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Neuroprotective Effects of Natural Antioxidants Against Branched-Chain Fatty Acid-Induced Oxidative Stress in Cerebral Cortex and Cerebellum Regions of the Rat Brain

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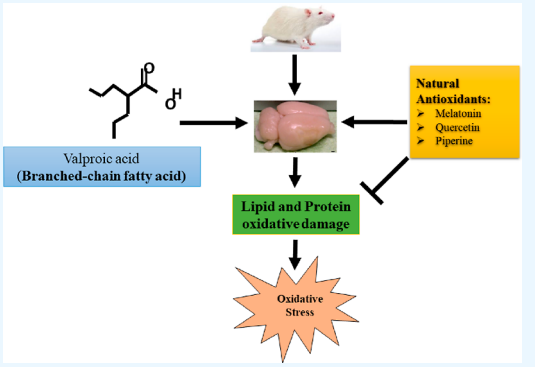
ABSTRACT: Valproic acid (VPA) is short branched-chain fatty acid (BCFA) derived from valeric acids which are naturally produced by *Valeriana officinalis* (flowering plant). Neurotoxicity caused by BCFA-like VPA may be mediated by oxidative stress, according to research involving the cerebral cortex and cerebellum. In the present study, we explored the possible protective effect of different antioxidants such as melatonin, quercetin, and piperine on VPA exposure by using a supernatant preparation of the cerebral cortex and cerebellum regions of the rat brain. The present study revealed that melatonin, quercetin, and piperine significantly prevented VPA-induced oxidative stress in the cerebral cortex and cerebellum regions. VPA was also observed to lower the level of reduced glutathione, and this effect was significantly mitigated by these antioxidants. Melatonin, quercetin, and piperine also ameliorated and altered the activities of AChE, Na^+, K^+ATPase, and MAO in the cerebral cortex and cerebellum. Results of this study also suggest that prior treatment of antioxidants like melatonin, quercetin, and piperine helps in combating the oxidative stress induced by VPA in the cerebral cortex and cerebellum region of the rat brain. Thus, sufficient dietary intake of these antioxidants by individuals at high risk of VPA exposure could prove beneficial in combating the adverse effect of VPA.



1. INTRODUCTION

Valproic acid (VPA) is a branched-chain fatty acid (BCFA) and also used as an anticonvulsant which is also supported by clinicians, but is challenging due to its side effect and induced toxicity.[1−3] VPA is considered safe; however, its higher concentration is associated with idiosyncratic neurotoxicity.[4] Despite the several therapeutic effects induced by VPA for many diseases, it is also known to be associated with various kinds of toxicity, the most serious of those being hepatotoxicity, teratogenicity, and neurotoxicity,[5−7] as evidenced in *in vitro* models. Reactive oxygen species (ROS) formation is majorly associated with VPA toxicity, which in turn constitutes an important risk factor for tissue damage and organ dysfunction.[8] Many studies have reported that VPA cytotoxicity was associated with mitochondrial dysfunction and oxidative stress. Several mechanisms have been proposed to explain mitochondrial dysfunction by VPA.[9,10] However, the underlying mechanisms of VPA are not well established, only the developmental neurotoxicity has been recognized. VPA has been discussed for a better understanding of neuronal signaling pathways in rodants.[11] The cerebral cortex and cerebellum are large and important regions of the brain and play a prominent role in memory and learning as well as coordination of motor movements.[12] Several studies have reported that cerebellum and cerebral cortex regions of the brain are highly prone to oxidative stress, due to their low capacity for an antioxidant as compared with other tissues.[13] Antioxidants are exogenous or endogenous molecules that act against oxidative stress by neutralizing ROS and other kinds of free radicals, thus exhibiting their therapeutic potential.[14] Thus, many antioxidants have been tested in various in vitro and in vivo neurodegenerative models.

