

Original Article

DOSE-DEPENDENT AMELIORATION OF EPIGALLOCATECHIN-3-GALLATE AGAINST SODIUM VALPROATE INDUCED AUTISTIC RATS

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ABSTRACT

Objective: Autism is a neurodevelopment related disorder with a range of clinical presentations attending serious behavioral and neurological disorders among young children that now occur at epidemic rates in developing countries, India included. The objective of this research was to study the effect of epigallocatechin gallate (EGCG) on sodium valproate-induced autism rats.

Methods: On the 12th day of gestation wistar rats were administered with a single intraperitoneal injection of sodium valproate (VPA) (600 mg/kg body weight), which induced autism. The rats were treated with EGCG in varying doses 1, 2 and 5 mg/kg body weight via oral administration. The neuroprotectivity effect of the EGCG was followed by assessing the neurotransmitters and neurobiochemical activities such as serotonin, glutamate and nitrite levels in hippocampus and cerebellum region of the brain.

Results: Early prenatal exposure to VPA provokes autistic symptoms. Induction of autism significantly impinged the neurotransmitters and neurochemicals such as serotonin, glutamate and nitrite levels in the brain (hippocampus and cerebellum) increased significantly in the rats exposed to VPA. After treatment with an effective dose of EGCG 2 mg/kg body weight the neurotransmitters and neurochemicals levels were decreased when compared with control and VPA-exposed rats.

Conclusion: EGCG ameliorates and reverses autistic attributes possibly due to its neuroprotective activity which could pave the way for future investigation for the possible therapeutic approach.

Keywords: Autism, Neurotransmitters, VPA, EGCG

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INTRODUCTION

Autism is a devastating neurodevelopmental disorder in modern days with core symptoms of impaired social interactions, deficits in verbal and non-verbal communication, and sometimes self-injurious behaviors [1]. It is increasing rapidly in India in the ratio of 1:88 children due to increased exposure of environmental insults such as thalidomide, ethanol, and valproic acid due to increased stress during critical periods of neuronal development especially among genetically predisposed children [2]. VPA is on the market as an anticonvulsant since 1974 and is used in many countries because of its efficiency against several types of epilepsy. Exposing pregnant rats to VPA has been reported to result in abnormalities in the frontal, parietal, and temporal lobes of the cerebral cortex, cerebellum, and hippocampal anomalies associated with autism [3, 4].

Neurotransmitters, the neuronal signaling molecules play an important role in the normal development of the brain and are also important for maintaining functions such as memory, learning, behaviour, motor activity, etc. Neurotransmitter-mediated signaling is initiated by binding of specific neurotransmitters to its receptor proteins on the postsynaptic membrane, the number of which varies for each neurotransmitter. Biochemical studies have focused on neurotransmitters such as serotonin, norepinephrine, and γ -amino butyric acid (GABA), glutamate and ubiquitin. Serotonin (5-hydroxytryptamine, 5HT) is an inhibitory neurotransmitter monoamine derived from tryptophan. Serotonergic neurones innervate virtually all parts of the central nervous system, but, like all monoamines, are most concentrated in the brainstem. It is responsible for regulating learning, memory, sensory perception, noise sensitivity, behaviour, sleep and appetite [5-7]. So it is a key brain chemical, identified in the physiological abnormalities in autism spectrum disorders (ASD).

Green tea, the second most consumed beverage all over the world after water, is characterised by being rich (30% of dry weight) in

polyphenols. These polyphenols consist of four main components: (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin gallate (EGCG) [8]. EGCG is a major component of green tea polyphenols which improves the bioavailability for the treatment of cancer [9], parkinson's disease [10], alzheimer's disease [11] and diabetes mellitus [12]. Till date, there is no specific treatment for autism. Currently available treatments mainly aim at symptomatic relief. Hence there is a need to find therapeutic drugs. We report on the effect of EGCG on VPA-induced neurobiochemical alterations in autistic rats.

MATERIALS AND METHODS

Chemicals and reagents

VPA and EGCG were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade obtained from Himedia laboratory Ltd., Mumbai, India. EGCG was administered orally by every day at the dose of 1, 2 and 5 mg/kg body weight (b.w.)

