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Resveratrol alone and its Combination with Pterostilbene amends Valproic Acid-Induced Autism in Swiss Albino Mice: Postnatal Model

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ABSTRACT

Objective: The present study was aimed to determine the therapeutic role of resveratrol and pterostilbene alone and combination in reversing the behavioral, biochemical, and histopathological alterations in valproic acid (VPA) induced oxidative stress and neuron damage in a postnatal model of autism.

Method: 13 days old Swiss albino mice pups were randomly divided into five groups of six each, vehicle-treated group (1 mg/mL CMC), autistic group (VPA 400 mg/kg, sc), resveratrol (20 mg/kg, po), pterostilbene (10 mg/kg, po), and combination of resveratrol (10 mg/kg, po) + pterostilbene (5 mg/kg, po) group. On postnatal day (PND) 14, valproic acid (VPA) 400 mg/kg, sc, was administered to all except vehicle treated group. Resveratrol and/or pterostilbene was administered daily from PND 14 to 40. During the treatment, period various behavioural parameters were analysed. At the end of study, animals were sacrificed for biochemical estimations and histopathological study.

Results: Single time administration of VPA at 400 mg/kg, sc, effectively induced autism. Treatment with resveratrol, pterostilbene, and the combination gave significant recovery in behavioral activity, biochemical, and histopathological alterations in mice when compared with the autistic group.

Conclusion: Resveratrol and pterostilbene are good nutraceuticals in reversing the valproic acid-induced autistic deficits, in this study combination of resveratrol and pterostilbene provide superior results on recovery over individual therapy, it is suggested that this combination therapy potentiates the benefits and is more suitable for autism therapy.

Keywords: Autism, Oxidative stress, Pterostilbene, Resveratrol, Valproic acid.

1. INTRODUCTION

Autism is a chronic neurodevelopmental disorder with complex neurobehavioral conditions.[1] Autism appears at an early age of 1 to 2 years, globally 21.7 million (1 in 68) peoples are affected by autism,[2,3] and the overall incidence of autism is believed to be consistent around the globe (CDC, 2014). In India, over 18 million people are with autism.[4] The government of India recognizes autism as a developmental disability, it occurs four to five times more often in boys than girls.[5] All autism disorders including autism, childhood disintegrative disorder, and pervasive developmental disorder-not otherwise specified (PDD-NOS) and Asperger syndrome has been merged into one umbrella diagnosis of autism spectrum disorder (ASD). Autism can be associated with impaired communication, social interaction, intellectual disability and difficulties in motor coordination, attention and physical health issues, such as, sleep, gastrointestinal disturbances, some persons with autism excel in visual skills, music, math, and art.[6]

Autism is mainly caused by deficits in nerve growth, development, and regulation. This affects the information processing in the brain to organize and communicate between nerve cells and their synapses leading to disturbances in communication between two neurons.[7] In

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current situations, rather than genetic and siblings, the main important factor for the contribution of autism is maternal conditions. Maternal illness, toxicants exposure (VPA, thalidomide, and misoprostol) deficiency of Vitamin D, folic acid during pregnancy, and certain difficulties during birth will also disturb the timing of brain development.[8] Numerous findings were reported underlying the biological abnormalities in autism; such as, irregularities in neurotransmitter (serotonin, dopamine, and acetylcholine); enzyme activities [MAO-A and B, Acetylcholinesterase (AchE)]; decreased cerebral blood flow; elevated markers of oxidative stress; altered intestinal microbial flora.[9]

Modeling of autism should provide a key link for treatment. Among all induction methods, valproic acid-induced pre and postnatal methods will establish perfect relation to autism. VPA is a widely used antiepileptic drug, it is a potent teratogen in humans that is associated with significantly increased risk of spina bifida, fetal syndrome with defective posterior neural tube closure, as well as, cardiac malformations, cleft palate, and limb defects.[10,11] Early exposure of VPA during the prenatal or postnatal period can serve as triggering factors for oxidative stress by lipid peroxidation which disrupt the neuron development.[12]

Stilbenes and their derivatives possess a vast number of applications. Many naturally occurring stilbene compounds, like resveratrol and pterostilbene have multi-functional effects, such as, antioxidant, anti-inflammatory, anti-cancer, and cardio-protectant actions.[13]

Resveratrol (3, 5, 4`-trihydroxystilbene) a naturally occurring non-flavonoid polyphenol belongs to the phytoalexin family.[22] Resveratrol is found in peanuts, the skin of grapes, blueberries, and senna, it has cardioprotection, anticancer, antidiabetic, antidepression, and neuroprotective effects against diabetes-induced oxidative damage.[14] Resveratrol exhibits antioxidant activities through inhibiting quinone reductase, which in turn up-regulates the expression of cellular antioxidant and detoxification of enzymes to improve cellular resistance to oxidative stress.[15,16]

