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Attenuative Effect of Zingerone on Behavioural Anomalies and Oxidative Stress in The Cerebellum of Prenatal Valproic Acid Induced Autism Spectrum Disorder Like Symptoms in F1 Female Pups: *In Vivo* Modelling

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Abstract
The pilot study aimed to evaluate the attenuative effect of zingerone (ZNO) against valproic acid (VPA)-induced autism spectrum disorder (ASD) like symptoms in the cerebellum of female rat pups. Pregnant female rats were administered with VPA (500mg/Kg/i.p) as a single dose on the 12th day of gestation to induce autistic phenotypes. The effect of ZNO at 30mg/Kg/p.o and 60mg/Kg/p.o on VPA-induced neurobehavioural anomalies (open field test, rotarod, forced swim test, and y-maze), oxidative stress (lipid peroxidation (LPO), total nitric oxide (NO)), altered antioxidative status (reduced glutathione (GSH), vitamin C (Vit-C) and catalase (CAT)), and histopathological staining (H&E staining) were assessed. Autistic behavioural anomalies were substantially diminished in a dose-dependent manner following ZNO administration. VPA-exposed female pups had elevated levels of LPO and total NO, which were significantly attenuated by ZNO. Treatment with ZNO also substantially promoted the levels of GSH, Vit-C, and CAT in the cerebellum. In the present study, for the first time, we report that ZNO showed promising results in attenuating VPA-induced symptoms, indicating a possible neuroprotective effect against the core symptoms of autistic female F1 rat pups.

Keywords
Neuroprotection, Autism Spectrum Disorder, Zingerone, Oxidative Stress, Anti-oxidant, H&E staining

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1. INTRODUCTION
Autism spectrum disorder (ASD) is a complex and composite neurodevelopmental disorder characterised by core features such as restrictive interests, reserved sociability, and repetitive behaviour [1], imposing a substantial challenge in everyday life [2], and societal experiences [3]. Recent studies have accounted for genetic predisposition

and prenatal environmental risk factors as potential etiologies for children being diagnosed with ASD [4], with males being diagnosed four times more often than females [1]. However, in clinical settings, females are strongly predicted to have a higher association with ASD comorbidities than males, contrary to what may be expected based on the higher male ratio prevalence [5]. Research has also highlighted the discrepancies in sex-associated manifestations in diagnosing and recognising females with ASD exclusive of other psychiatric conditions, due to predominantly male-associated presentations [6]. This poses an increased need to study and understand the disposition of ASD in females in pre-clinical models and clinical studies.

Exposure to valproic acid (VPA), a notable drug used to treat migraines, bipolar disorder and an antiepileptic drug administered as a monotherapy or in combination with other antiepileptic drugs during the critical pregnancy window, is associated with autistic features in the foetus [7] and also 'foetal valproate syndrome' [8]. This has been validated through several studies that describe that prenatal VPA induction [9-11] on the 12th day of gestation in pregnant female rodents has been associated with autistic phenotypes in F1 rat pups [12,13]. This model has been considered the gold standard in proposing and evaluating various therapeutics against autistic phenotypes and their signalling pathways [13-18].

Therapeutic strategies have been of substantial interest in attenuating ASD core symptoms. Only risperidone and aripiprazole are approved by the Federal Drug Administration (FDA) in the United States for autistic individuals and irritability. Designing and assessing drugs that attenuate the heterogeneous core behavioural symptoms associated with ASD and its comorbidities are in progress [19]. Zingerone (ZNO), present in dried ginger, belongs to the family of methoxyphenols and its derivatives. ZNO has been comprehensively studied for its anti-oxidant [20-22], anti-inflammatory [20,21,23], and anti-apoptotic properties [23-27]. The neuroprotective effect of ZNO has been previously analysed and reported for several neurological disorders and diseases of the CNS, such as Parkinson's disease [24], focal transient ischemia [28], status epilepticus [29], manic-like behaviour [30], cadmium neurotoxicity [31], lead-induced brain injury [32], and nickel neurotoxicity [33]. Herewith, to translate the neuroprotective effects of ZNO in neurodevelopmental disorders, to the best of our knowledge, for the first time, we report a pilot study on the alleviative effect of postnatal oral administration of ZNO on prenatal VPA-induced ASD-like symptoms in F1 female rat pups.

2. MATERIALS AND METHODS

2.1. Drugs and Reagents

ZNO under the name 4-(4-Hydroxy-3-methoxyphenyl)-2-butanone (CAS RN: 122-48-5) and VPA under the name sodium valproate (CAS RN: 1069-66-5) were purchased from Tokyo Chemical Industry (India) Pvt. Ltd. All other chemicals used in the experiments were of analytical grade. ZNO and VPA were dissolved in 0.9% physiological saline as vehicle as per the requirement.

2.2. Ethics Statement

The current investigation and all protocols pertaining were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) (Approval No: 01/01/2024) and housed in the Central Animal House Facility of Dr ALM. Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai, Tamil Nadu, India.

2.3. Establishing an *in vivo* model of ASD

Sexually matured, healthy adult male and female Wistar rats weighing 250g to 300g were randomly assigned and mated (trios) overnight under controlled conditions of temperature and humidity with a 12:12 hours of light: dark cycle. A constant supply of fresh water, clean bedding and standard pellets were made available during housing. Strict prior habitualization periods were introduced to rats of both sexes to avoid aggression during mating. The following day, a vaginal smear was used to confirm the presence of sperm. A positive indication was considered as day 0 of gestation (GD 0) [34]. The pregnant rats were then separated from the males and housed individually.

2.3.1. Dosage fixation and experimental grouping

The dosage for ZNO oral administration was based on previously reported dosages for neuroprotection as per the various studies mentioned earlier. It was found that prenatal exposure to VPA (500mg/Kg) through the intraperitoneal route (i.p) [35] in pregnant female rats on GD 12 [13] successfully induced ASD like phenotypes in F1 pups. Based on the above-mentioned study, the pregnant rats were divided into two sets:

Set 1: The pregnant female rats remained untreated. The F1 female pups born from this set were grouped for control and drug alone (control pups).