Induction of Autism

All the animals (Wistar rats) were fed with the standard pellet diet (Hindustan Lever Ltd., Bangalore, India) and water was made available *ad libitum*. Food and water were replenished daily. The animals were housed in polycarbonate cages in a room with a 12 h day-night cycle, the temperature of 22 ± 2 °C and humidity of 45–64%. The experimental protocol was approved by the committee for research and animal ethics, Esma institute of technology laboratory, Karur (Vide No: DIC (2008/33/014/00049/BEYA/TN/1695(BSS)/2014) and were in accordance with the guidelines of Indian Council of Medical Research (ICMR) [7].

On the 12th d of gestation, female rats were divided into two groups.

Group I: Treated: Received a single intraperitoneal injection of VPA 600 mg/kg (n =12).

Group II: Control: Received physiological saline (n =12).

VPA was dissolved in saline at concentrations of 250 mg/ml [6]. Both VPA-treated and controlled female rats were housed individually and allowed to raise their own litters. The offspring is weaned on postnatal day (PND 20) and rats of either sex housed separately. Experiments are carried out only on male offspring.

Experimental design

On PND 20, male pups were divided into Eight groups (n=6).

Group 1 (Control): Normal offspring received saline PND 21-90.

Group 2 (EGCG 1 mg/kg): Normal offspring received EGCG PND 21-90.

Group 3 (EGCG 2 mg/kg): Normal offspring received EGCG PND 21-90.

Group 4 (EGCG 5 mg/kg): Normal offspring received EGCG PND 21-90.

Group 5 (VPA): Autistic offspring received normal saline from PND 21-90.

Group 6 (VPA+EGCG 1 mg/kg): Autistic offspring received EGCG from PND 21-90.

Group 7 (VPA+EGCG 2 mg/kg): Autistic offspring received EGCG from PND 21-90.

Group 8 (VPA+EGCG 5 mg/kg): Autistic offspring received EGCG from PND 21-90.

Animals were sacrificed on PND 90 by cervical dislocation and brains were isolated on ice and weighed.

Neurotransmitters and neurobiochemical parameters

Serotonin

The hippocampus and cerebellum were separated from brains were isolated on ice and 100 mg of tissue was homogenized with 5 ml of acidified butanol (0.68 ml of 0.01N Hydrochloric acid (HCl)). After centrifugation for 5 min at 3,000 rpm, 2.5 ml supernatant was pipetted into a glass tube and shaken mechanically for 5 min with 5 ml of n-heptane and 0.4 ml of 0.1N HCl. The phases were separated by centrifugation as before. To determine 5-HT, 0.4 ml aliquots of the aqueous phase were pipetted into test tubes and 2.4 ml 0.004% o-phthalaldehyde (OPT) in 10 N HCl was added.

It was kept for heating in boiling water bath 15 min. The tubes were cooled in water and fluorescence was measured using spectrofluorometer. Activation and fluorescent wavelengths were 360 and 470 nm respectively. Blanks were prepared by reacting 0.6 ml of the OPT solution with 0.1N HCl. Standards were prepared with 60 µg/ml in deionized water, diluted 1:100 for use with 0.1N HCl containing and 0.1 ml reacted with 0.6 ml of 0.004% OPT in HCl solution [13, 14].

Nitrite

Nitrite levels estimation is a measure of nitric oxide produced. The brain tissues (hippocampus and cerebellum) was weighed and homogenized in 50 mmol/l potassium phosphate buffer (pH 7.8) and centrifuged at 11,000 g for 15 min at 4°C. 200 µl of supernatant was mixed with 200 µl saturated vanadium trichloride in 1N HCl and immediately 200µl Griess reagent (0.1% N-(1-naphthyl) ethylene diamide dihydrochloride, 1% sulfanilamide in 5% phosphoric acid) were added and mixed with it, and incubated at 37°C for 30 min. The absorbance was measured at 540 nm using autoanalyzer. The concentration was calculated from a standard curve of potassium nitrite and expressed as micromoles of nitrate/nitrite [15, 16].

Glutamate

The weighed brain tissues (hippocampus and cerebellum) were taken in ice cold 10% trichloro acetic acid and homogenized. The homogenate was then centrifuged at 10,000 g for 10 min at 0°C. An aliquot (0.1 ml) of the tissue extract was taken in 0.2 ml of 0.014 M ninhydrin solution in 0.5 M carbonate-bicarbonate buffer. This was kept in a water bath at 60°C for 30 min. The tubes were then cooled and treated with 5 ml of alkaline copper tartrate reagent. After 10 min, the fluorescence was read at 377/451 (excitation/emission) in a spectrofluorometer. The amount of glutamate was expressed µmole/g of tissue [17].

