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Remediate Effect of Ryanodine Receptor Antagonist in Valproic-Acid Induced Autism

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Autism spectrum disorder (ASD) mainly diagnosed with social behavioral problems, lack of communication, social interaction, and repetitive behavior along with cognitive dysfunction. Ryanodine receptors are involved in various neurological and behavioral impairments in different conditions. The role of Ryanodine receptors has not been explored in experimental ASD. The present study explicates the role of ryanodine receptor antagonist; ruthenium red (RR) in prenatal valproic acid (Pre-VPA) administered experimental ASD phenotypes. Three chamber social behavior, Y-Maze were utilized to assess social interaction, spontaneous alteration, respectively. Hippocampus and Prefrontal cortex (PFC) were utilized for various biochemical assessments, whereas cerebellum was used for assessments of blood brain barrier (BBB) permeability. Pre-VPA rats showed reduction in spontaneous alteration, social interaction. Pre-VPA administration were decreased PFC levels of IL-10, and GSH along with hippocampus cAMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF). Also, the animals have shown increase in PFC levels of IL-6, TNF-a, TBARS, Evans blue leakage and water content. Daily administration of R Red considerably diminished Pre-VPA administered reduction in spontaneous alteration, social interaction, CREB, BDNF and increase in inflammation, oxidative stress, BBB permeability. Conclusively, Pre-VPA has induced autistic phenotype, which were attenuated by ryanodine receptor antagonist. Ryanodine receptor antagonists may further test for their pharmacological effects in ASD phenotypes.

Keywords: Blood Brain Barrier; CREB; IL-6, BDNF; Ryanodine; Social Interaction.

A neurodevelopmental disorder, autism spectrum disorder (ASD) is currently being diagnosed with communication problems, repetitive behavior along with social behavior dysfunction. Shockingly, there is no affirmed treatment accessible till presently. This is following an upward example and overall budgetary weight. Males are increasingly inclined to ASD when contrasted with females. More than one percent of the populace overall is experiencing ASD[1]. Several environmental and genetic factors seem to be responsible but the exact cause still unknown. Autism has diverse genetic relations along with different environmental causes including infection, pesticides and drugs[2,3]. Valproic acid is a typically used antiepileptic drug and it is used to treat depressive disorders, migraine, anxiety, and neuropathic pain. Prenatal valproic acid (Pre-VPA)

treatment is one of the best characterized models of autism rats with solid choice, face and predictive validity[4]. Our lab and several researchers have reported that Pre-VPA exposure leads to neuro-inflammation, oxidative stress and mitochondrial dysfunction[5–9]. The autistic individuals have some genetic defects in calcium channel genes, which regulates the neurodevelopment[10].

Elevation of the concentration of cytosolic calcium induces the release of neurotransmitters at synaptic junctions, leads to dendritic function, regulates behavior-dependent changes in synaptic plasticity and gene expression[11]. Calcium homeostasis is generally regulated by various kind of calcium channels and their receptors. ryanodine receptor is one of the calcium Ruthenium red is being known as selective intracellular ryanodine receptor (RyR) antagonist. Antagonism of the RyR results into constructive effect in different brain conditions such as Alzheimer's disease and Huntington's disease[12–14]. It has been documented that antagonism of ryanodine receptor exert neuro-protective effect along with decreases infarct volume and decrease levels of intracellular calcium in brain[15]. Some previous studies also reported that ruthenium red gives useful impacts by declining brain inflammation[16] and oxidative stress[17]. The antagonistic role of ryanodine receptors in ASD still not explored. This manuscript explored the novel job of ryanodine receptors antagonist; ruthenium red in Pre-VPA administered autistic phenotypes in rodents.

MATERIALS AND METHODS

Animals

Adult albino wistar rats were kept in polypropylene cages in Amity University (Reg No. 1327/PO/ReBi/S/10/CPCSEA) at a temperature of 25 ± 2°C with relative humidity of 50 ± 5%. The animals had free access to water and standard laboratory pellet chow diet (Ashirwad Industries, Punjab, India). Animals were exposed to the natural light and dark cycle with 12 hours of light (starting at 07:00 hrs. and ending at 19:00 hrs.) followed by 12 hours of dark (starting at 19:00 hrs. and ending at 07:00 hrs.). The Institutional Animal Ethics Committee of Amity University, Uttar Pradesh, India, has approved all experiments.

Drugs chemicals and reagents

Analytical and laboratory grade chemicals and reagents were used in the present study. Sodium salt of valproic acid was taken from Sun Pharma Pvt. Ltd. Evans blue, ethylene glycol tetra acetic acid (EGTA) was purchased from SISCO Research Laboratory Pvt. Limited, Mumbai, India. Lowry's reagent, N-naphthylethylenediamine and 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma-Aldrich, India. Hydrogen peroxide and pyridine was obtained from Rankem Laboratories Pvt. India, Ltd.

Administration of drugs

Drugs and their solutions were prepared before the procedure. For autism induction, sodium valproate was dissolved in 0.9 % saline (3.3 ml/kg) and given single dose (500 mg/kg; *i.*p; on12.5th day of embryonic phase) to the pregnant female rats[7]. Ruthenium Red was dissolved in 0.9 % saline (3.3 ml/kg; i.p.) and given orally (1 or 3 mg/kg; from postnatal day 21st to 50th) between 9.00 and 18.00 h by oral gavage to the rats[18].