Set 2: The pregnant female rats were induced with VPA 500mg/Kg/i.p on GD 12. The F1 female pups born from this set were grouped for induced, treatment – I and treatment – II (experimental pups). All pregnant females delivered the neonates in the early hours of GD 21. The day the F1 pups were

delivered was considered postnatal day 0 (PND 0). The dams and their litter were left undisturbed until PND 15. On PND 15, the F1 pups were weighed and sexed based on their anogenital distance [36]. Only female F1 rat pups were considered for the current study.

2.3.2. Treatment Schedule

The female F1 rat pups (within their sets) were randomly assigned to various control and experimental groups. ZNO was administered as treatment through the oral route (p.o). The treatment schedule lasted for 20 days from PND 15 to PND 35.

Group I: Control – Healthy F1 female pups were considered as the control. They remained untreated with vehicle, inducer, and treatment from PND 0 to PND 35.

Group II: VPA (500mg/Kg/i.p) induced – Pregnant female rats were induced with VPA 500mg/kg b.w/i.p as a single dosage on GD 12. Post delivery, the F1 female pups were kept untreated from PND 0 to PND 35.

Group III: Treatment – I (VPA (500mg/Kg/i.p) + ZNO (30mg/Kg/p.o) - Pregnant female rats were induced with VPA 500mg/kg b.w/i.p as a single dosage on GD 12. Post delivery, the F1 female pups were treated with ZNO 30mg/Kg/p.o from PND 15 to PND 35.

Group IV: Treatment – II (VPA (500mg/Kg/i.p) + ZNO (60mg/Kg/p.o) - Pregnant female rats were induced with VPA 500mg/kg b.w/i.p as a single dosage on GD 12. Post delivery, the F1 female pups were treated with ZNO 60mg/Kg/p.o from PND 15 to PND 35.

Group V: Drug Control (ZNO 60mg/Kg/p.o) - Healthy F1 female pups were treated with ZNO 60mg/Kg/p.o alone from PND 15 to PND 35.

2.4. Postnatal developmental tail deformities in VPA-induced female rat pups

A well-established tail deformity commonly observed in gestational VPA induction was assessed [37].

2.5. Body weight gain of F1 female rat pups

The body weight gain of F1 female pups was evaluated from PND 15 to PND 35. The pups were gently removed from the dams, weighed and returned to their home cages.

2.6. Behavioural Assessments

On PND 35, after the final day of drug administration, the F1 female pups were assessed for various behavioural paradigms. All standard steps and precautions pertaining to behavioural testing protocols were followed.

2.6.1. Open Field Test (OFT) - assessment of spontaneous locomotory activity

The OFT measures the spontaneous locomotor activity and autonomous instinct in pups as described by Elgamal [38] with suitable modifications. Briefly, an opaque circular arena measuring 60cm in diameter was divided into several small squares and placed in a dimly lit room. The pups were novel to the environment of the arena until the day of testing. The number of squares successfully crossed by the pups within the 5mins cut-off time was recorded and manually evaluated by an observer concealed from the experimental grouping. When a pup completely moves from one square to another using both forelimbs and hindlimbs, the incident was calculated as '1 square crossed'. The arena was cleaned between trials using 70% ethanol to eliminate any odour-related discrepancies. After 10mins the next pup was placed in the arena for valuation. The total no. of squares crossed is considered as a measure of 'ambulatory behaviour', indicating the ability of the animal to spontaneously explore the novel arena and have intact autonomous behavioural instinct.

2.6.2. Rotarod Test - assessment of motor dysfunction

Evaluation of sensory motor dysfunction was performed using the rotarod test as described by Mehta [39] with minor modifications. All animals were trained to accustom themselves to the apparatus 24hrs before the day of the test to eliminate novelty-induced behaviour. Briefly, the pups were gently placed on a rotating rod and allowed to run at 20rpm for 60secs, kept constant throughout the experiment. The 'time of fall' was noted for each trial. The area was cleansed with 70% ethanol after each trial. The experiment assessed the vital motor coordination and balance of an animal. The longer the 'latency to fall', the longer the animal could coordinate and balance itself on the rotarod, thereby indicating intact sensory motor function.

2.6.3. Forced Swim Test (FST) - assessment of anxiousness

The FST was used to evaluate anxiousness in pups as described by Mehta [39] with minor modifications adapted from Can [40]. Habitualization was conducted 24hrs before the day of the test. Briefly, a plexiglas tank of height 40cm, length and breadth 20cm was considered. Lukewarm water was filled up to 30cm and kept consistent throughout the experiment. On the day of the test, the pups were gently lowered into the tank containing lukewarm water and allowed to swim for 4mins. Each trial was recorded. During the course of the experiment, the pups underwent phases of 'active swimming' and 'passive immobility'. At the end of the cut-off period, the pups were gently removed from the water, patted dry and kept in a warm environment before returning to their home cage. The apparatus was

washed between each trial using sodium dodecyl sulfate solution as a detergent to eliminate any lingering odours from previous trials. An observer blind to the experimental grouping assessed the 'time of immobility' of each pup. An elevated 'time of immobility' is taken as a measure of anxiety-like behaviour.

Time of immobility (secs) = Total time of each trial (secs) - Time of 'active swimming' (secs)

2.6.4. Y-maze – assessment of repetitive behaviour

Repetitive behaviour is a core symptom in ASD and can be evaluated by the Y-maze [34]. The Y-maze apparatus (INCO- Medicraft (Semiautomated)) consists of 3 opaque identical arms, A, B, C, with a centre junction connecting the 3 arms. The rat pups, naïve to the apparatus, were placed in the centre of the Y-maze and allowed to explore each of the 3 arms freely for 8mins. The number of times each arm was visited was recorded directly from the automated circuit box. An entry is considered valid when the pup subsequently visits the arms without repetition, e.g., B, C, A. Each successful subsequent arm entry into any of the 3 arms is considered a 'spontaneous alteration'. The total no. of arm entries and the total no. of successful spontaneous alterations were recorded to calculate the '% spontaneous alteration'. Pups with virtuous spatial memory and recognition tend to have a higher '% spontaneous alteration' than pups with autistic features, indicating a sign of repetitive behaviour.