In the present study, we have evaluated the effect of natural antioxidants like melatonin, quercetin, and piperine against VPA-induced cytotoxicity in the cerebellum and cerebral cortex regions of the rats. Melatonin is endogenously produced by the pineal gland during darkness and exogenously present in a variety of food products such as cereals, vegetables, nuts, fruits, and seeds.[15] The beneficial effects of melatonin on oxidative damage have also been evaluated in several in vitro and in vivo studies. In several studies, it has been investigated

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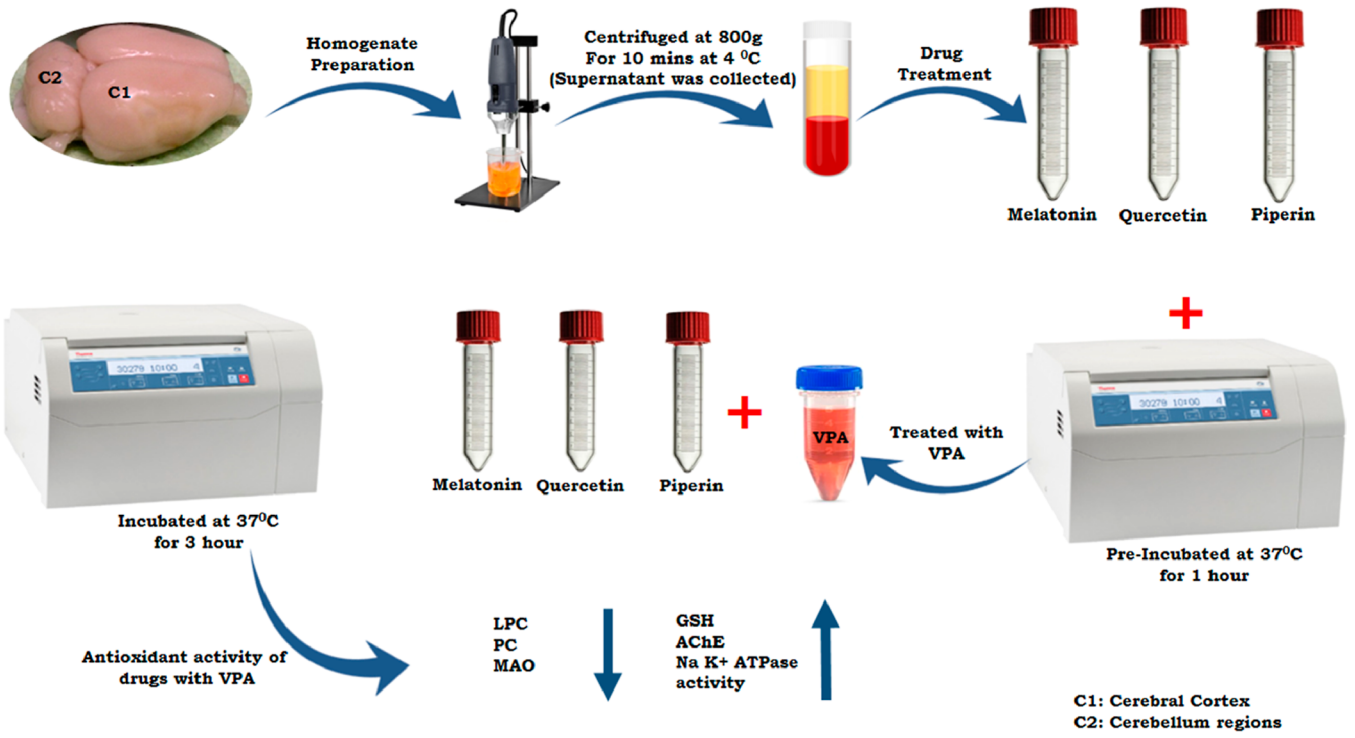

Figure 1. Sketch of the sample preparation procedure and experimental design.