ASD induced by Pre-VPA

Pregnancy was identified by the existence of a vaginal plug on the embryonic day 1st. A single dose of sodium valproate with concentration of 500 mg/kg injected through intra-peritoneal route to pregnant females on embryonic 12.5th day received sodium valproate. These sodium valproate treated pregnant females were accommodated weaning of offspring (post-natal day 20) The only male offspring born were recruited for multiple tests for the analysis[6,19].

Protocol design

In total five groups of animals, with each group containing eight (n=8) animals were used for the protocol of the study. Choice of animals was based on research already published, effectively using albino Wistar rats to model experimental ASD like condition (7). The timeline, groups, and parameters assessed for present study are seen in Figure 1.

Fertilization was ascertained using a vaginal examination for the presence of sperm cells, this day was considered as gestational day 1. Pregnant dams were housed individually till the day of parturition and weaning of the pups. The pups were weaned and then distributed randomly into their respective groups on post-natal day 20[7,19].

Group I—Control group- Pregnant dams were injected with single dose of 0.9 % saline (3.3 ml/kg) on gestational 12.5th day. The received male pups were taken as control group. Group II and III — Ruthenium Red (R Red) D1 and D2 per *se*: Male pups born to untreated pregnant dams were randomly selected for these group, received R Red D1 and R Red D2 (3 mg/kg and 6 mg/kg, i.p.) respectively from post-natal (PND) 21st to PND 50th ; Group III—Prenatal valproic acid (Pre- VPA) group: Pregnant dams were injected with single dose of sodium valproate (500 mg/kg, *i.p;*) on gestational 12.5th day; Group IV and V —Pre-VPA + R Red D1 and D2 group: Males exposed to Pre-VPA were randomly selected and received R Red D1 (3 mg/kg, i.p.) and R Red D2 (6 mg/kg, i.p.) from PND 21st to PND 50th (Figure 1).

Social interaction assessment

On postnatal day 48th - 49th, social interaction was evaluated by Three-Chamber Sociability and Social Novelty Test apparatus (30 cm long and 70 cm wide which divided into three identical chambers) for the rat. We have used this mehod in our previous research adopted from Kim et al,. 2011 with slightly modification[20]. Concisely, the test arena measured 76 cm x 30 cm x 35 cm and was divided into three equal chambers with an access point between each chamber. Animals were given free access to all the chambers and Each trial started with the animal placed in the central chamber. To encourage exploration of the side chambers, all animals were accustomed to the apparatus for 5 min prior to starting of the test trial. After ending of the habituation period, the rats were tested in the sociability phase lasting for 10 min. Animals to be placed under a wire cage were spent 30 minutes at the wire cage, prior to initiation of the sociability phase. In the sociability phase, a stranger animal was placed under the wired cage in either (left or right) side chamber, while in the other chamber an empty cage would be placed. In order to avoid side preferences, the placement of wired cages was randomized and the chamber with the stranger animal and the empty cage were called as stranger chamber and empty chamber, respectively. Upon conclusion of the sociability phase the social preference phase was initiated 2 hrs after the last animal trial. In the social preference phase each animal was allotted 10 min to look at the complete arena. During this phase, the animal earlier considered as stranger, was now rendered familiar and introducing another novel animal into the paradigm, along with the familiar animal. The two chambers now would be called as familiar and novel chamber. The time spent by test animals in both the side chambers was measured. Sociability index (SI) and social preference index (SPI) were calculated according to the following formula[5,6,21].

$$SI = \frac{Total\ time \text{ in } stranger\ chamber}{Total\ time \text{ in } empty\ chamber}$$

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

Repetitive behavior assessment

Repetitive behavior measured through Y-Maze by observing the decrease in percentage spontaneous alternations[5]. The rats were exposed to the starting arm during an 8-minute trial with free exploration in the three arms of the maze. The series of Arm entries were noted down to calculate spontaneous alternations numbers made. The alternation was noted as the rat came successively into the three different arms. The following formula was used to calculate

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

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). Prefrontal cortex, hippocampus and cerebellum are the main brain regions implicated in ASD pathogenesis and neurochemical disorders[7,22] So, for this study, we selected prefrontal cortex and cerebellum brain regions.

Oxidative stress assessments

Glutathione (GSH) and lipid peroxidation (TBAR) are known as oxidative stress markers, so in this study, we evaluated these markers in prefrontal cortex[5,6]

Glutathione (GSH)

The quantitative estimation of reduced form of glutathione (GSH) was assessed using

spectrophotometer (PerkinElmer) at 412 nm[7]. In a test tube, PFC supernatant and trichloroacetic acid (10 per cent w / v) were combined at 1:1 ratio and the test tubes were centrifuged (1000 g at 4 ° C for 10 min). 0.3 M disodium hydrogen phosphate (2 mL) and supernatant (0.5 mL) is mixed. The spectrophotometric absorbance was observed at 412 nm following the addition of .25 mL of freshly prepared DTNB (DTNB dissolved in 1 percent w / v sodium citrate). 10-100 ìM of the reduced form of glutathione was used for plotting of standard curve. The effects of reduced glutathione consistent with mg of protein were expressed as micromoles.