% Spontaneous alteration: [(total no. of spontaneous alternations)/(total no. of arm entries - 2)]*100

2.7. Tissue Collection

Following the conclusion of the behavioural paradigm on PND 35, the pups were sacrificed under the influence of diethyl ether. They were further cervically decapitated, and the whole brain was isolated. 0.9% physiological saline was used to clear any excess blood. The cerebellum was dissected and stored at -80℃ until further experiments. About 100mg of the cerebellum tissue was weighed and homogenised in 0.1M phosphate buffered saline (pH = 7.4). The homogenate was centrifuged at 10,000rpm at 4℃ for 15 mins, and the supernatant was aliquoted for biochemical assay.

2.8. Biochemical Assay

2.8.1. Measurement of lipid peroxidation (LPO)

LPO was estimated as a measure of oxidative stress in the cerebellum of F1 female rat pups as described by Fernandes [41] with minor modifications. Malondialdehyde (MDA) is a stable aldehyde generated as a result of LPO. Therefore, the total MDA levels present in the homogenate supernatant were taken as an indication of oxidative stress due to LPO. Briefly, 0.5mL of the supernatant was added to 1mL of 10nM thiobarbituric acid (TBA) and heated at 95℃ for an hour till the formation of pink coloured MDA-TBA complex. The test tubes were then cooled on ice. A calibration curve was plotted with different concentrations of 1,1,3,3 tetramethoxypropane, ranging from 4nmol/well to 20nmol/well. Once the test tubes reached room temperature, 300µL of the final product, along with reagent blank and standards, was plated in a 96-well plate and read at 535nm. The levels of MDA were reported as nmols of MDA/mg of protein.

2.8.2. Measurement of total nitric oxide (NO)

The total NO levels were determined by the method of Mehta [36] with slight modifications. The reaction mixture contains equal volumes of supernatant and Griess reagent. The mixture was incubated for 10mins at room temperature in the dark. The end-product was the production of a pinkish-purple azo dye. The absorbance was read at 540nm spectrophotometrically. Sodium nitrite was used to perform a standard curve. The levels of NO as an endpoint for oxidative stress were reported as µM of total NO/mg of protein.

2.8.3. Estimation of reduced glutathione (GSH)

The levels of GSH were measured based on a previously described protocol by Anand [42], with minor modifications. Briefly, an equal quantity of homogenate supernatant and 10% trichloroacetic acid was mixed and centrifuged at 1000g at 4℃. To 0.5mL of the supernatant, 2 mL of 0.3M phosphate buffer (pH 8.4), 0.5 mL of 0.001M Ellman's reagent, and 0.4 mL of double-distilled water were added. The mixture was vortexed, and 300µL of the end-product produced was plated in a 96-well plate and read at 415nm in a microplate reader. A standard GSH calibration curve was plotted, and the concentration of GSH was expressed as µM of GSH/mg of protein. The levels of GSH were estimated as a measure of endogenous antioxidant status in the cerebellum of female pups. Reduced levels of GSH may indicate potential oxidative stress and compromised antioxidant defence.

2.8.4. Assay of catalase (CAT)

The CAT assay was measured using the previously described protocol by Sinha [43], with slight modifications. Briefly, 1mL of the homogenate supernatant was added to the assay mixture containing 1mL of 0.01M phosphate buffer (pH 7.0) and 0.5mL of 0.2M hydrogen peroxide (H_2O_2). The addition of 2mL of dichromate-acetic acid reagent arrested the reaction. The test tubes were kept in a heat block for 10mins at 95℃. After cooling, the absorbance was read at 610nm using a double-beam UV-spectrophotometer. A control without the homogenate was also simultaneously run. The

activity of CAT was expressed as µmols of H_2O_2 consumed/min/mg of protein.

2.8.5. Assessment of Vitamin C (Vit-C)

The levels of Vit-C were measured based on the previously detailed protocol of Mochnik [44]. The absorption maxima of the coloured product were read at 520nm using a double-beam UV-spectrophotometer. The levels of Vit-C present in the sample were expressed as 'Vit C units/mg of protein'.

2.9. Histopathological studies

After euthanasia, the isolated whole brain from each group was carefully cleaned several times with 0.9% NaCl to remove traces of blood and hair thoroughly. The brain was then fixed in 10% formaldehyde solution for 1 to 2 weeks, which reduces distortion of cellular structures. The tissues were then embedded in molten paraffin wax, sectioned at 4µm thickness, followed by staining with haematoxylin and eosin (H&E) using the protocol described by Zhang [45]. The slides were then viewed at 400x total magnification at a scalebar of 360µm.

2.10. Statistical Analysis

The statistical analysis was conducted using GraphPad Prism version 8.0.2 for Windows, and the data were represented as mean ± standard deviation (SD). Descriptive statistics were conducted, followed by identification of any outliers. Two-way ANOVA was performed to analyse the variance between the groups for body weight gain, and Ordinary One-way ANOVA was conducted for all other parameters. When ANOVA was found significant, Tukey's post-hoc analysis was conducted to analyse the significance between each group.

3. RESULTS

3.1. Visual assessment of the postnatal developmental tail deformity on VPA induction

On prenatal VPA (500mg/Kg/i.p) induction on the 12th day of gestation in pregnant female rats yielded F1 female pups with developmental tail deformity, which was recorded on PND 5. A 'simple bent in the middle' of the tail was observed as the prominent tail malformation characteristic of successful VPA induction (Figure 1).

