wei and colleagues, IL-6 increases the creation of excited synapses in the cerebellum of autistic subjects and rodents. In mice and autistic patients, higher IL-6 levels affect the excitatory and inhibitory (E / I) balance by increasing glutamate excitatory neurotransmission (Wei et al., 2011, 2012). Anxiety, social deficiencies, repeated behavior, and hy-

peractivity have all been associated with E/I imbalances (Kim et al., 2014; Kumar et al., 2015; Wei et al., 2012). In rodents, neuroinflammation of the prefrontal cortex has resulted in elevated anxiety and decreased social behavior (Codagnone et al., 2015) and ameliorated using anti-IL-6 antibodies treatment (Smith et al., 2007). Clinically, low anti-inflammatory marker (IL-10) levels along with higher pro-inflammatory marker levels (IL-6 & TNF-α) are correlated with key autism symptoms such as repetitive behavior and social deficits (Ross et al., 2013). The IL-10 induces down-regulation of microglial pro-inflammatory pathways followed by anti-inflammatory action. While one of the main inflammatory features related to reduced IL-10 is increased inflammatory tracts of the microglia (Norden et al., 2014). As a result, PPA-related autistic symptoms may be connected to neuroinflammation caused by elevated TNF-α and IL-6 levels in the brain (El-Ansary et al., 2012; MacFabe et al., 2007, 2008). Neuro-inflammation impairs the plasticity of dendritic spines and morphology responsible for cognitive function (Zou et al., 2016). Few studies have shown that ryanodine receptor antagonists decrease inflammatory markers such as interleukin 2, and TNF-α and increase the anti-inflammatory markers such as IL-10 (Dadsetan et al., 2008; López-Neblina et al., 2007) including the inflammatory response after ischemia and reperfusion. The pharmacological mechanism of dantrolene is associated with the inhibition of the release of

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Ca^{2+} from the skeletal muscle sarcoplasmic reticulum (SR. Alternatively, ryanodine receptor antagonists efficiently prevent cell death by blocking calcium mobilization (Luciani et al., 2009). Previously, it was discovered that ryanodine antagonists limit calcium inflow through the NMDA receptors and reverse glutamate-induced excitability (Mody, 1995; Makarewicz et al., 2003). Impaired BBB permeability was believed to be essential for cognitively impaired vascular function (Taheri et al., 2011). As a result, we investigated the BBB's permeability using Evans Blue. BBB dysfunction is caused by the release of ryanodine-connected intracellular calcium via the endoplasmic reticulum (Kuhlmann et al., 2009). Higher levels of neuroinflammatory markers may modulate social interaction, repetitive behavior, and anxiety, as well as BBB function, in rats with PPA-mediated autism, according to the above lines.

In both clinical and experimental investigations, oxidative stress is one of the causes of autistic characteristics (Fry et. al., 2011; Nadeem et al., 2018; Rossignol & Frye, 2014). Glutathione deficiency, as well as other linked antioxidant mechanisms like the redox system, the methionine cycle, and transculturation, have been found in autistic individuals (James et al., 2004). The cerebellar damage along with abnormal glutathione redox status is associated with communication problems and social deficits in rodents and autistic subjects (Frye et al., 2013; Morakotsriwan et al., 2016) most individuals with ASD and MD do not have a specific genetic mutation to explain the MD, raising the possibility of that MD may be acquired, at least in a subgroup of children with ASD. Acquired MD has been demonstrated in a rodent ASD model in which propionic acid (PPA. Oxidative homeostasis dysregulation induced by Wnt/-catenin pathway stimulation was observed to affect sociability, repetitive behavior, and anxiety in rats (Zhang et al., 2012). Ryanodine antagonist reverses increased oxidative stress signals in different brain conditions (Crouzin et al., 2007; Singh & Sharma, 2016) we have studied whether this \"hyposensitivity\" to oxidative stress could be linked to an altered Ca^{2+} homeostasis. Fe^{2+} ions triggered a Ca^{2+} entry which was required for Fe^{2+} ion-induced toxicity. This influx was sensitive to blockers of TRP-like nonspecific Ca^{2+} channels, including Ruthenium Red, La^{3+}, and Gd^{3+} ions which also prevented the Fe^{2+} ion-induced toxicity and oxidative stress as revealed by protein carbonylation status. The pretreatment with α-tocopherol resulted in a reduction of the Ca^{2+} increase induced by Fe^{2+} ions and masked the blocking effect of La^{3+} ions. Moreover, such a pretreatment reduced the capacitive Ca^{2+} entries (CCE. Endogenous oxidative stress markers are reversed by ryanodine receptor antagonists, followed by lipid peroxidation product content (TBARS), and therefore oxidative stress is alleviated (Jain & Sharma, 2016) we have reported the induction of vascular dementia by experimental diabetes. This study investigates the efficacy of a ruthenium red, a ryanodine receptor antagonist and pioglitazone in the pharmacological interdiction of pancreatectomy diabetes (PaD. The ryanodine receptor's antagonistic activity has been found to reduce the production of reactive oxygen species caused by glycation end products (Kuhlmann et al., 2009). The above line of discussion that may conclude, ruthenium red possibly modulates the endogenous antioxidant pathways and results in decreased oxidative stress in PPA-exposed rats.

Autism has been linked to BBB disruption, which reinforces the gut barrier concept. Postmortem of the cerebellum of ASD altered gene expression responsible for BBB integrity (Fiorentino et al., 2016). Interestingly, in our previous research, Pre- VPA exposed rats were found with increased BBB permeability measured by Evans blue leakage (Kumar et al., 2015b). Altered cytokines levels, endogenous redox mechanism, mitochondrial function, and elevated neurodermatitis levels may contribute to BBB dysfunction (Dong et al., 2019; Sorby-Adams et al., 2017) encompassing traumatic brain injury (TBI. Increased oxidative stress may be responsible for the increased BBB permeability (Lochhead et al., 2010). The intra-cellular tight junctions between neighboring cells determine BBB permeability and both low and high amounts of Ca^+ have been found to have a deleterious effect on these endothelial junctions. Collective intracellular Ca^+ levels have been shown to affect in tight junction structures through apoptosis and protein kinase C-alpha (Rakkar & Bayraktutan, 2016). PPA can modify the BBB permeability via changes in the neuroinflammatory and oxidative stress pathways. Ruthenium red modulates water homeostasis and blood-brain barrier permeability possibly via maintaining Ca^+ homeostasis within the brain.

Based on the preceding discussion, ruthenium red, followed by GSH, CREB, and BDNF, could alleviate PPA-induced significant decreases in social behavior. Furthermore, ruthenium red was found to counter PPA-associated increases in anxiety, PFC TNF-α, IL-6, IL-10, and TBARS. PPA increased BBB permeability and water content, which was ameliorated by ruthenium red treatment in rats. There is a need for more research into the role of ryanodine receptor antagonists in autism-like conditions.

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Ethical Considerations

Compliance with ethical guidelines

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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