Pterostilbene (trans-3, 5-dimethoxy-4-hydroxystilbene) is a natural dimethylated analog of resveratrol derived compound found primarily in *Pterocarpus marsupium* (PM) heartwood and blueberries, it is a potent analog of resveratrol. Compared to resveratrol, pterostilbene has good absorption and bioavailability.[17,18] It has therapeutic properties in a vast range of human diseases that include neurological (Alzheimer's), cardiovascular, metabolic, and hematologic disorders. Clinical potency in neuroprotection, antioxidant capabilities, and high bioavailability makes it a potentially advantageous, therapeutic agent for the present study.[19-21]

2. MATERIALS AND METHODS

2.1. Drugs and Chemicals

Samilabs, Hyderabad: Resveratrol (95%) and Pterostilbene (90% Silbinol); Sun Pharmaceuticals, Mumbai: Sodium Valproate injection (Encorate); Sigma Aldrich, USA: DTNB [5, 5'-dithiobis (2-nitro benzoic acid)], Eserine, Acetylthiocholine iodide, reduced Glutathione; Himedia laboratories Pvt. Ltd., Mumbai: 2-Thiobarbituric acid; S. D. Fine Chemicals Ltd., India: All other reagents of analytical grade.

2.2. Experimental Animals

Swiss albino mice pups, weighing 5 to 10 grams, at the age of 2 weeks were used in the study. They were placed along with the mother, who was maintained on standard laboratory pellet chow diet of Provimi Limited (India), provided water *ad libitum*, and were kept under standard conditions at 23 to 25°C, 35 to 60% humidity, and 12 hours light /dark cycle. The experimental protocol was duly approved by Institutional Animal Ethics Committee (IAEC) and care of the animals was carried out as per the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA) (Protocol. No: I/IAEC/LCP/018/2014/SM-30♂ or ♀)

2.3. Experimental Design

Female mice were regularly monitored for the estrous cycle. On an estrous day, mice were kept for mating with the male mice for a whole night. The presence of sperms in vaginal smears on the next day morning indicates pregnancy day 0. From the first day of pregnancy, mice were checked daily for health and weight. When the pups were born, the day of birth was recorded as PND 1. On PND 13, pups were randomly divided into five groups, each group with six mice (n = 6), *viz.*,

Group I:	Vehicle treated group (1 mg/mL CMC, po) from PND 14 to 40.
Group II:	Autistic group (VPA 400 mg/kg, sc) only on PND 14.
Group III:	VPA + resveratrol (20 mg/kg, po) PND 14 to 40.
Group IV:	VPA + pterostilbene (10 mg/kg, po) PND 14 to 40.
Group V:	VPA + resveratrol (10 mg/kg, po) + pterostilbene (5 mg/kg, po) PND 14 to 40.

During PND 14 to 40, mice pups were subjected to various behavioral testing to assess motor coordination, nociceptive response, locomotion, anxiety, and cognition on various postnatal days up to PND 40 shown in

Figure 1. At the end of the study, animals were sacrificed; the brain was isolated for biochemical estimations and histopathological examination.

2.4. Behavioral Studies

2.4.1. Negative Geotaxis

Negative geotropism was tested on postnatal days 14 to 19 by placing the mouse face down along a 45° incline in a temperature-controlled environment. Latency to turn 180° such that the head was facing upward along the incline was recorded with a maximum of 30 seconds for each trial.[22]

2.4.2. Swimming Performance

An aquarium filled with water (28–29°C) was used for swimming tests on PNDs 16, 18, and 20. Each animal was put at the center of the aquarium and was observed for 5 to 10 seconds. The swimming performance was evaluated according to the position of nose and head (angle) on the surface of the water. The angle of swimming was rated as follows: 0-head and nose below the surface; 1-nose below the surface; 2-nose and top of the head at or above the surface but ears still below the surface; 3-the same as two except that the water line was at mid-ear level; and 4-the same as three except that the water line was at the bottom of ears. Thereafter, the test pups were dried and returned to the home cages. Swimming is a measure of motor development and the integration of a coordinated series of reflex responses.[23]

2.4.3. Motor Coordination Test

This utilizes a rotarod maintained at a speed of 40 rotations per minute (RPM). On PND 24, 25, and 26, mice were placed individually on the rotating rod and the time taken by each animal to maintain its balance on the rod over a 5 minutes period was recorded.[24]

2.4.4. Locomotor Activity using Actophotometer

Spontaneous locomotor activity was measured daily on PND 34 to 37 in an activity cage having dimensions of 39 × 28 × 26 cm. The breakage of photo beams was monitored with infrared sensors and automatically recorded for 5 minutes.[25]

2.4.5. Nociception Test

Nociception activity was observed through Eddy's hot plate method. On PND 37, 38, and 39, mice were placed individually on a hot plate (55 ± 0.3°C) and latency of the first hind-paw response was recorded. The hind-paw response was defined as either a foot shake or a paw lick. The cut-off time of 30 seconds was maintained.[22]

2.4.6. Open Field Habituation

The exploratory behavior of the mice was evaluated by the open-field habituation task method. Mice were placed in a 40 × 50 × 60 cm open field whose brown linoleum floor was divided into 12 equal squares by white lines and left to explore it freely for 5 minutes on PND 40. The number of line crossings and head dipping was counted.[22]

2.4.7. Elevated Plus-Maze Test for Anxiety

The elevated plus-maze consisted of two open arms (25 × 5 cm) and two enclosed arms of the same size, with 15 cm high opaque walls. The maze elevated to a height of 55 cm above the floor. Each mouse was placed on PND 40 in the central square of the maze (5 × 5 cm), facing one of the closed arms. Mouse behavior was recorded during a 10 minutes test period on PND 40. The number of entries into the open/closed arms and time spent in each of them was utilized for data analysis.[23,26]

2.5. Biochemical Parameters

Mice brains were isolated washed with ice-cold 0.1M phosphate buffer pH 7.4 to remove the blood and homogenized with 0.1M phosphate buffer saline solution. Then homogenate and the resultant supernatant were used for further biochemical estimations, such as, AChE and antioxidants.