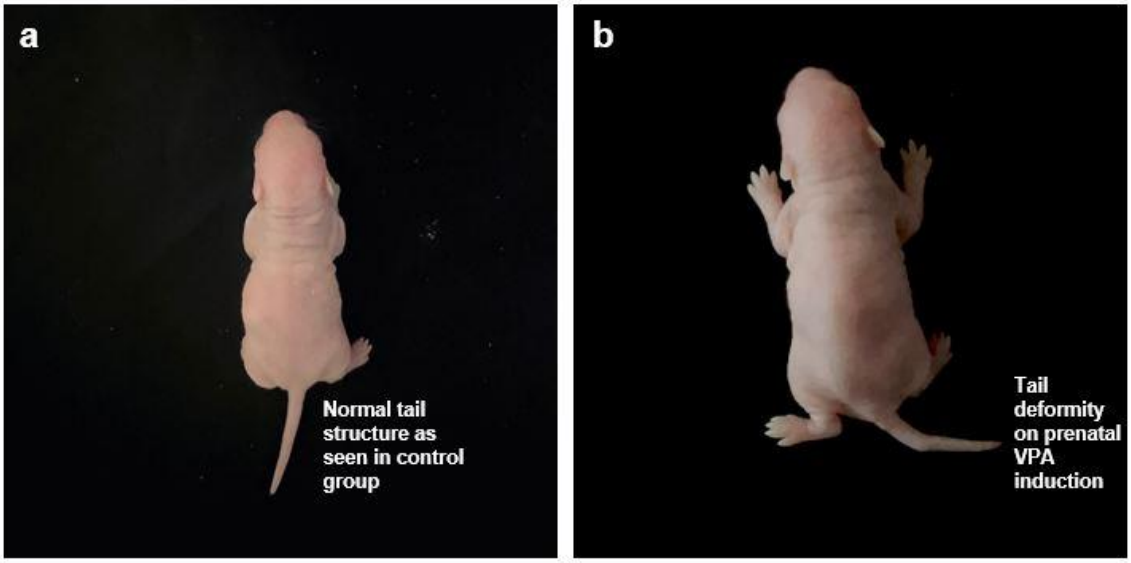

Figure 1: Visual assessment of the postnatal developmental tail deformity on VPA induction. On PND 5, the pups were visually assessed for their developmental abnormalities to evaluate the successful induction of VPA. A characteristic bend in the tail is widely reported in F1 pups of both sexes due to prenatal VPA-induced developmental deformity. a) control rat pups on PND5 showing normal developmental morphology of the tail. b) Prenatal VPA (500mg/Kg/i.p.) on the GD 12 induced F1 pups showing a characteristic bend in the tail due to developmental deformity.

3.2. Assessment of body weight gain in VPA-induced F1 female rat pups from PND 15 to PND 35

All F1 female rat pups showed an increase in body weight as recorded from PND 15 to PND 35. However, there were no statistically significant findings in the body weight gain between the control and treated groups on PND 15, PND 25 and PND 35 (Figure 2).

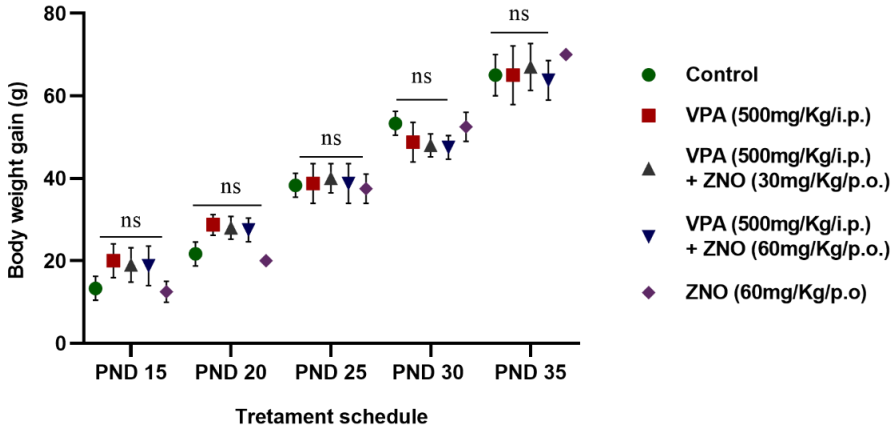

Figure 2: Assessment of body weight gain in VPA-induced F1 female rat pups from PND 15 to PND 35. The body weight gain was assessed from PND15 to PND35. It was consistently found that, though there was a gain in the body weight of F1 female rats across the experimental group, there was no statistically significant increase in body weight.

3.3. Effect of postnatal oral ZNO administration on prenatal VPA-induced changes in general locomotor activity of F1 female rat pups

The experimental F1 female rat pups were subject to the open field test on PND 35 after the final dose of ZNO treatment. One-way ANOVA substantially showed a difference in general locomotory behaviour between the control and experimental groups (p < 0.001). Prenatal VPA (500mg/Kg/i.p) induced rats showed reduced no. of squares crossed in the open field (p < 0.0001) as compared to the control group. On treatment with ZNO 30mg/Kg/p.o (p < 0.05) and 60mg/Kg/p.o (p < 0.01), the F1 female rat pups showed improved time in exploratory behaviour when compared to the prenatal VPA induced group, indicating a substantial increase in no. of square crossings in the open field. However, the ZNO 60mg/Kg/p.o only administered group did not show statistically significant findings (Figure 3a).