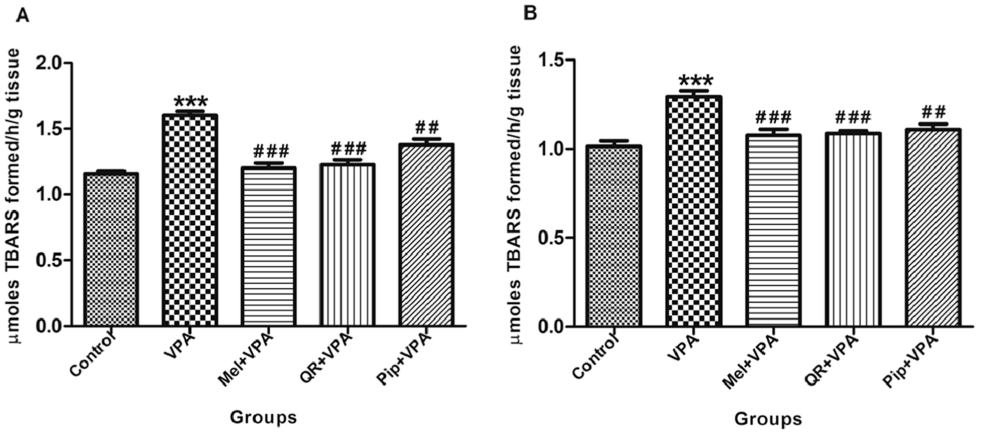

Figure 2. Effect of VPA and antioxidants on the LPO level in (A) cerebral cortex and (B) cerebellum region of rat brain. Values were expressed as means ± S.E.M. ($n = 6$). The level of LPO was measured as μ moles TBARS formed/h/g tissue. ***$p < 0.001$ VPA versus control, $^{###}p < 0.001$ + melatonin + VPA versus VPA, $^{###}p < 0.001$ quercetin + VPA versus, $^{###}p < 0.01$ piperine + VPA versus VPA.

that melatonin inhibits oxidative stress and apoptosis in the cerebral cortex and cerebellum.[16−18] Quercetin is a plant-derived flavonoids possessing properties as a free radical scavenger and neuroprotection from oxidative injury by its ability to modulate intracellular signals promoting cellular survial.[19] Reports have indicated that quercetin enhances the resistance of neurons against oxidative stress and excitotoxicity by modulation of the cell death mechanism.[20,21] The therapeutic effects of quercetin against various oxidative stress-related diseases have been documented, but no study has revealed its antioxidant effects against VPA exposure in the cerebellum and cerebral cortex. Therefore, it is important to determine the protective effects of these antioxidant against VPA-induced neurotoxicity. Additionally, another antioxidant, piperine, is a major component of the piper species and an alkaloid of black paper.[22] It has also been traditionally used as a food flavoring agent. Many studies have suggested that piperine has been associated with neuroprotective effects against oxidative stress and apoptotic signaling cascade.[23,24] However, the paucity of literature was striking toward the antioxidant effects of melatonin, quercetin, and piperine against BCFA like VPA in the cerebellum and cerebral cortex of the rat brain.

2. RESULTS

The antioxidant activity of melatonin, quercetin, and piperine were evaluated against VPA, as shown in Figure 1.

The effect of VPA on lipid peroxidation (LPO) was investigated by assessing TBARS levels in the supernatant of cerebral cortex and cerebellum of the rat brain. TBARS levels were significantly increased (***$p < 0.001$) in the VPA-exposed group when compared to the control group (Figure

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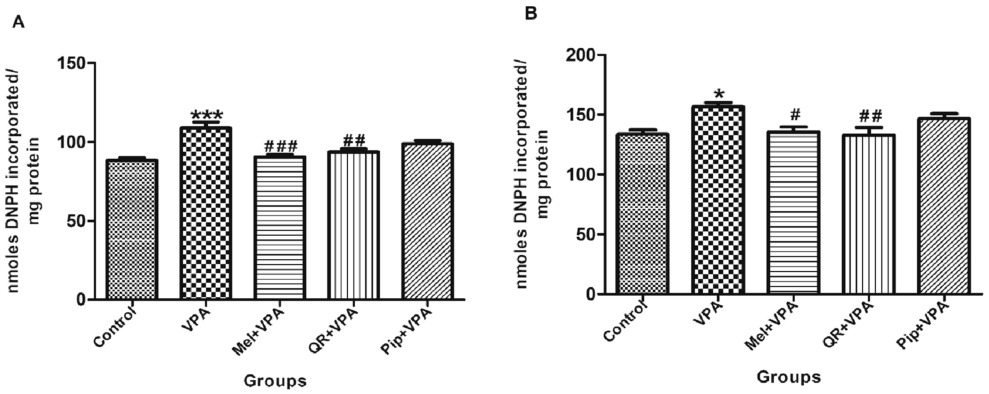