2.5.1. Estimation of Superoxide Dismutase

Superoxide dismutase (SOD) activity was determined by the pyrogallol oxidation method. One unit SOD activity is defined as the amount of enzyme that inhibits the rate of auto-oxidation of pyrogallol by 50%. The reaction is initiated by adding pyrogallol and the change in optical density was recorded at 420 nm.[27]

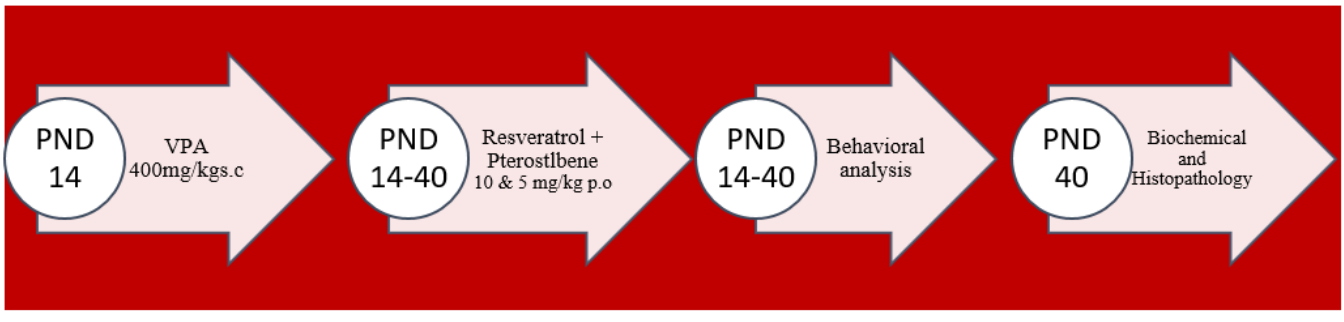


Figure 1: Experimental design representation

2.5.2. Estimation of Catalase

The rate of decomposition of H_2O_2 to water and molecular oxygen is proportional to the activity of catalase. The sample containing catalase is incubated in the presence of a known concentration of H_2O_2. After incubation for exactly one minute, the reaction is stopped with ammonium molybdate. The amount of H_2O_2 remaining in the reaction is then determined by the oxidative coupling reaction between molybdate and H_2O_2.[28]

2.5.3. Estimation of Reduced Glutathione (GSH)

GSH was determined by its reaction with 5, 5-dithiobis (2-nitrobenzoic acid) (Ellman's reagent) to yield a yellow chromophore which was measured spectrophotometrically. The brain homogenate was mixed with an equal amount of 10% trichloroacetic acid (TCA) and centrifuged (Remi cold centrifuge) at 2,000 × g for 10 minutes at 4ºC. To 0.1 mL of a processed tissue sample, 2 mL of phosphate buffer (pH 8.4), 0.5 mL of 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), and 0.4 mL of double-distilled water were added and the mixture was shaken. The absorbance was read at 412 nm within 15 minutes.[29]

2.5.4. Estimation of AChE Activity

The AChE activity was measured in brain tissue by the reaction of thiocholine with dithiobisnitrobenzoate ions. The rate of formation of thiocholine from acetylcholine iodide in the presence of brain cholinesterase was measured using a spectrophotometer (Shimadzu 1800) at a wavelength of 412 nm.[30]

2.5.5. Estimation of MDA

The level of malondialdehyde was determined by the procedure described by Ohkawa *et al*., 2% tissue homogenate was prepared by weighing 100 mg of tissue appropriately and homogenizing in 5 mL of 0.1M phosphate buffer pH 7.4 with Remi motor at a speed of 2,500 rpm for 2 minutes in ice-cold surrounding environment. The homogenate was centrifuged at 200 rpm for 2 minutes. 500 µl of 2% tissue homogenate in 0.15 mol/L KCl was mixed with 200 µl 8.1% SDS and then incubated at room temperature for 5 minutes. 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL 0.8% thiobarbituric acid was added. The reaction mixture was heated at 95ºC for 90 minutes. The mixture was allowed to cool and 1 mL of distilled water was added 0.5 mL butanol/pyridine (15:1) solution was then added under agitation using a vortex. This solution was centrifuged at 1,000 x g for 15 minutes, and the resultant colored layer was separated and measured at 532 nm using spectrophotometer.[31]