Lipid peroxidation

Lipid peroxidation was assessed as close to our previous investigations[7]. The PFC supernatant sample (0.2 mL) was taken in test tube. In addition, 8.1 per cent sodium dodecyl sulphate (0.2 mL), 30 per cent acetic acid pH 3.5 (1.5 mL) and 0.8 per cent thiobarbituric acid (1.5 mL) were added, followed by distilled water up to 4 mL.. The test tubes were put in the incubator at 950C for 1 hour. The incubated mixture was then cooled by adding distilled water (1 mL) followed by adding n-butanol-pyridine mixture (15:1 v / v) to 5 mL. The centrifuge tubes were handled at 4000 g for 10 min. The spectrophotometric absorbance was taken on developing pink color. 1, 1, 3, 3-tetra methoxy propane (1-10 nM) was used for plotting standard calibration curve. Nano moles per mg of protein were used for expression of result.

Elisa Method for Iterleukin-6 (IL-6), interleukin-10 (IL-10) and tissue necrosis factor-á (TNF-á) , Brain-derived neurotrophic factor (BDNF) and cAMP response element-binding protein (CREB)

These were assessed by enzyme-linked immune-sorbent assay- sandwich method in PFC and hippocampus area of brain. The IL-6, IL-10, TNF-á, BDNF and CREB were estimated by using RayBio® Rat ELISA kits. 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 final calculation of concentration. Briefly, IL-6, IL-10, TNF-á, BDNF and CREB quantitation was made in PFC supernatant at 450 nm on 96-well percolated to specific antibodies plates. Supernatant and standard were taken out in to well. The immobilized antibody was bound with IL-6, IL-10, TNF-á, BDNF and CREB present in sample, respectively. Washing was given to the tubes, and the biotinylated anti-Rat IL-6, IL-10, TNF-á, BDNF and CREB antibodies were applied to the plates. Unbound biotinylated antibody was removed by washing the well. The HRP conjugated streptavidin was added to the wells. After washing a TMB substratum solution was applied to the wells. A blue color formation showed the IL-6, IL-10, TNF, BDNF and CREB have linked in sample. The stop solution was pipette to the wells which changed the color to yellow. Results were expressed in pg/ml and ng/ml[23].

Blood-brain barrier (BBB) permeability and water content

BBB permeability and water content performed by the concentration of Evans blue dye in the cerebellum as previously reported methods[6,24]. Concisely, 4% of dye /Evans blue (intra-peritoneal; 4 ml/kg) was injected to subject and was permitted to circulate for 2 hours. Before collecting the samples, the subjects (anesthetized) were trans-cordially perfused with saline to flush out the remaining dye in the vessels. Weighed cerebellum for quantitative spectroscopic calculation. In short, the cerebellum precisely measured. The cerebellum processed at pH 7.4 dye extraction in 3.5 mL of 0.1 mol / L phosphate buffer saline by a homogenizer. Then, for protein precipitation, 6 ml of 60 per cent trichloroacetic acid was added. This processed cerebellum was vortexed after 30 minutes of cooling for 2 minutes. The cerebellum was processed for 40 minutes through a centrifuge at 4000 rpm to obtain pellets. The dye levels were measured at 610 nm via a spectrophotometer. The findings were expressed using a standardized curve of Evans blue as ìg of Evans blue / g of cerebellum tissue. For 48 hours wet-weighted cerebellum tissue was put in the oven at 105 ° C. Weighted out after 48 hours of dry cerebellum. Wet-dry method was used to calculate the volume of water in the cerebellum. The percentage water content was calculated as: (wet weight - dry weight)/wet weight × 100%. and results shown in percentage of water content[24].

Statistical analysis

Sigma Stat (v3.5) was used for statistical analysis. The findings were demonstrated in the form of mean ± standard deviation. Statistics for

all variables were analyzed using two-way ANOVA followed by a post-test by Bonferroni. This at $p<0.05$ was considered statistically important.

RESULTS

Social behavior

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

Pre-VPA has decreased time went through in stranger chamber and expanded time went through in empty chamber, with contrast to control rats, which shows lower sociability in Pre-VPA treated rats. Administration of R Red has significantly mitigated Pre-VPA associated reduction of time went through in stranger chamber followed by increased time went through in the empty chamber. Pre-VPA treated rats, has shown lower sociability index when compared to control rats, which was markedly attenuated by R Red (Figure 2).

Pre-VPA administration has decrease time went through in novel chamber and expand time went through in the familiar chamber, with contrast to control rats, which shows lower social preference in Pre-VPA treated rats. Administration of R Red has significantly mitigated Pre-VPA associated reduction of time went through in novel chamber and increased time went through in the familiar chamber (Figure 2). Pre-VPA treated rats have shown lower the social preference index, contrast to control rats, which was significantly mitigated by R Red.