Statistical analysis

The data were subjected to one-way analysis of variance followed by Duncan's multiple range test (DMRT) using SPSS software 12.0. Results were expressed as mean±SD for six rats in each group. P values<0.05 were considered significant.

RESULTS

Administration of VPA on the 12th d of gestation induced autism manifested by the following neurotransmitters. VPA exposure induces neurobiochemical alterations such as an increase in serotonin (5-HT) levels in both hippocampus and cerebellum abnormalities when compared with control rats on PND 90, these alternations have been considered to support the validity of the rat model of autism, because serotonin is the key neurotransmitter for autism induction. On treatment with EGCG (2 mg/kg body weight), there was a significant reduction in their serotonin levels as compared to other doses (1 mg/kg and 5 mg/kg body weight) in hippocampus and cerebellum regions of the brain in VPA-exposed rats (table 1).

Table 2 represents the induction of autism by VPA significantly (p<0.05) increased total nitrite levels compared to control group. Treatment with EGCG (2 mg/kg body weight) VPA-exposed rats significantly reversed the total nitrite (p<0.05), levels compared to other doses (1 mg/kg and 5 mg/kg body weight) in the brain (hippocampus and cerebellum).

Table 1: Effect of EGCG on serotonin in rats prenatally exposed to VPA

Groups	Brain tissue (µg/g of tissue)	
	Hippocampus	Cerebellum
Normal Control	0.48±0.05	044.50±24.34
EGCG (1 mg/kg)	0.47±0.04	045.61±24.10
EGCG (2 mg/kg)	0.46±0.03	045.53±24.00
EGCG (5 mg/kg)	0.45±0.03	044.10±23.21
VPA Control (250 mg/kg)	0.97±0.09 ^a	110.50±28.42 ^a
VPA (250 mg/kg)+EGCG (1 mg/kg)	0.87±0.07 ^b	090.12±25.62 ^b
VPA (250 mg/kg)+EGCG (2 mg/kg)	0.63±0.06 ^b	065.12±24.98 ^b
VPA (250 mg/kg)+EGCG (5 mg/kg)	0.77±0.07 ^b	079.12±24.99 ^b

Each column is mean±SD for six rats in each group. Significance was determined by one-way ANOVA followed by DMRT. ^aP<0.05 versus normal control, ^bP<0.05 versus VPA control.

Table 2: Effect of EGCG on nitrite levels in rats prenatally exposed to VPA

Groups	Brain tissue ($\mu\text{mol/g}$ of tissue)	
	Hippocampus	Cerebellum
Normal Control	069.72 \pm 3.99	090.51 \pm 4.5
EGCG (1 mg/kg)	068.62 \pm 3.98	089.67 \pm 4.4
EGCG (2 mg/kg)	068.98 \pm 3.97	088.34 \pm 4.4
EGCG (5 mg/kg)	068.83 \pm 3.98	088.17 \pm 4.3
VPA Control (250 mg/kg)	122.20 \pm 8.34 ^a	140.08 \pm 6.2 ^a
VPA (250 mg/kg)+EGCG (1 mg/kg)	090.11 \pm 6.57 ^b	120.86 \pm 5.3 ^b
VPA (250 mg/kg)+EGCG (2 mg/kg)	075.67 \pm 4.31 ^b	098.83 \pm 4.9 ^b
VPA (250 mg/kg)+EGCG (5 mg/kg)	085.43 \pm 4.99 ^b	110.61 \pm 4.8 ^b

Each column is mean \pm SD for six rats in each group. Significance was determined by one-way ANOVA followed by DMRT. ^aP<0.05 versus normal control, ^bP<0.05 versus VPA control.

Altered hippocampal and cerebellum glutamate levels were measured on PND 90. VPA rats had shown significantly increased glutamate levels in hippocampus and cerebellum compared to control group. EGCG (2 mg/kg body weight)

treatment partially decreased glutamate level in the brain (hippocampus and cerebellum) when compared with other doses (1 mg/kg and 5 mg/kg body weight) in VPA-induced autistic rats (table 3).