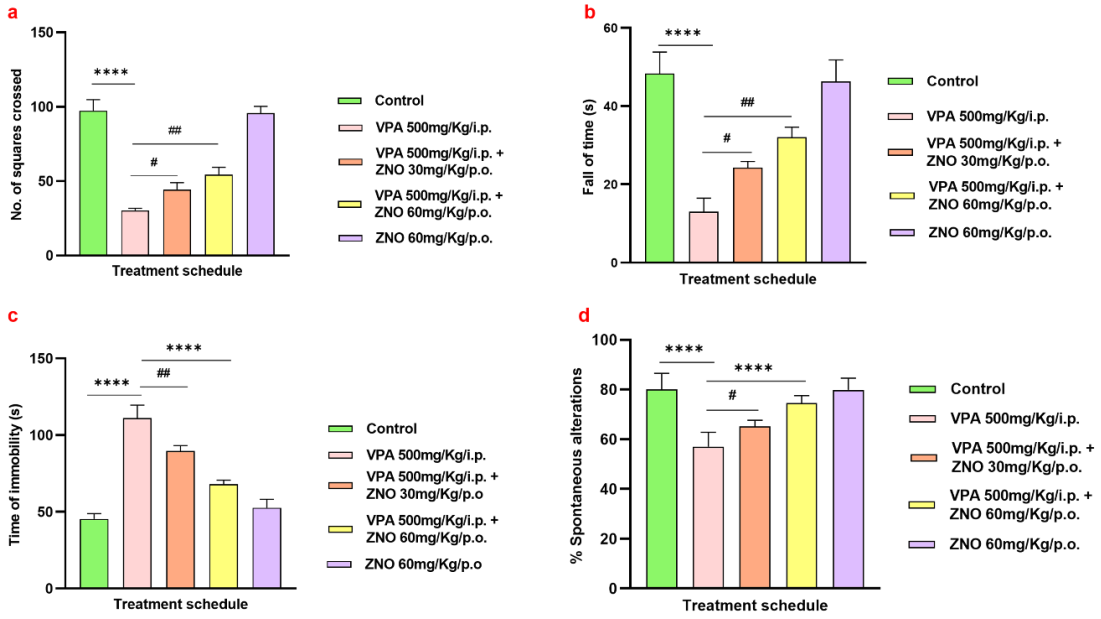

Figure 3: Effect of postnatal oral ZNO administration on prenatal VPA-induced behavioural anomalies in F1 female rat pups. All behavioural paradigms were conducted on PND 35. a) and b) represent the effect of postnatal oral ZNO administration on prenatal VPA-induced changes in general locomotor activity and motor coordination, respectively. The total no. of squares crossed was evaluated to assess general locomotor activity as an endpoint of OFT, and the fall of time was evaluated to assess motor coordination and balance as an

endpoint of Rotarod analysis. Statistical differences were analysed and represented as induced group (VPA 500mg/Kg/i.p) ***$p < 0.0001$ as compared to control, ZNO oral treatment at dosage – 1 (30mg/Kg) $^{\#}p < 0.05$ and dosage – 2 (60mg/Kg) $^{\#\#}p < 0.01$ as compared to the prenatal VPA-induced group. c) represents the time of immobility in control and experimental F1 female pups. Anxious-like behaviour was taken as an endpoint in the FST. Statistical differences were analysed and represented as induced group (VPA 500mg/Kg/i.p) ***$p < 0.0001$ as compared to control, ZNO oral treatment at dosage – 1 (30mg/Kg) $^{\#\#}p < 0.01$ and dosage – 2 (60mg/Kg) ***$p < 0.0001$ as compared to the prenatal VPA-induced group. d) repetitive behaviour, a core symptom of ASD, was assessed by the Y-maze and %spontaneous alteration was plotted. Statistical analysis was carried out and represented as VPA-induced females ****$p < 0.0001$ as compared to the control, treatment – 1 ZNO (30mg/Kg) $^{\#}p < 0.05$ as compared to the induced group and treatment- 2 ZNO (60 mg/Kg) ****$p < 0.0001$ as compared to the induced group.

3.4. Effect of postnatal oral ZNO administration on prenatal VPA-induced changes in motor co-ordination activity of F1 female rat pups

The experimental F1 female rat pups yielded statistically significant variance between the groups on One-way ANOVA ($p < 0.0001$). Prenatal VPA (500mg/Kg/i.p) induced F1 female pups showed longer latency to fall by losing balance in the rotating rotarod ($p < 0.0001$) as compared to the control group, indicating compromised motor coordination. On treatment with ZNO at 30mg/Kg/p.o ($p < 0.05$) and 60mg/Kg/p.o ($p < 0.01$), the F1 female rat pups presented with shorter latency to fall by an increase in 'fall of time' as compared to the VPA induced group. ZNO 60mg/Kg/p.o alone treated group did not show any statistically significant variance (Figure 3b).

3.5. Effect of postnatal oral ZNO administration on prenatal VPA-induced changes in anxiety-like behaviour of F1 female rat pups

A statistically significant variance was observed between the groups performing FST on One-way ANOVA ($p < 0.0001$). Post-hoc analysis revealed that gestational VPA (500mg/Kg/i.p) exhibited increased anxiousness behaviour in the FST ($p < 0.0001$), indicating increased time of immobility as compared to the control F1 female pups. Treatment with ZNO 30mg/Kg/p.o ($p < 0.01$) and 60mg/Kg/p.o ($p < 0.0001$) showed a dose-dependent attenuation in the levels of anxiousness by diminution in the time of immobility during FST as compared to the VPA alone induced group. Oral administration of ZNO at 60mg/Kg alone did not confirm any statistically significant variance (Figure 3c).

3.6. Effect of postnatal oral ZNO administration on prenatal VPA-induced repetitive behaviour in F1 female rat pups

The results of the current study indicated statistically significant variance in spontaneous alterations in the Y-maze test among control and experimental groups ($p < 0.0001$). F1 female rat pups induced with prenatal VPA (500mg/Kg/i.p) on the 12th day of gestation showed increased repetitive arm entries ($p < 0.0001$), thereby indicating a decline in the %spontaneous behaviour as compared to the control group. ZNO administration was able to attenuate the VPA-induced repetitive behaviour in a dose-dependent manner – 30mg/Kg/p.o ($p < 0.05$) and 60mg/Kg/p.o ($p < 0.0001$), resulting in a relative recuperation effect on the %spontaneous alterations in the treatment groups. Postnatal oral ZNO administration at 60mg/Kg alone did not yield any statistically significant difference between groups (Figure 3d).

3.7. Effect of postnatal oral ZNO administration on prenatal VPA-induced changes in the levels of MDA in the cerebellum of F1 female rat pups

Statistical analysis of MDA levels in the cerebellum of F1 female rat pups generated a substantial difference between groups as determined by conducting One-way ANOVA ($p < 0.0001$). The data demonstrated that VPA (500mg/Kg/i.p) induction on the 12th day of gestation showed elevated levels of MDA in the cerebellum ($p < 0.0001$) as compared to the control group. Postnatal ZNO administration at 30mg/Kg/p.o ($p < 0.01$) and 60mg/Kg/p.o ($p < 0.001$) showed considerable attenuation in elevated MDA levels in the cerebellum in a dose-dependent manner. ZNO 60mg/Kg/p.o alone administration did not show a substantial difference in variation (Figure 4a).