Repetitive behavior

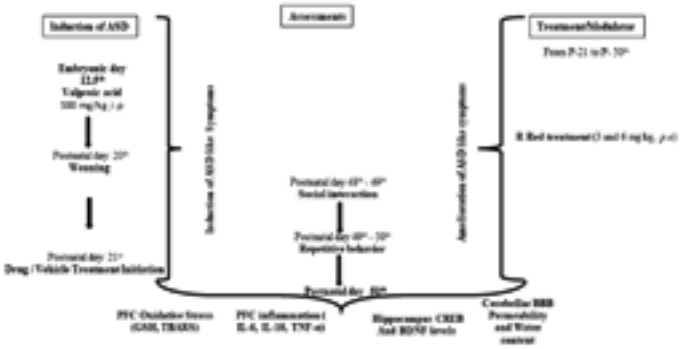
Pre-VPA treatment has considerably reduced the % spontaneous alteration. This shows repetitive behavior of Pre-VPA exposed rats. Treatment with R Red suggestively mitigate Pre-VPA associated decreased % spontaneous alteration (Figure 3), which suggests a decrease of Pre-VPA induced repetitive behavior.

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

Pre-VPA has increased PFC inflammation (IL-6 levels, TNF-á levels and decreased IL-10 levels), oxidative stress (decrease GSH levels and increase TBARS levels) along with decreased hippocampus CREB and BDNF. R Red treatment has significantly mitigated Pre-VPA induced increased PFC inflammation (IL-6 levels, TNF-á levels and decreased IL-10 levels), oxidative stress (decrease GSH levels and increase TBARS levels) along with decreased hippocampus CREB and BDNF (Figure 4, Figure 5 and Figure 6).

Blood brain barrier permeability and water content in the cerebellum

A considerably higher Evans blue concentration and water content were present in the cerebellum of Pre-VPA exposed rats in contrast to control rats. Treatment with R Red expressively mitigates Pre-VPA induced increased concentration of Evans blue and water content in the cerebellum (Figure 7), which suggest amelioration in Pre-VPA induced BBB dysfunction.