Figure 3. Effect of VPA and antioxidants on the PC level in (A) cerebral cortex and (B) cerebellum regions of rat brain. Values were expressed as means ± S.E.M. (n = 6). The level of PC was measured as n moles 2,4 dinitrophenylhydrazine (DNPH) incorporated/mg proteins. $^{*}p < 0.05$ $^{***}p < 0.001$ VPA versus control, $^{*}p < 0.05$ $^{***}p < 0.001$ melatonin + VPA versus VPA, $^{\#\#}p < 0.01$ quercetin + VPA versus VPA.

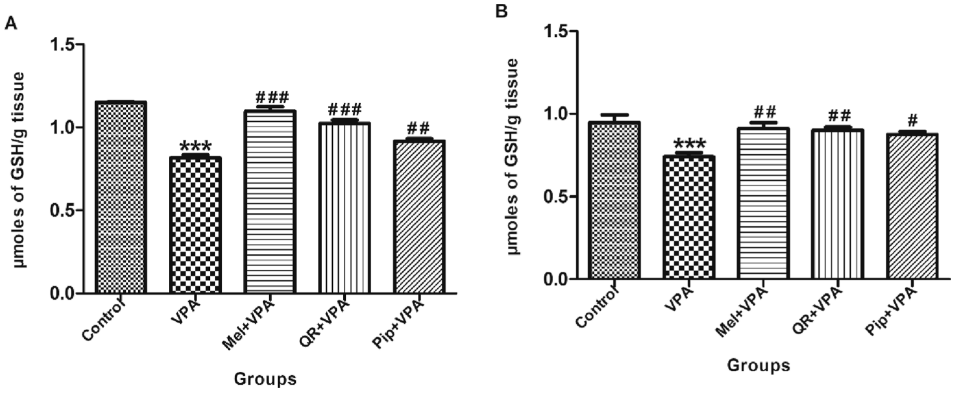

Figure 4. Effect of VPA and antioxidants on the GSH level in (A) cerebral cortex and (B) cerebellum region of the rat brain. Values were expressed as mean ± S.E.M. (n = 6). The level of GSH was measured as μ moles GSH/g tissue. $^{***}p < 0.001$ VPA versus control, $^{\#\#}p < 0.01$ $^{\#\#\#}p < 0.001$ melatonin + VPA versus VPA, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ quercetin + VPA versus VPA, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ piperine + VPA versus VPA.