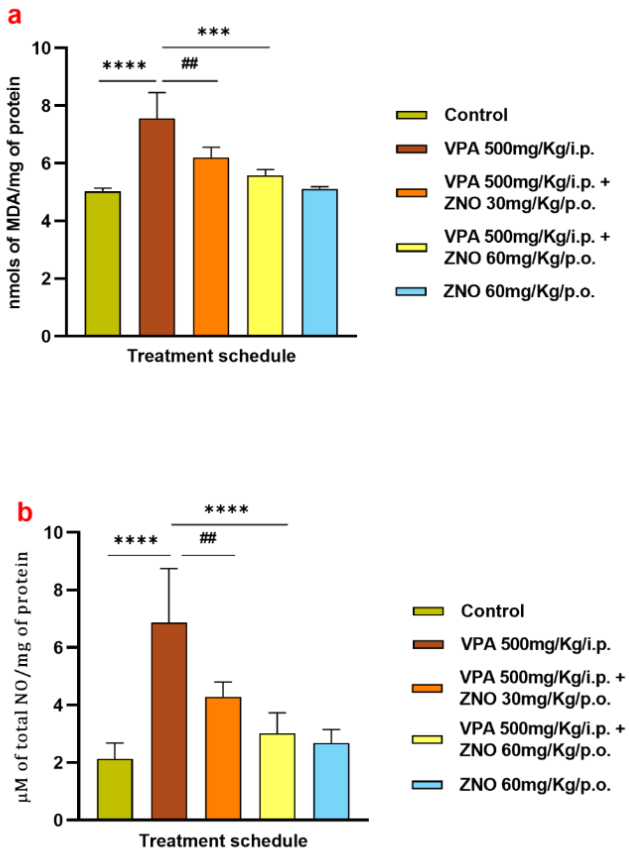

Figure 4: Effect of postnatal oral ZNO administration on prenatal VPA-induced reactive oxygen species in F1 female rat pups. a) represents the expression of MDA levels, an end-point of lipid peroxide estimation, indicating the levels of oxidative stress in the cerebellum. The data were statistically analysed and represented as VPA 500mg/Kg/i.p ****$p < 0.0001$ as compared to the control, ZNO treatment at 30mg/Kg/p.o $^{##}p < 0.01$ and at 60mg/Kg/p.o ***$p < 0.001$ as compared to the VPA alone-treated group. b) represents the expression of total NO levels, an indication of elevated reactive oxygen damage in the cerebellum. The data were statistically analysed and represented as VPA 500mg/Kg/i.p ****$p < 0.0001$ compared to the control, and ZNO treatment at 30mg/Kg/p.o $^{##}p < 0.01$ and at 60mg/Kg/p.o ****$p < 0.0001$ as compared to the VPA alone treated group

3.8. Effect of postnatal oral ZNO administration on prenatal VPA-induced changes in the levels of NO in the cerebellum of F1 female rat pups

The study's findings indicated a significant difference in the levels of NO in the cerebellum on conducting One-way ANOVA ($p < 0.0001$). The data revealed that gestational VPA (500mg/Kg/i.p) induction elevated the levels of NO ($p < 0.0001$) in the cerebellum of F1 female rat pups as compared to the control group. ZNO mitigated the augmented levels of NO in the cerebellum at 60mg/Kg/p.o ($p < 0.0001$) as compared to the VPA alone induced group, than ZNO administration at 30mg/Kg/p.o ($p < 0.01$) as compared to the VPA alone induced group, signifying a dose-dependent effect. The ZNO 60mg/Kg/i.p alone administered group did not show a substantial difference between groups (Figure 4b).

3.9. Effect of postnatal oral ZNO administration on prenatal VPA-induced changes in the levels of GSH in the cerebellum of F1 female rat pups

The data from the present study implied statistically significant alteration in the levels of GSH in the cerebellum on conducting One-way ANOVA (F ($p < 0.0001$). Prenatal VPA (500mg/Kg/i.p) induction on the 12th day of gestation prominently abridged the levels of GSH ($p < 0.0001$) in the cerebellum of F1 female rat pups as compared to the control. Results from ZNO treatment for 20 days at 30mg/Kg/p.o ($p < 0.01$) and 60mg/Kg/p.o ($p < 0.0001$) showed a dose-dependent attenuation in the level of GSH in the cerebellum as compared to the VPA alone induced group. The ZNO at 60mg/Kg/p.o administration did not show a meaningful difference between the groups (Figure 5a).

3.10. Effect of postnatal oral ZNO administration on prenatal VPA-induced changes in the levels of CAT in the cerebellum of F1 female rat pups

The data from the present study implied statistically significant alteration in the levels of CAT in the cerebellum on conducting One-way ANOVA (p < 0.0001). Prenatal VPA (500mg/Kg/i.p) induction on the 12th day of gestation prominently abridged the levels of CAT (p < 0.0001) in the cerebellum of F1 female rat pups as compared to the control. Results from the ZNO treatment at 30mg/Kg/p.o (p < 0.05) and 60mg/Kg/p.o (p < 0.0001) showed a dose-dependent attenuation in the level of CAT in the cerebellum as compared to the VPA alone induced group. ZNO at 60mg/Kg/p.o administration did not show a statistically significant difference between groups (Figure 5b).