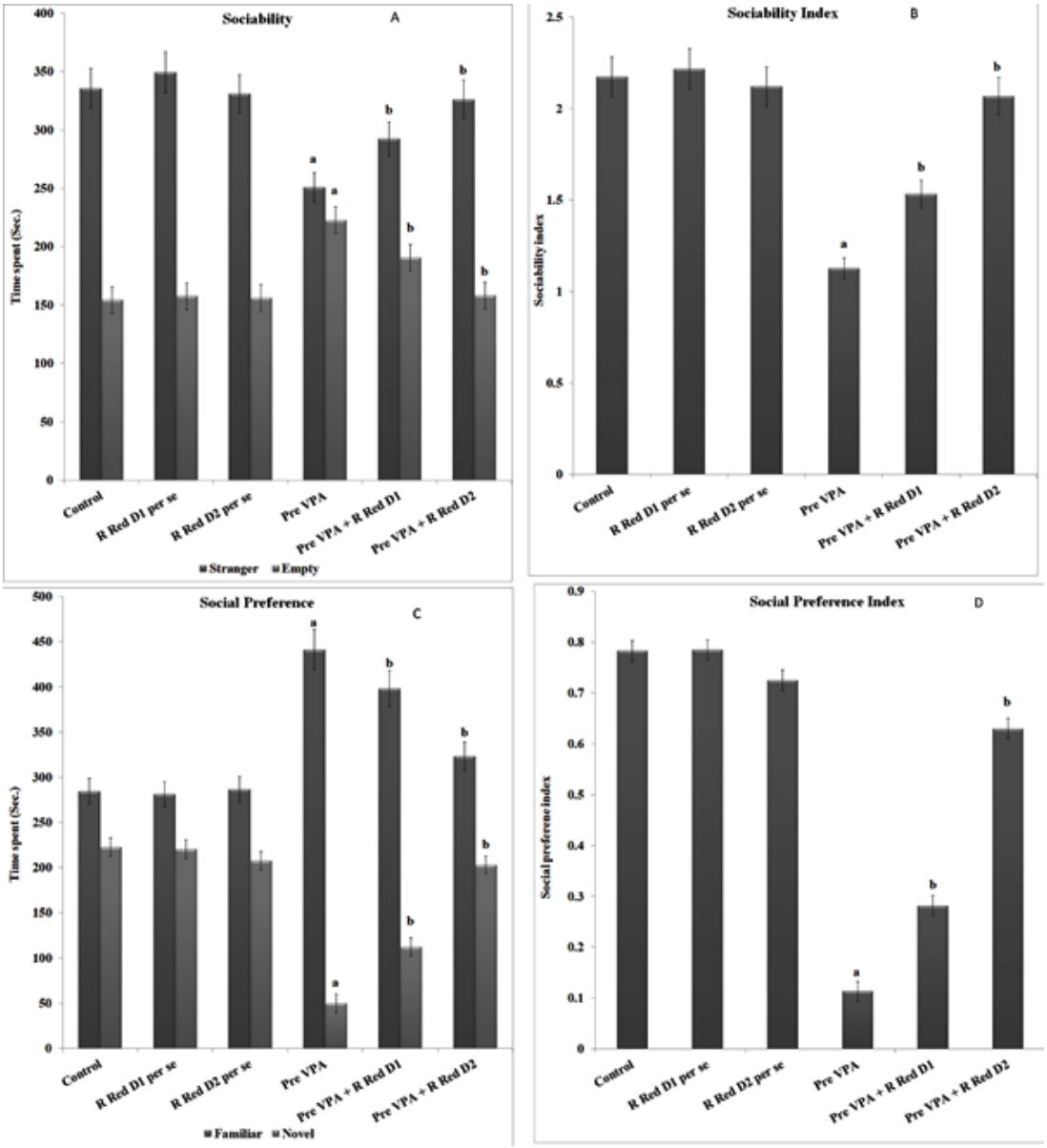


E- Embryonic day; PFC- Prefrontal cortex; P- Postnatal day; Pre-VPA - Prenatal valproic acid; R Red – Ruthenium Red; D1- dose 1 (3 mg/kg); D2- dose 2 (6mg/kg).

Fig. 1. Schematic representation of experimental protocol

DISCUSSION

Oxidative stress is one of the sponsors for autistic characteristic in clinical as well pre-clinical findings (9,25,26). We found decreased levels of GSH along with increased TBARS in Pre-VPA exposed animals. In prior studies, pre-VPA results in increased levels of oxidative



Results are mean ± standard deviation, two-way ANOVA followed by Bonferroni's post-test. [a] $p<0.05$ vs control rats, b $p<0.05$ vs Pre-VPA treated rats

A-Stranger: [a] $F_{1, 42} = 106.277$; [b] $F_{2, 42} = 31.543$; Empty: [a] $F_{1, 42} = 228.979$; [b] $F_{2, 42} = 68.632$;

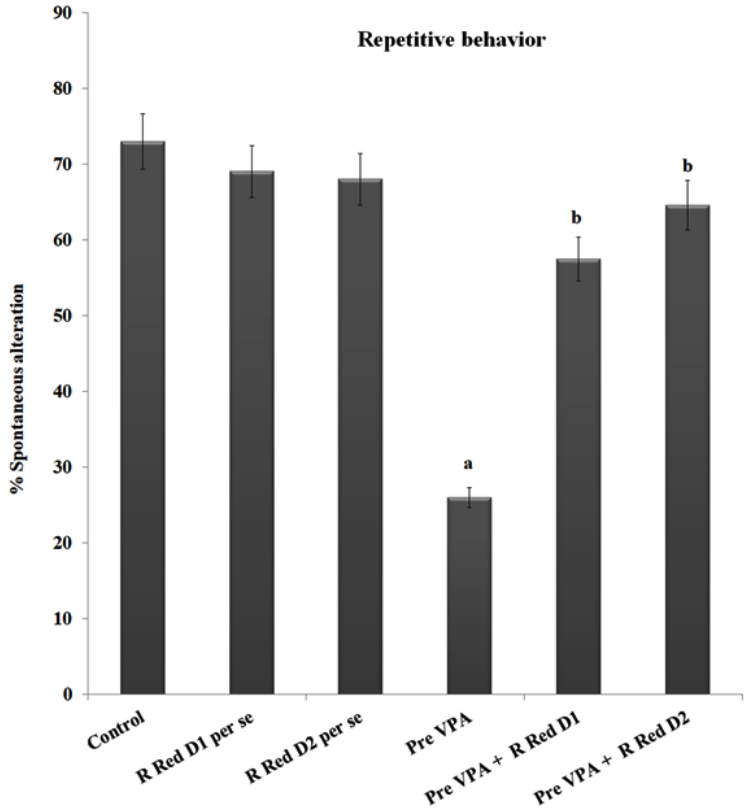
B-Sociability Index: [a] $F_{1, 42} = 502.727$; [b] $F_{2, 42} = 131.026$; C-Familiar: [a] $F_{1, 42} = 502.086$; [b] $F_{2, 42} = 60.493$; Novel: [a] $F_{1, 42} = 1477.037$; [b] $F_{2, 42} = 421.444$; D-Social preference Index: [a] $F_{1, 42} = 2585.419$; [b] $F_{2, 42} = 444.524$.

Pre-VPA - Prenatal valproic acid; R Red – Ruthenium Red; D1- dose 1 (3 mg/kg); D2- dose 2 (6mg/kg).

Fig. 2. Sociability, sociability index social preference and social preference index on Three-Chamber Sociability and Social Novelty Test apparatus

markers in rats[7]. In the current study, Ruthenium Red successfully counteracted the oxidative stress produced by Pre-VPA. There are several pathways which are implicated in oxidative stress. In autism, Impaired glutathione associated antioxidant pathways such as methionine cycle, redox system, and transculturation has already been reported[27]. Furthermore, previous research has suggested that autism manifests communication problems, repetitive behavior and social deficits due to chronic cerebellar damage, abnormal glutathione metabolism, and Wnt/â-catenin activation[28–30]. The current study also found that repetitive and impaired social behavior was evident. Few previous research indicates ryanodine receptor antagonist reverse increased oxidative stress markers in different brain condition[31,32]. Ryanodine receptor antagonist reverse Ca+ exito-toxicity associated endogenous oxidative stress markers, followed by lipid peroxidation products content (TBARS) and therefore amelioration of oxidative stress[18]. It has been expressed that antagonism of ryanodine receptor lower the advanced glycation end products dependent generation of reactive oxygen species[33]. Following the above discussion, it may be concluded that ruthenium red modulates the endogenous antioxidant pathways and results in reduced oxidative stress, which is followed by social and repetitive behavior corrections among rats exposed to pre-VPA.