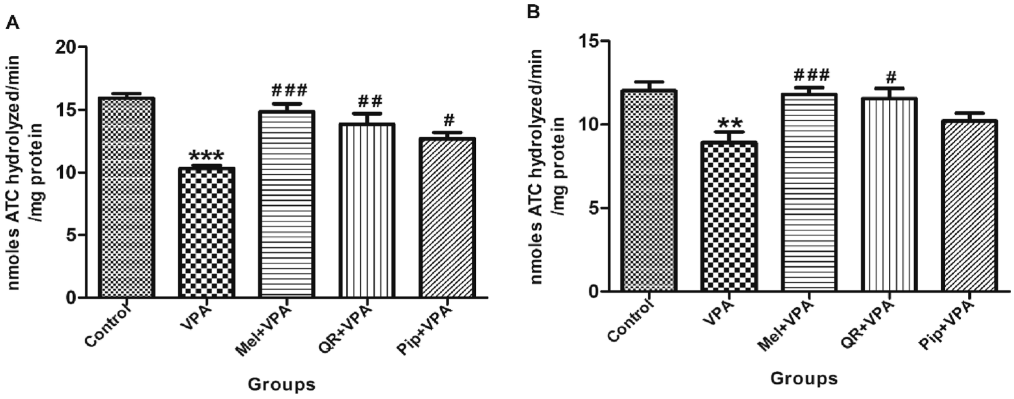

Figure 5. Effect of VPA and antioxidants on AChE activity in (A) cerebral cortex and (B) cerebellum region of rat brain. Values were expressed as mean ± S.E.M. (n = 6). The activity of AChE was measured as n moles acetylthiocholine iodide (ATC) hydrolyzed/min/mg protein. The bars represents the mean ± SE n = 6. $^{***}p < 0.01$, $^{***}p < 0.001$ VPA versus control, $^{\#\#\#}p < 0.001$ melatonin + VPA versus VPA, $^{\#\#}p < 0.01$ $^{\#}p < 0.05$ quercetin + VPA versus VPA, $^{\#}p < 0.05$ piperine + VPA versus VPA.

2A,B). Also, the pre-treatment activity of melatonin, quercetin, and piperine showed significant decrease in the ($^{\#\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$) level of LPO as compared with the VPA-treated group.

Protein carbonyl (PC) content in the supernatant was investigated in the cerebral cortex and cerebellum, as shown in Figure 3A. VPA significantly raised PC contents in the cerebral cortex ($^{***}p < 0.001$) and cerebellum ($^{*}p < 0.05$) supernatant in comparison to the control group. Pre-treatment of melatonin and quercetin restored the level of PC content significantly in both cerebral cortex ($^{\#\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$) and cerebellum ($^{\#}p < 0.01$, $^{\#\#}p < 0.001$) supernatant, when compared to VPA. However, no significant changes in the level of PC were observed with pre-treatment of piperine.

Figure 4A,B, shows the toxic potential of VPA as indicated by the decrease in reduced glutathione (GSH) activity in the supernatant of the cerebral cortex ($^{***}p < 0.001$) and

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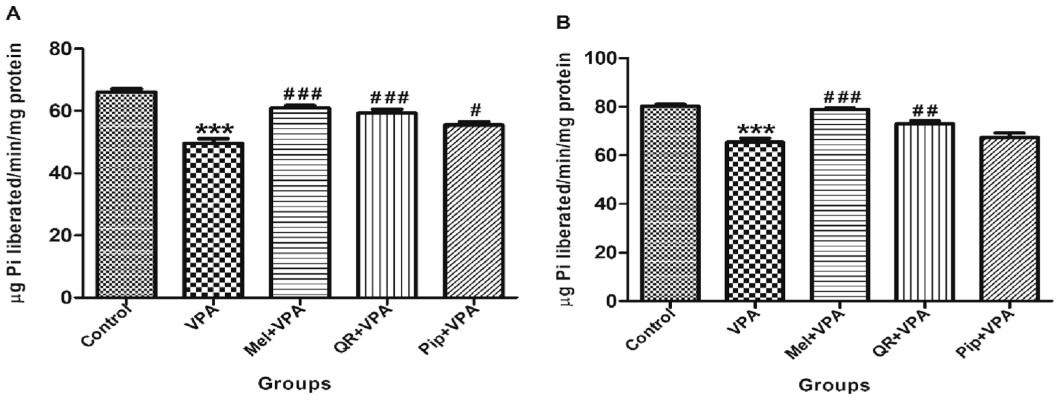

Figure 6. Effect of VPA and antioxidants on Na^+ K^+ -ATPase activity in (A) cerebral cortex and (B) cerebellum region of the rat brain. Values were expressed as mean ± S.E.M. (n = 6). The activity of Na^+ K^+ -ATPase was measured as ng Pi liberated/min/mg protein. The bars represent the mean ± SE n = 6. $^{***}p < 0.001$ VPA versus control, $^{###}p < 0.001$ melatonin + VPA versus VPA, $^{##}p < 0.01$ $^{#}p < 0.001$ quercetin + VPA versus VPA, $^{#}p < 0.05$ piperine + VPA versus VPA.