2.5.6. Estimation of Nitrite Level

Nitrite and nitrate determinations in biological material are increasingly being used as markers of NO production. Nitric oxide production was quantified by measuring nitrite, a stable oxidation end product of NO. Briefly, nitrite production was determined by mixing 50 mL of the assay buffer with 50 mL of Griess reagent [1.5% sulfanilamide in 1M HCl plus 0.15% N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water, v:v]. After 10 minutes of incubation at room temperature, the absorbance at 540 nm was determined and nitrite concentrations were calculated from the sodium nitrite standard curve. All measurements were performed in triplicate.[32]

2.6. Histopathology

At the end of the study, on PND 40, mice were sacrificed, brains were isolated, placed immediately in 10% neutral formalin solution, and processed and embedded in paraffin. Sagittal sections of the cerebellum (5 µm thick) were stained with hemotoxylin and eosin (HandE) and analyzed using a light microscope for changes in the cerebellum.

2.7. Statistical Analysis

All data are presented as mean ± S.E.M. The significance of difference among the groups was assessed using a one-way analysis of variance (ANOVA) followed by Tukey's test, using GraphPad Prism software, and $P < .05$ was considered significant.

3. RESULTS

3.1. Behavioral Parameters

3.1.1. Effect of Resveratrol and Pterostilbene on Negative Geotaxis

In the autistic group, time taken to re-orient along the inclined plane was increased when compared with the vehicle-treated group $^{###}P < .001$. Treatment with resveratrol and pterostilbene alone and combination showed a decrease in the time taken to re-orient $^{***}P < .001$, $^{**}P < .01$, and $^{*}P < .05$, when compared with the autistic group on PND 14, 15, 16, 17, 18, and 19. The results are shown in Figure 2.

3.1.2. Effect of Resveratrol and Pterostilbene on Swimming Performance

There was a decrease in swimming performance in the autistic group compared with the vehicle-treated group $^{###}P < .001$. Treatment with resveratrol and pterostilbene alone and combination showed an increase in swimming performance $^{***}P < .001$, $^{**}P < .01$, and

*$P < .05$, when compared with the autistic group on PND 16, 18, and 20. The results are shown in Table 1.

3.1.3. Effect of Resveratrol and Pterostilbene on Motor Activity using the Rotarod Test

Autistic mice expressed a shorter time to fall from the rotating rod than the vehicle-treated group $^{###}P < .001$. Treatment with resveratrol and pterostilbene alone and in combination showed improved motor activity and grip strength $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$, than autistic group on PND 26, 27, and 28. The results are shown in Table 2.

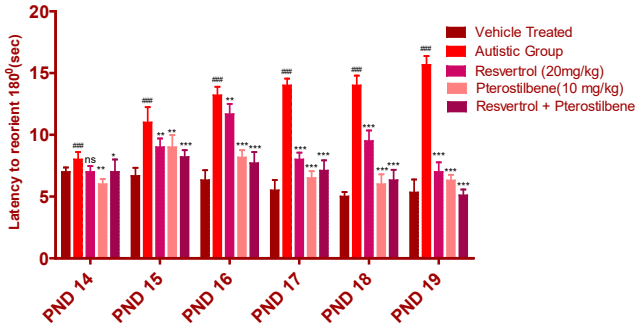


Figure 2: Effect of resveratrol and pterostilbene on negative geotaxis
Values are expressed as mean ± SEM, n = 6. (ANOVA + Tukey's tests) $^{###}P < .001$ comparisons of vehicle treated *vs.* autistic group, $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$ comparison of autistic group *vs.* treatment groups.

3.1.4. Effect of Resveratrol and Pterostilbene on Locomotor Activity using Actophotometer

Actophotometer was used to evaluate locomotor activity in mice. In autistic mice, there was increased locomotion due to hyperactive nature compared with the vehicle-treated group $^{###}P < .001$. Treatment with resveratrol and pterostilbene alone and in combination showed decreased in hyperactivity $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$ than autistic group on PND 26, 27, and 28. The results are shown in Table 3.

3.1.5. Effect of Resveratrol and Pterostilbene on Thermal Nociception

There was a significant increase in latency to withdraw the hind paw was observed in the autistic group compared with the vehicle-treated group $^{###}P < .001$. Treatment with resveratrol and pterostilbene alone and in combination showed decreased in latency to withdraw the hind paw

Table 1: Effect of resveratrol and pterostilbene on swimming performance

	Swimming performance in the aquarium		
Groups	*PND 16*	*PND 18*	*PND 20*
Group I (vehicle)	3.83 ± 0.66	3.6 ± 0.21	3.83 ± 0.16
Group II (VPA 400 mg/kg)	2.30 ± 0.21$^{###}$	2.16 ± 0.16$^{###}$	2.33 ± 0.21$^{###}$
Group III (VPA + resveratrol 20 mg/kg)	2.61 ± 0.21	2.83 ± 0.3	3.33 ± 0.3*
Group IV (VPA + pterostilbene 10 mg/kg)	3.5 ± 0.22**	3.66 ± 0.21***	3.4 ± 0.11***
Group V (VPA+ resveratrol 10 mg/kg + pterostilbene 5 mg/kg, po)	3.83 ± 0.16***	3.5 ± 0.22***	3.67 ± 0.21****