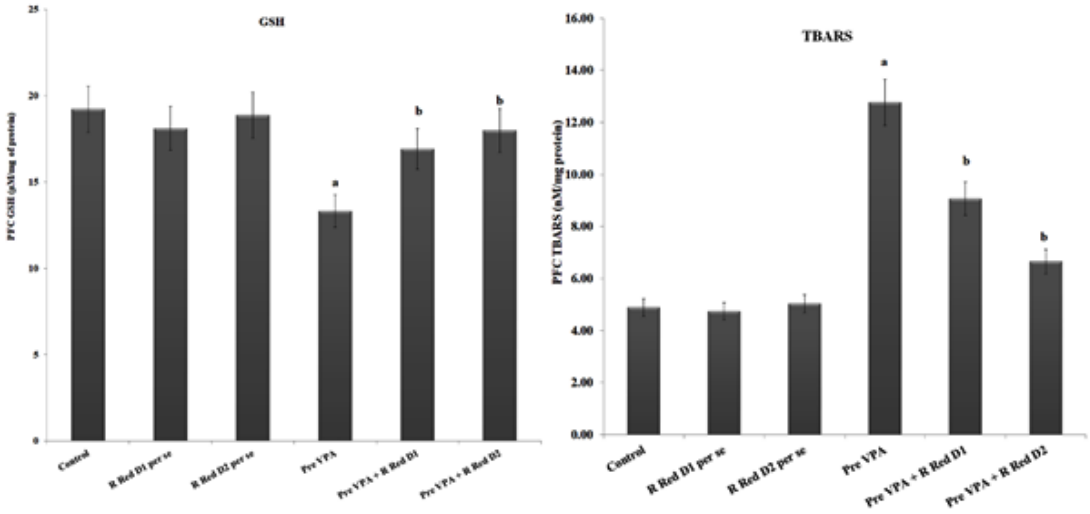
TNF-á and IL-6 were increased, while IL-10 was decreased in Pre-VPA exposed animals, while R Red 3 mg/kg and 6 mg/kg counteracted the levels of neuroinflammatory markers in the current study. Previous research has also revealed that in Pre-VPA and autism, neuroinflammatory



The results are a mean ± standard deviation, two-way ANOVA with Bonferroni's post-test.
[a] $p<0.05$ vs control rats, b $p<0.05$ vs Pre-VPA treated rats
[a] $F_{1, 42} = 623.665$; b $F_{2, 42} = 274.682$
Pre-VPA - Prenatal valproic acid; R Red – Ruthenium Red; D1- dose 1 (3 mg/kg); D2- dose 2 (6mg/kg).

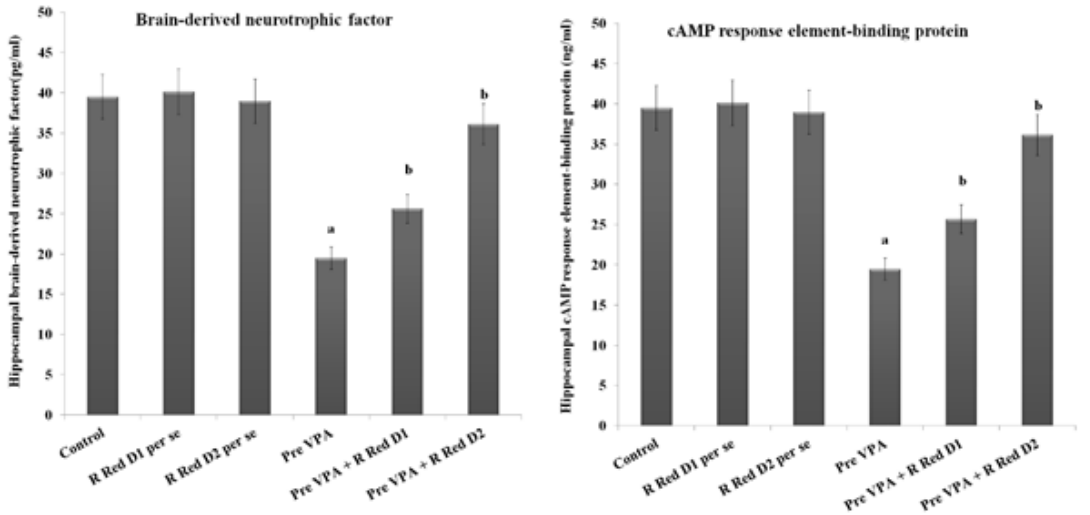
Fig. 3. Repetitive behavior measured as % spontaneous alteration on Y maze

markers are higher, followed by neuroinflammation (5,26,34,35). Social behavior impairment can be caused by neuroinflammation followed by neuronal disorganization and reactive gliosis in frontal cortex, which has already been demonstrated[36]. Additionally, we found impaired social behavior in the rats exposed to Pre-VPA. Social behavior impairments and repetitive behavior in autism are thought to be due to neuroinflammatory pathways. It has proposed that increased neuro-