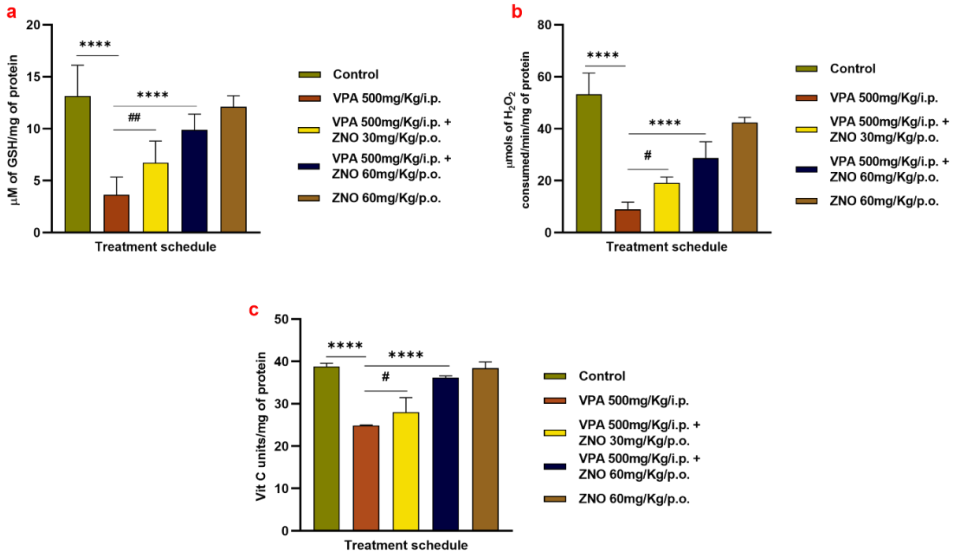

Figure 5: Effect of postnatal oral ZNO administration on prenatal VPA-induced changes in the antioxidant status in the cerebellum. a) represents the levels of GSH in the cerebellum of F1 female rat pups as an indicator of antioxidant status. The data were statistically analysed and represented as VPA 500mg/Kg/i.p ****p < 0.0001 as compared to the control, ZNO treatment at 30mg/Kg/p.o $^{\#\#}p$ < 0.01 and at 60mg/Kg/p.o ****p < 0.0001 as compared to the VPA alone treated group. b) and c) represent the levels of H_2O_2 consumed in the assay of catalase and Vit C levels as an end-point of anti-oxidant status evaluation in the cerebellum. The data were statistically analysed and represented as VPA 500mg/Kg/i.p ****p < 0.0001 as compared to the control, ZNO treatment at 30mg/Kg/p.o $^{\#}p$ < 0.05 and at 60mg/Kg/p.o ****p < 0.0001 as compared to the VPA alone treated group.

3.11. Effect of postnatal oral ZNO administration on prenatal VPA-induced changes in the levels of Vit-C in the cerebellum of F1 female rat pups

The data obtained from the current study indicated substantially significant alterations in the levels of Vit-C on conducting One-way ANOVA (p < 0.0001). Prenatal VPA (500mg/Kg/i.p) induction as a single dose prominently lowered the levels of Vit-C (p < 0.0001) in the cerebellum of F1 female rat pups as compared to the control. A dose-dependent oral administration of ZNO at 30mg/Kg (p < 0.05) and 60mg/Kg (p < 0.0001) significantly elevated the levels of Vit-C in the cerebellum of F1 female rat pups as compared to the VPA alone induced group. ZNO at 60mg/Kg/p.o treatment alone did not yield statistically significant variation (Figure 5c).

3.12. Effect of postnatal oral ZNO administration on prenatal VPA-induced histopathological changes in the cerebellum of F1 female rat pups using H&E staining

The coronal section of the H&E-stained cerebellum was analysed for the neuronal architecture, and the loss of pyramidal neurons was visualised at 400X microscopic fields. Photomicrographs of the control group showed distinctively intact and clear differentiation of the outer molecular layer, Purkinje layer and granular layer. a) normal neuronal matrix was exhibited (green arrow) with normal capillary blood vessels (pink arrow). Normal Purkinje cell neuronal architecture was observed (yellow) with normal granular layer morphology (white arrow). b) The prenatal VPA (500mg/Kg/i.p) induced group showed mild neurodegeneration in the Purkinje layer (red arrow), accompanied by pyknotic irregular

hyperchromatic nuclei (light blue colour) and vascular proliferation (black arrow) when compared to the control group. c) The prenatal VPA (500mg/Kg/i.p) induced rat pups when orally administered with ZNO at 30mg/Kg showed normal blood capillaries (pink arrow), with few pyknotic irregularities (light blue arrow). d) ZNO administration at 60mg/Kg/p.o showed normal neurofibrillary matrix, normal blood capillaries (pink arrow). Purkinje cellular architecture was normal with intact nuclei (yellow arrow) when compared with the VPA alone-induced group. e) The ZNO 50mg/Kg/p.o alone administered group showed no substantial changes in its neuronal architecture from the control (Figure 6).

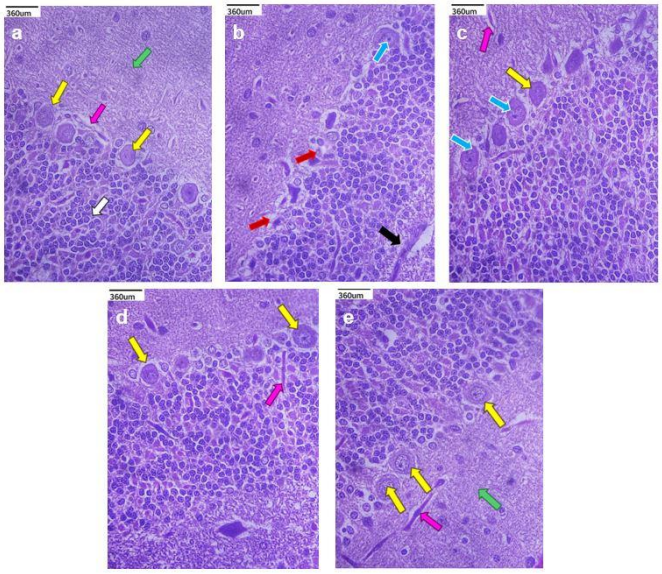

Figure 6: Effect of postnatal oral ZNO administration on prenatal VPA-induced histopathological changes in the cerebellum using H&E staining. The coronal sections are visualised at 400x total microscopic fields at a scale of 340µm. a) represents the control with a normal granular layer (white arrow), normal blood vessels (pink arrow), healthy Purkinje cells (yellow arrow), with a normal neurofibrillary matrix (green arrow). b) represents the VPA 500mg/Kg/i.p induced group with mild neurodegeneration in the Purkinje cells (red arrow), with irregular pyknotic hyperchromatic nuclei (light blue colour), and vascular proliferation (black). c) represents the VPA 500mg/Kg/i.p + ZNO 30mg/Kg/p.o group with few hyperchromatic nuclei and normal blood structures. d) represents the VPA 500mg/Kg/i.p + ZNO 60mg/Kg/p.o group with normal Purkinje cells and normal blood capillaries. e) represents the ZNO 60mg/Kg/p.o alone administered group.