Values are expressed as mean ± SEM, n = 6. (ANOVA + Tukey's tests) $^{###}P < .001$ comparisons of vehicle-treated *vs.* autistic group, $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$ comparison of autistic group *vs.* treatment groups

Table 2: Motor activity in rotarod test

	Latency to fall (sec)		
Groups	*PND 24*	*PND 25*	*PND 26*
Group I (vehicle)	5 ± 0.36	5.5 ± 0.42	6.83 ± 0.47
Group II (VPA 400 mg/kg)	2.5 ± 0.22$^{###}$	2.83 ± 0.3$^{###}$	2.5 ± 0.22$^{###}$
Group III (VPA + resveratrol 20 mg/kg)	4 ± 0.36*	4.66 ± 0.42*	5 ± 0.36**
Group IV (VPA + pterostilbene 10 mg/kg)	4.66 ± 0.33***	5.83 ± 0.4***	5.5 ± 0.42***
Group V (VPA+ resveratrol 10 mg/kg + pterostilbene 5 mg/kg, po)	7 ± 0.36***	6.16 ± 0.47***	7 ± 0.44***

Values are expressed as mean ± SEM, n = 6. (ANOVA + Tukey's tests) $^{###}P < .001$ comparisons of vehicle-treated *vs.* autistic group, $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$ comparison of autistic group *vs.* treatment groups.

Table 3: Effect of resveratrol and pterostilbene on locomotor activity

	Locomotor activity (no.of beam splits)		
Groups	*PND26*	*PND27*	*PND28*
Group I (vehicle)	191.2 ± 2.81	211.2 ± 6.64	175 ± 5.66
Group II (VPA 400 mg/kg)	363.8 ± 5.75$^{###}$	348 ± 9.41$^{###}$	400.3 ± 8.91$^{###}$
Group III (VPA + resveratrol 20 mg/kg)	331.7 ± 4,025**	311.2 ± 4.45**	343.3 ± 8.88***
Group IV (VPA + pterostilbene 10 mg/kg)	281.5 ± 0.94***	259.2 ± 8.404***	237.8 ± 5.67***
Group V (VPA+ resveratrol 10 mg/kg + pterostilbene 5 mg/kg, po)	203 ± 5.63***	205.5 ± 4.28***	192.8 ± 5.7***

Values are expressed as mean ± SEM, n = 6. (ANOVA + Tukey's tests) $^{###}P < .001$ comparisons of vehicle-treated *vs.* autistic Group, $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$ comparison of autistic group *vs.* treatment groups.

***$P < .001$, **$P < .01$, *$P < .05$ compared to the autistic group on PND 37, 38, and 39. The results are shown in Table 4.

3.1.6. Effect of Resveratrol and Pterostilbene on Social Interaction and Open Field Activity

Social interaction and open field test is used to evaluate the patterns of exploration and behavior with other mice. In the autistic group, there was lower in exploration activity compared to the vehicle-treated group $^{###}P < .001$. Treatment with resveratrol and pterostilbene alone and in combination showed significant improvement in exploratory activity ***$P < .001$, **$P < .01$, and *$P < .05$ compared to the autistic group on PND 26 to 28. The results are shown in Table 5.

3.1.7. Effect of Resveratrol and Pterostilbene on Anxiety using Elevated Plus-Maze Test

Open arm exploration/ time spent on open arms of the elevated plus-maze was less in the autistic group compared with the vehicle-treated group $^{###}P < .001$. Treatment with resveratrol and pterostilbene alone and combination showed an increase in the number of open arm exploration and time spent in the open arm ***$P < .001$, **$P < .01$, *$P < .05$ compared to the autistic group on PND 40. The results are shown in Table 6.

3.2. Biochemical Parameters

3.2.1. Effect of Resveratrol and Pterostilbene on Antioxidants (SOD, Catalase, GSH)

In autistic group, decreased levels of antioxidants (SOD, catalase, and GSH) were seen when compared with the vehicle-treated group $^{###}P < .001$. Treatment with resveratrol and pterostilbene alone and in combination showed increases antioxidant levels ***$P < .001$, **$P < .01$, *$P < .05$ compared to autistic group. The results are shown in Table 7.

3.2.2. Effect of Resveratrol and Pterostilbene on AchE, MDA, Nitrite Level

In autistic group, increased levels of AchE, MDA, and nitrite level were seen when compared with the vehicle-

Table 4: Effect of resveratrol and pterostilbene on thermal nociception

	Latency to paw withdrawal (sec)		
Groups	*PND 37*	*PND 38*	*PND 39*
Group I (vehicle)	3.5 ± 0.22	54.33 ± 0.33	4.16 ± 0.3
Group II (VPA 400 mg/kg)	7.5 ± 0.56$^{###}$	10 ± 0.44$^{###}$	8 ± 0.51$^{###}$
Group III (VPA + resveratrol 20 mg/kg)	5.16 ± 0.4**	7.83 ± 0.4**	5.66 ± 0.42**
Group IV (VPA + pterostilbene 10 mg/kg)	4.5 ± 0.22***	6.33 ± 0.49***	4.5 ± 0.42***
Group V (VPA+ resveratrol 10 mg/kg + pterostilbene 5 mg/kg, po)	4.5 ± 0.42***	4.16 ± 0.3***	3.83 ± 0.3***

Values are expressed as mean ± SEM, n = 6. (ANOVA + Tukey's tests) $^{###}P < .001$ comparisons of vehicle-treated *vs.* autistic group, ***$P < .001$, **$P < .01$, *$P < .05$ comparison of autistic group *vs.* treatment groups.