Table 3: Effect of EGCG on glutamate levels in rats prenatally exposed to VPA

Groups	Brain tissue ($\mu\text{mol/g}$ of tissue)	
	Hippocampus	Cerebellum
Normal Control	10.23 \pm 0.33	12.65 \pm 0.49
EGCG (1 mg/kg)	10.19 \pm 0.33	12.23 \pm 0.48
EGCG (2 mg/kg)	10.17 \pm 0.32	12.34 \pm 0.47
EGCG (5 mg/kg)	10.18 \pm 0.32	12.33 \pm 0.47
VPA Control (250 mg/kg)	17.20 \pm 0.16 ^a	16.45 \pm 0.21 ^a
VPA (250 mg/kg)+EGCG (1 mg/kg)	15.11 \pm 0.29 ^b	15.82 \pm 0.33 ^b
VPA (250 mg/kg)+EGCG (2 mg/kg)	12.67 \pm 0.25 ^b	14.23 \pm 0.35 ^b
VPA (250 mg/kg)+EGCG (5 mg/kg)	14.43 \pm 0.26 ^b	15.35 \pm 0.36 ^b

Each column is mean \pm SD for six rats in each group. Significance was determined by one-way ANOVA followed by DMRT. ^aP<0.05 versus normal control, ^bP<0.05 versus VPA control.

DISCUSSION

Autism is a neurological developmental disorder defined by the presence of a triad of communication, social and stereotypical behavioural characteristics with onset before 3 y of age in children. The development of the human brain is a series of precisely timed events which results in neural circuits. The circuits are critical in the integration of sensory information in cognitive functions and movements [18]. The neurotransmitters and the neuropeptides not only play an important role in the function of neuronal circuits but they are also involved in the regulation of the neuronal development of these structures. They appear in the embryos before the neurons are differentiated and change their role during the brain development [19].

In our present study prenatal exposure to VPA evokes changes in some neurotransmitters and neurochemicals such as serotonin, glutamate and nitric oxide levels were increased, nearly every area of the brain has been shown to abnormalities have been found in autistic brain and the regions are frontal, parietal, temporal lobes of the cerebral cortex and the cerebellum [20]. The cerebellum has long been considered of primary importance to the initiation of movements, coordination, sensory and learning [21]. Serotonin regulates several aspects of brain development, including regulation of cell division, differentiation, neurite outgrowth and synaptogenesis. Earlier reports VPA rats had shown increased serotonin levels in the hippocampus [6]. Early abnormality of the 5-HT system might be followed by postnatal abnormalities such as abnormal 5-HT concentration and abnormal neural network formation [21]. In our present study EGCG treatment partially decreased hippocampus and cerebellum serotonin levels. It may attribute to up-regulated serotonin transporter (SERT) expression that enhances the reuptake of released serotonin [22].

Hippocampal glutamate aids uptake from rats after prenatal exposure to VPA, with a significant increase in glutamate uptake at

P120. Glutamate signaling defects causing an imbalance in excitatory and inhibitory neuronal circuits are implicated in autism; were found to cause abnormal trafficking of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor receptors in neurons. AMPA is a non-NMDA (N-methyl-D-aspartate receptor) type ionotropic trans membrane receptor for glutamate that mediates fast synaptic transmission in the central nervous system (CNS). EGCG modulates AMPA receptor functions in the VPA exposed rats. These results will provide valuable insights into the regulatory mechanisms of neurotransmitters and social behaviors and guide the development of novel AMPA receptor-based therapies to correct social deficits in autism [23-26]. EGCG significantly reduced delayed neuronal damage and it has been demonstrated to pass the blood-brain barrier and reach brain parenchyma in an animal study [27], shows a significant increase in the number of surviving neurons in the hippocampal region [28].

In the present study, increased total nitrite levels indicate the involvement of oxidative stress in VPA-induced autism. Nitric oxide-induced cell death in the nervous system is a major concern in conditions such as brain ischemia, neurodegeneration and inflammation [29]. Treatment with EGCG significantly reversed the altered oxidative stress markers in hippocampal and cerebellum brain region, due to its antioxidant properties exert to scavenge Reactive oxygen species (ROS) would have played a major role in preserving the antioxidant system [30, 31].

CONCLUSION

In the present study, we found administration of VPA on the 12th d of gestation significantly inducing autism. Oral administration of EGCG significantly protects the autism by maintaining the neurotransmitters and neurochemicals in the brain. These effects could be due to its potent antioxidant and also good radical scavenging; in addition to its ability to invoke a range of cellular

mechanisms it modulates signaling pathways in the neurotransmitters of hippocampus and cerebellum.

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CONFLICT OF INTERESTS

Declared none

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