Results are mean ± standard deviation; two-way ANOVA followed by Bonferroni's post-test.
[a] p<0.05 Vs control rats, b p<0.05 Vs Pre-VPA treated rats
GSH- [a] $F_{1, 18}$ = 163.265; b $F_{2, 18}$ = 685.007; TBARS: [a] $F_{1, 18}$ = 1257.099; [b] $F_{2, 18}$ = 175.039.
Pre-VPA - Prenatal valproic acid; R Red – Ruthenium Red; D1- dose 1 (3 mg/kg); D2- dose 2 (6mg/kg).

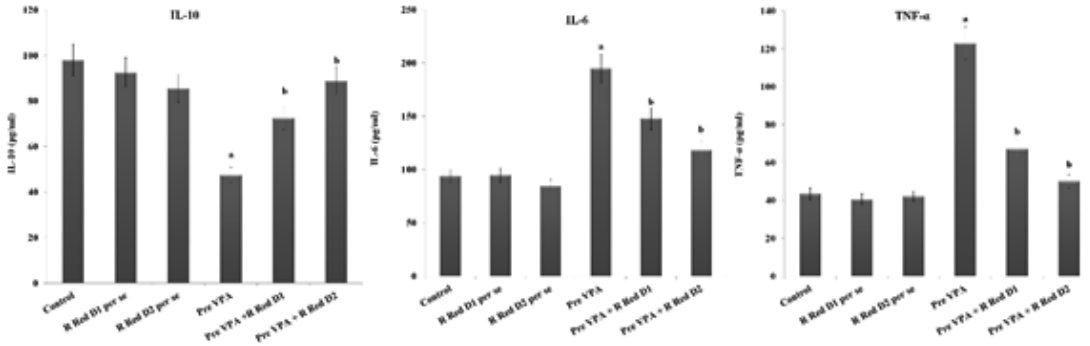
Fig. 4. Effect of various agents on prefrontal cortex oxidative stress



Results are mean ± standard deviation; two-way ANOVA followed by Bonferroni's post-test.
[a] p<0.05 vs control rats, b p<0.05 vs Pre-VPA treated rats
BDNF [a] $F_{1, 18}$ = 476.678; [b] $F_{2, 18}$ = 78.896: CREB $F_{1, 18}$ = 199.643; [b] $F_{2, 18}$ = 29.509
Pre-VPA - Prenatal valproic acid; R Red – Ruthenium Red; D1- dose 1 (3 mg/kg); D2- dose 2 (6mg/kg).

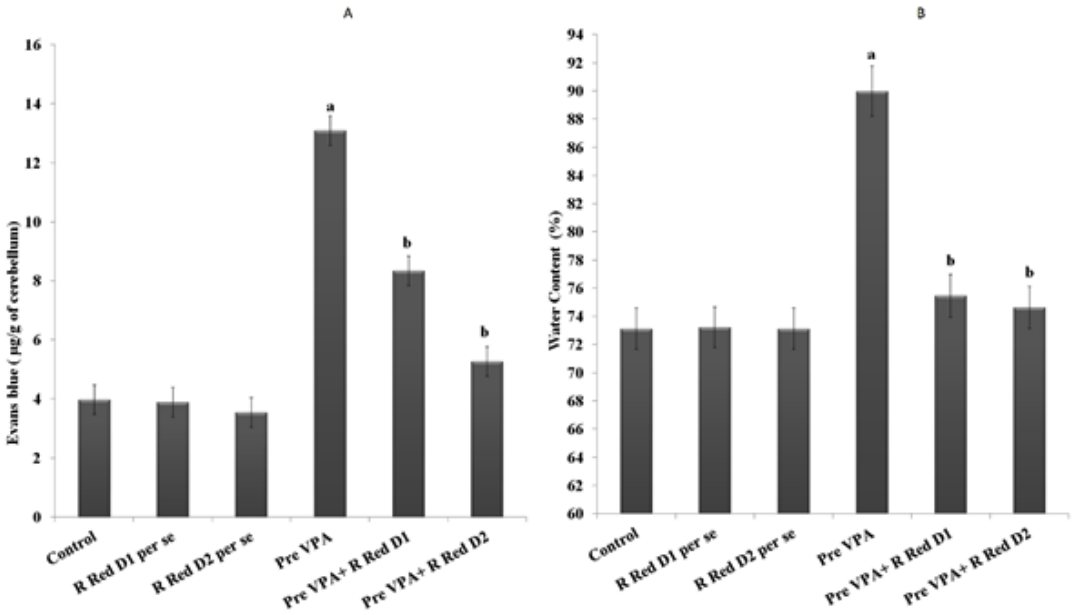
Fig. 5. Effect of various agents on hippocampal brain-derived neurotrophic factor (BDNF) and cAMP response element-binding protein (CREB)

inflammatory markers like TNF-á and IL-6 may modulate social and repetitive behavior by altering the brain microvascular system[37] and equilibrium of excitatory and inhibitory state responsible for glutamate signaling[35,36,38]. Earlier researchers have reported that antagonism of ryanodine receptor counter inflammatory marker viz. interleukin 2, interleukin-10, TNF-á[39,40]. Further, ryanodine receptor antagonist administration effectively block the calcium mobilization and hinder cell death[41].