Table 5: Effect of resveratrol and pterostilbene on social interaction and open field activity

	Social interaction (time in seconds)	*Open field habituation*	
Groups		*Line crossings*	*Head dipping's*
Group I (vehicle)	360.7 ± 9.28	84.17 ± 2.05	25.67 ± 1.626
Group II (VPA 400 mg/kg)	207.8 ± 7.12$^{###}$	183.3 ± 4.64$^{###}$	13.5 ± 0.67$^{###}$
Group III (VPA + resveratrol 20 mg/kg)	258.7 ± 11.33**	156.7 ± 4.64**	20 ± 0.96**
Group IV (VPA + pterostilbene 10 mg/kg)	357.5 ± 9.61***	136.5 ± 5.892***	21.5 ± 0.46***
Group V (VPA+ resveratrol 10 mg/kg + pterostilbene 5 mg/kg, po)	362 ± 7.8***	87.17 ± 2.18***	26.17 ± 1.35***

Values are expressed as mean ± SEM, n = 6. (ANOVA + Tukey's tests) $^{###}P < .001$ comparisons of vehicle-treated *vs.* autistic group, ***$P < .001$, **$P < .01$, *$P < .05$ comparison of autistic group *vs.* treatment groups.

Table 6: Effect of resveratrol and pterostilbene on elevated plus-maze test

Groups	*No. of entries in open arms arms*	*Time spent in open arms (sec)*	*No. of entries in enclosed arm*	*Time spent in enclosed arms (sec)*
Group I (vehicle)	14 ± 0.63	186.5 ± 4.08	6.5 ± 0.61	412.2 ± 7.79
Group II (VPA 400 mg/kg)	4.66 ± 0.49$^{###}$	86.5 ± 2.86$^{###}$	11 ± 0.44$^{###}$	513.5 ± 7.302$^{###}$
Group III (VPA + resveratrol 20 mg/kg)	9.66 ± 1.05***	131.4 ± 2.48***	7.5 ± 0.42***	468.6 ± 9.41***
Group IV (VPA + pterostilbene 10 mg/kg)	12 ± 0.73***	157.7 ± 5.97***	6.83 ± 0.6***	442.3 ± 3.29***
Group V (VPA+ resveratrol 10 mg/kg + pterostilbene 5 mg/kg, po)	14.17 ± 0.7***	188.3 ± 3.87***	5.66 ± 0.33***	411.7 ± 6.1***

Values are expressed as mean ± SEM, n = 6. (ANOVA + Tukey's tests) $^{###}P < .001$ comparisons of vehicle-treated *vs.* autistic group, ***$P < .001$, **$P < .01$, *$P < .05$ comparison of autistic group *vs.* treatment groups.

Table 7: Effect of resveratrol and pterostilbene on antioxidants

Groups	SOD (U/mg protein)	Catalase (U/mg protein)	Glutathione (U/mg protein)
Group I (vehicle)	15.34 ± 0.55	1.07 ± 0.051	36.54 ± 2.651
Group II (VPA 400 mg/kg)	6.79 ± 0.36###	0.318 ± 0.013###	27.15 ± 1.655###
Group III (VPA + resveratrol 20 mg/kg)	9.072 ± 0.5*	0.722 ± 0.023***	39.71 ± 1.653**
Group IV (VPA + pterostilbene 10 mg/kg)	12.25 ± 0.39***	0.94 ± 0.063***	47.2 ± 1.54***
Group V (VPA+ resveratrol 10 mg/kg + pterostilbene 5 mg/kg, po)	14.65 ± 0.24***	1.108 ± 0.045***	41.07 ± 3.175***

Values are expressed as mean ± SEM, n = 6. (ANOVA + Tukey's tests) ###P <.001 comparisons of vehicle-treated *vs.* autistic group, ***P <.001, **P <.01, *P <.05 comparison of autistic group *vs.* treatment groups.

Table 8: Effect of resveratrol and pterostilbene on AchE, MDA, and nitrite levels

Groups	AchE (μM/min/mg tissue)	MDA (μg/mg protein)	Nitrite (μg/mg protein)
Group I (vehicle)	1.3 ± 0.15	36.38 ± 2.07	78.86 ± 2.73
Group II (VPA 400 mg/kg)	7.15 ± 0.38###	133.1 ± 7.21###	105.1 ± 4.14###
Group III (VPA + resveratrol 20 mg/kg)	5.2 ± 0.44**	108.5 ± 5.63**	87.98 ± 5.3*
Group IV (VPA + pterostilbene 10 mg/kg)	2.254 ± 0.25***	75.89 ± 2.35***	68.64 ± 1.47***
Group V (VPA+ resveratrol 10 mg/kg + pterostilbene 5 mg/kg, po)	1.33 ± 0.144***	33.67 ± 1.9***	73.28 ± 2.108***

Values are expressed as mean ± SEM, n = 6. (ANOVA + Tukey's tests) ###P <.001 comparisons of vehicle-treated *vs.* autistic group, ***P <.001, **P <.01, *P <.05 comparison of autistic group *vs.* treatment groups.

treated group ###P < .001. Treatment with resveratrol and pterostilbene alone and combination showed decrease in the elevated levels of AchE, MDA, and nitrite level, ***P < .001, **P < .01, *P < .05 compared to autistic group. The results are shown in Table 8.