4. DISCUSSION

Clinical studies have shown that administration of VPA monotherapy (≥ 1000 mg/day) as an anti-seizure drug during pregnancy has been linked to infants being more prone to be diagnosed with ASD-like symptoms [46]. VPA acid exposure on the 12th day of the rodent gestational period, which corresponds to the 1st trimester in humans, has been shown to elicit autistic features in F1 pups similar to those in clinical autistic patients [12,47]. The VPA-induced model has been extensively validated and is considered a gold standard for assessing phenotypes closely associated with ASD, as well as for assaying and evaluating possible therapeutics for the attenuation of core symptoms [14,15,48,49]. In a study conducted by Elnahas [10], they found that prenatal VPA exposure showed enhanced autistic phenotypes, such as decreased sociability index, increased repetitive behaviour, higher anxiousness and a significant increase in grooming behaviour, memory impairment, delay in achieving typical neurodevelopmental targets, increased oxidative stress, accompanied by reduced antioxidant status, validated by changes in histopathological architecture in the prefrontal cortex, hippocampus and cerebellar cortex, than in postnatal VPA exposure. Several studies have described male pups as being predominantly affected by prenatal VPA exposure than females, indicating the possibility of sex-specific phenotypes [50-53], however, it may be argued that, due to the apparent lack of extensive studies in autistic female pups (primarily due to DSM-V criteria), sex-based discrepancies might also have contributing factors.

Structural abnormalities in the cerebellum have been described in ASD. Several neuroimaging studies have shown that reduced density in Purkinje cells, disorganised cerebellar microarchitecture, and

structural and functional alterations in lobules VI (involved in executive functions and language processing) and VII, which may contribute towards altered motor control and coordination, cognition and language processing. However, the exact mechanism of how cerebellar dysfunction leads to ASD-like symptoms is yet to be elucidated [54].

Our current study evaluated the role of ZNO against VPA-induced autistic features in F1 female pups. ZNO belongs to the family of methoxyphenols and its derivatives and is primarily the active constituent of dried ginger [55]. It has been well established for its anti-oxidant properties [56-58]. Based on the previous studies, ZNO has been studied as a potential treatment compound against several neurological diseases [29,30,59] and neurotoxicity [33,60] at varying dosages. For the first time, our study has reported the attenuative effect of ZNO in the cerebellum of F1 female pups with VPA-induced autistic features. The OFT conducted on PND 35 revealed diminished levels in general locomotory behaviour in prenatal VPA (500mg/Kg/i.p) induced F1 autistic females. Indicating a reduced liking to explore the open arena, which may further direct towards compromised ambulatory behaviour. This was in accordance with the study conducted by Sandhu [18]. The study also established that VPA induction at 600mg/Kg/i.p significantly hampered the locomotor ability of the F1 female pups in the OFT, with F1 female pups demonstrating a substantial decrease in the time spent in the central zone of the open arena. In our present study, ZNO administration at 30mg/Kg/p.o and 60mg/Kg/p.o was statistically significantly able to attenuate the VPA-induced changes in a dose-dependent manner when administered from PND 15 to PND 35. Loss of sensory balance was an indication of diminished motor coordination in prenatal VPA-induced autistic F1 female pups, as evident from the rotarod test. In a study conducted by Hussein [35], they reported that prenatal VPA induction at 500mg/Kg/i.p subsided the motor coordination of the pups along with spatial memory defects. This was in accordance with the results of our current study. Anxiety and repetitive behaviour were also assessed and found to be compromised on prenatal VPA 500mg/Kg/i.p induction on the 12th day of gestation. F1 rat pups showed diminished %spontaneous alterations in the Y maze test and increased latency to be immobile in the FST, indicating core-autistic features. ZNO was able to mitigate these changes in a dose-dependent manner at 30mg/Kg/p.o and 60mg/Kg/p.o.

Owing to the antioxidant properties of ZNO oral treatment, in our current study, it was shown that ZNO was significantly able to attenuate the oxidative stress markers – MDA due to LPO and total NO content in the cerebellum of F1 female pups, in a 20-day treatment schedule. Prenatal VPA-induction had increased the levels of oxidative stress in the cerebellum, which were mitigated in a dose-dependent manner on ZNO administration at 30mg/Kg/p.o and 60mg/Kg/p.o. The endogenous antioxidants GSH and catalase were abridged on VPA-induction, which was partially restored on ZNO administration. Vit-C, an exogenous antioxidant, was also significantly reduced on prenatal VPA induction. ZNO at optimally 60mg/Kg/p.o was able to mitigate the VPA-induced reduction in the cerebellum as compared to the 30mg/Kg/i.p. These findings were also in accordance with previously reported literature [16,35,61].

5. CONCLUSION

In conclusion, the current pilot study evaluated the neurological nuances associated with prenatal VPA induced ASD like symptoms in F1 female rat pups and the attenuative properties of oral administration of ZNO. The behavioural, biochemical and histopathological studies were able to substantiate that prenatal VPA induces ASD like manifestation in F1 female rat pups in accordance with various previously reported literature studies. For the first time, we report that ZNO was able to alleviate the ASD like symptoms by diminishing the levels of reactive oxygen species and increasing the levels of antioxidant status.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contribution

The corresponding author, Dr Sumathi Thangarajan, contributed towards the conceptualisation, design, and investigation of the current study, followed by the scrutiny of the final data, review and approval of the final manuscript. Ms Shruthi Sampatkumar contributed towards conducting all experiments, statistical data analysis, and the original draft of the manuscript.