Results are mean ± standard deviation; two-way ANOVA followed by Bonferroni's post-test.
[a] $p<0.05$ vs control rats, b $p<0.05$ vs Pre-VPA treated rats
IL-6 levels: [a] $F_{1, 18} = 973.189$; [b] $F_{2, 18} = 84.89$; IL-10 levels [a] $F_{1, 18} = 219.928$; [b] $F_{2, 18} = 138.116$; TNF-á levels [a] $F_{1, 18} = 1105.281$; [b] $F_{2, 18} = 364.755$.
Pre-VPA - Prenatal valproic acid; R Red – Ruthenium Red; D1- dose 1 (3 mg/kg); D2- dose 2 (6mg/kg), IL-6- interleukins-6; IL-10- interleukins 10; TNFá -Tumor necrosis factor-á

Fig. 6. Effect of various agents on prefrontal cortex inflammation (IL- 6, IL-10 and TNF-á levels)



Results are mean ± standard deviation, two-way ANOVA followed by Bonferroni's post-test.
[a]$p<0.05$ Vs control rats, [b] $p<0.05$ Vs Pre-VPA treated rats.
A- [a] $F_{1, 18} = 971.701$; [b] $F_{2, 18} = 163.993$; B- [a] $F_{1, 18} = 181.92$; [b] $F_{2, 18} = 94.856$
Pre-VPA - Prenatal valproic acid; R Red – Ruthenium Red; D1- dose 1 (3 mg/kg); D2- dose 2 (6mg/kg).

Fig. 7. Effect of various agents on blood brain barrier permeability measured by Evans blue concentration and water content in cerebellum

It has been expressed that ryanodine receptor antagonism inhibits calcium entry via the NMDA receptors and backs glutamate-induced excite-toxicity[42,43]. Because of this reason, we have tried to assess the permeability of blood brain barrier via Evans blue leakage. Ryanodine-mediated intracellular calcium discharge by the endoplasmic reticulum contributes to BBB distraction[33]. On the above lines this may be suggested that increased levels of neuro-inflammatory markers could modulates social behavior, repetitive behavior along with BBB function in Pre-VPA induced autism in rats.

Ruthenium red increased CREB and BDNF levels in Pre-VPA treated rats in the current study. Researchers have found that CREB/ BDNF signalling has a significant impact on synaptic plasticity, morphology, and behaviour[44,45], Therefore, this would be one of the major pathway in autism. It has already been established that the loss of BDNF causes abnormal learning, memory formation, and plasticity[46,47], As a consequence, Pre-VPA animals exhibited impaired social and repetitive behaviors, possibly due to BDNF/ CREB-associated synaptic plasticity. BDNF and CREB have also been found to be decreased in animals exposed to Pre-VPA[23]. It has suggested, CREB activity plays role in double cortin, dendritic development, expression of neuronal microtubule associated protein and BDNF that could result in autistic characteristic features such as altered synaptic plasticity and neuronal survival[48,49]. CREB and BDNF levels were significantly reduced in the hippocampus of the rats exposed to Pre-VPA, which was in line with previous findings[50] and ruthenium red counter the effect of Pre- VPA associated BDNF and CREB. Ruthenium red might have increased levels of BDNF/CREB in the pre-VPA exposed rats through neuronal survival, calcium influx and discharge with activity-dependent calcium signals, synaptic versatility, and calcium signals that influence synaptic plasticity.

A previous study suggested BBB impairment in autistic condition, postmortem with ASD cerebellum exhibited altered gene expression responsible for BBB integrity[51]. An interesting finding of our previous study was that rats pre-exposed to VPA had increased BBB permeability measured by Evans blue dye[7]. In line with this, we found that in Pre-VPA exposed rats, Evans blue leakage and water content increased. The abnormal levels of cytokines, endogenous redox mechanisms, mitochondrial function, elevated levels of neurodermatitis, and oxidative stress may cause dysfunction in the BBB[52–54]. Ruthenium red may affect cellular Ca+ levels in tight junctions through apoptosis and protein kinase C-alpha[55], Thus, in addition to being able to modulate water homeostasis, ruthenium red can also maintain Ca+ homeostasis in the brain in autistic individuals.

CONCLUSIONS

On the basic of the above line of discussion, ryanodine receptor antagonist; ruthenium red ameliorates Pre-VPA induced remarkable decrease in social behavior followed by GSH, IL-10, CREB and BDNF levels. Furthermore, ruthenium red was found to counter Pre-VPA associated increase in repetitive behavior, PFC IL-6, and TNF-á and TBARS. Pre-VPA has been found to increase in BBB permeability and water content, which was ameliorated by ruthenium red treatment in rats. Further studies can explore the molecular mechanisms of ryanodine receptors in autism.

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Conflicts of Interest Statement

None

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None.

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