3.3. Histopathology

In this study, histopathological findings of a sagittal section of the cerebellum (HandE × 200) revealed the severe degeneration of neurons in the molecular and granular layer, loss of integrity of Purkinje fibers were seen in the cerebellum of the autistic group Figure 3b. Severe degeneration of neurons and loss of Purkinje fibers indicates VPA severely affected the development of neurons. Treatment with resveratrol and pterostilbene alone treatment, Figures 3c and d, clearly showed that there is a decrease in the degeneration (vacuole number and size) than in the autistic group. A combination of resveratrol and pterostilbene (Figure 3e). The tissue has a good recovery rate than alone treatment might be a summative effect. By this finding, it reveals that resveratrol and pterostilbene have the protective ability in VPA induced oxidative stress neurodegeneration by its potent anti-oxidants and neuroprotective effects.

4. DISCUSSION

The present study revealed that VPA at a dose of 400 mg/kg, sc, effectively induced autism in mice pups, behavioral symptoms are similar to autism in children. The postnatal method is most suitable to evaluate the potency of many therapeutic agents. Administration of sodium valproate on PND 14 significantly developed autistic symptoms in groups III, IV, and V.[23]

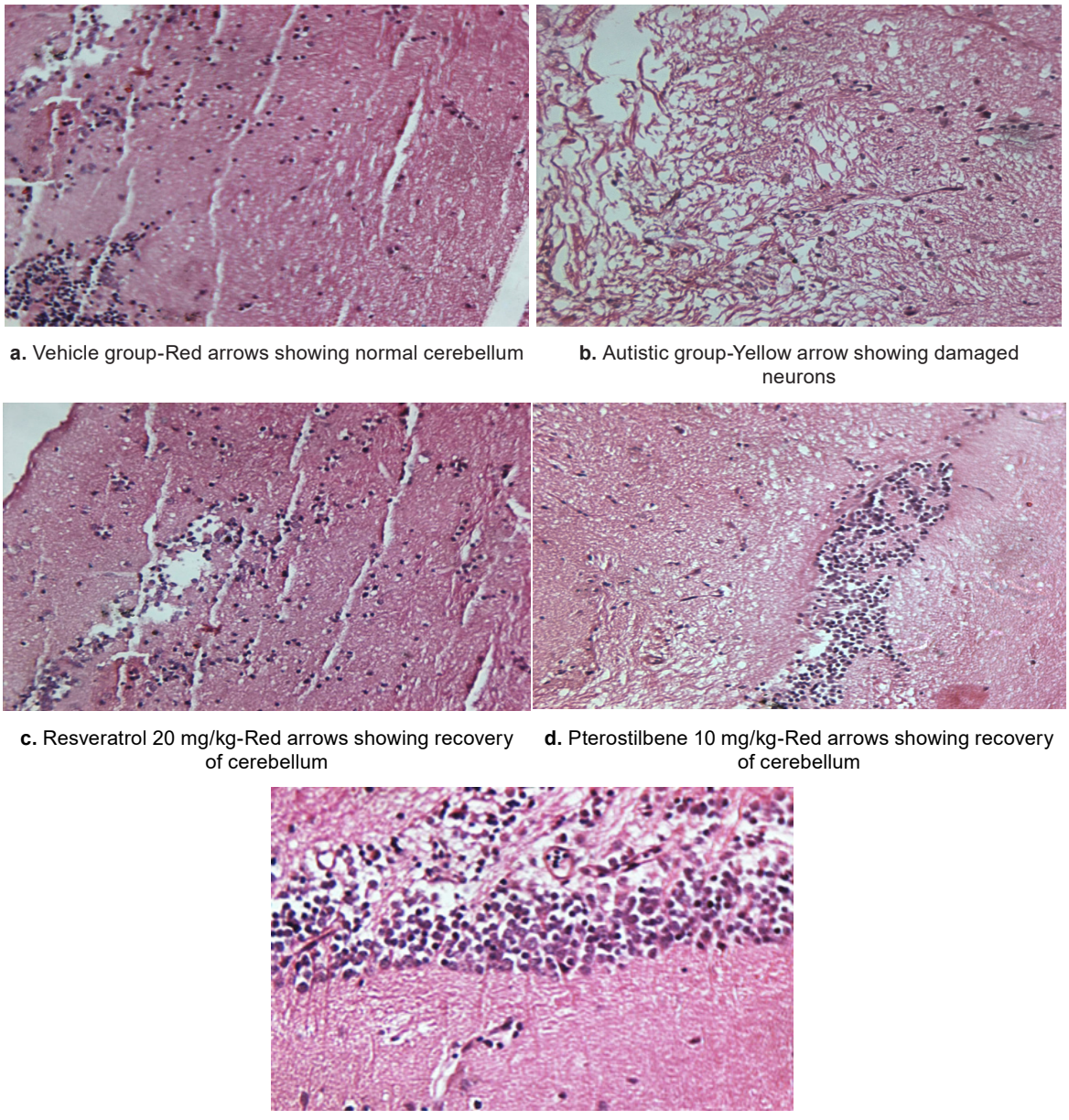
Negative geotaxis is to assess motor skill development and motor performance of cerebellar development and function. An increase in the time taken to reorient on the inclined plane was seen in the autistic group.[23] After the treatment with resveratrol and pterostilbene alone and combination, the reorientation time was effectively decreased from PND 17 onwards.

Swimming performance was evaluated to measure of motor development and integration of coordinated series of reflex responses. According to the position of nose and head (angle) on the surface of the water,[22] poor swimming performance was seen in the autistic group. Treatment with resveratrol, pterostilbene, and combination increased the rate of swimming score from PND 18 onwards and also restored the motor deficits.

Neuromuscular coordination that is regulated by the cerebellum can be evaluated by the Rotarod test.[33] Decreased time of retention in sodium valproate treated mice on a rotating rod can be due to the cerebellar damage.[34] Resveratrol and pterostilbene treatment resulted in an increase in latency to fall from the rotating rod.

Increased motor activity in actophotometer in autistic mice is due to hyperactivity/hyperexcitability which is seen in the autistic group may be caused by increased glutaminergic transmission.[36] Treatment with resveratrol and pterostilbene significantly reduced hyperactivity in mice.

Elevated plus-maze was used to analyze the anxiety and fear attributed to enhanced amygdala function.[37]



a. Vehicle group-Red arrows showing normal cerebellum

b. Autistic group-Yellow arrow showing damaged neurons

c. Resveratrol 20 mg/kg-Red arrows showing recovery of cerebellum

d. Pterostilbene 10 mg/kg-Red arrows showing recovery of cerebellum

e. Resveratrol + pterostilbene-Red arrows showing recovery of cerebellum. Restoration of cerebellar neurons and Purkinje fibers

Figure 3: Effect of resveratrol and pterostilbene on histopathological changes; **a.** Vehicle treated; **b.** Autistic group; **c.** Resveratrol 20 mg/kg group; **d.** Pterostilbene 10 mg/kg; **e.** Resveratrol + pterostilbene

Autistic group mice exhibited close arm explorations and open arm entry was less compared to the vehicle-treated group. Treatment with resveratrol and pterostilbene improves open arm exploration by its anxiolytic action.

Social deficits and lack of communication are seen in autistic people. Decreased social exploration was observed in mice with autism.[37] Treatment with resveratrol and pterostilbene improves social behavior in mice. This amelioration of autistic behavioral alterations with resveratrol and pterostilbene is possibly due to their protective role of the brain.

Neuronal cells at the early stages of development are more vulnerable to the effect of oxidative stress. Increased oxidative stress is one of the main factors for neurodevelopmental disturbances in autism. Postnatal exposure to environmental pro-oxidants, such as, valproic acid triggers the formation of reactive oxygen species (ROS).[38] ROS will interfere with the neurodevelopmental process by damaging lipids and proteins in cellular membranes and DNA in the cells.[39]

Postnatal exposure to valproic acid triggers the formation of reactive oxygen species (ROS). Oxidative stress causes peroxidation of lipids, they destroy the developing neurons.[33] Thus, antioxidant levels were declined in the autistic group. After the treatment with stilbenes (resveratrol and pterostilbene) antioxidants levels (SOD, catalase, and GSH), were restored to normal.

Acetylcholine is concerned with the learning and memory process, and it is hydrolyzed by enzyme acetylcholinesterase (AchE).[40] Due to VPA up-regulation of acetylcholinesterase enzyme seen in neuron synapse, reduces the concentration of acetylcholine. In autistic group, AchE activity reportedly increased, treatment with stilbenes (resveratrol and pterostilbene) reversed the levels of excess enzyme.

Due to oxidation, NO is converted into nitrite (NO_2^-). Increased NO_2^- in the brain could disrupt normal brain synaptic connections and neurodevelopment.[41] Increased nitrite levels were seen in autism. Treatment with resveratrol and pterostilbene decreased both MDA and nitrite levels contributing to protection from oxidative stress.

Histopathology of the cerebellum reveals VPA causes severe oxidative stress and neurodegeneration reported in the autistic group, which became normal after the treatment with resveratrol and pterostilbene. Thus, a combination of resveratrol and pterostilbene has a good recovery rate over alone treatment.

5. CONCLUSION

By these findings, we conclude that resveratrol and pterostilbene alone and the combination has a good protective ability in VPA induced oxidative stress, behavioral deficits, and cerebellar neurodegeneration by its powerful antioxidant, cognition-enhancing, and neuroprotective effects. It is suggested that this combination therapy potentiates the benefits and is more suitable for autism therapy.

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