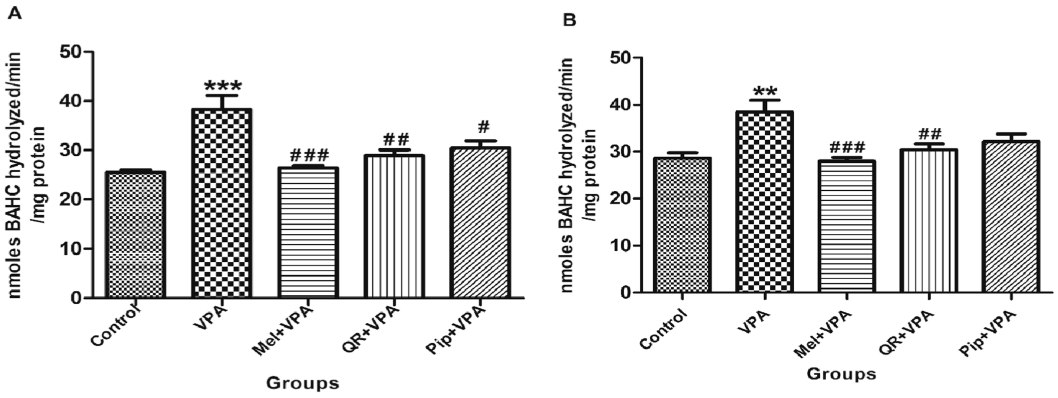

Figure 7. Effect of VPA and antioxidants on MAO activity in (A) cerebral cortex and (B) cerebellum region of the rat brain. Values were expressed as mean ± S.E.M. (n = 6). The activity of MAO was measured as μ moles hydrolyzed/min/mg protein. The bars represent the mean ± SE n = 6. $^{**}p < 0.01$ $^{***}p < 0.001$ VPA versus control, $^{###}p < 0.001$ melatonin + VPA versus VPA, $^{##}p < 0.01$ quercetin + VPA versus VPA, $^{#}p < 0.05$ piperine + VPA versus VPA.

cerebellum ($^{***}p < 0.001$) in comparison to the control group. Pre-exposure of melatonin, quercetin, and piperine have displayed significant increases in the cerebral cortex ($^{###}p < 0.01$, $^{###}p < 0.001$) and cerebellum ($^{#}p < 0.05$, $^{##}p < 0.01$) in the GSH level when compared with VPA.

Figure 5 A,B shows the activity of acetylcholinesterase (AChE) in the supernatant of the cerebral cortex and cerebellum. The activity of AChE was significantly decreased in the supernatant of both the cerebral cortex ($^{***}p < 0.001$) and cerebellum ($^{***}p < 0.01$) as compared to the control. Pre-treatment of melatonin, quercetin, and piperine significantly mitigated ($^{#}p < 0.05$, $^{##}p < 0.01$, $^{###}p < 0.001$) the activity of AChE in the supernatant of the cerebral cortex but piperine pre-exposure did not express any significant effect on the activity of AChE when compared to the VPA-exposed group.

The activity of Na^+, K^+ -ATPase is shown in Figure 6A,B in the supernatant of the cerebral cortex and cerebellum regions and found a significant inhibition ($^{***}p < 0.001$) in the activity of Na^+, K^+ -ATPase in exposed groups of VPA when compared with the control group. Melatonin, quercetin, and piperine pre-treatment has shown a significant reversal in the activity of Na^+, K^+ -ATPase in cerebral cortex ($^{#}p < 0.05$, $^{###}p < 0.001$) and cerebellum ($^{##}p < 0.01$, $^{###}p < 0.001$) when compared with VPA. Pre-treatment of piperine did not show any significant changes on the activity of Na^+, K^+ -ATPase in the cerebellum comparison with the VPA-exposed group.

Figure 7A,B indicates the activity of MAO. VPA treatment significantly enhanced the activity of MAO in the supernatant of the cerebral cortex ($^{***}p < 0.0001$) and cerebellum ($^{**}p < 0.01$) regions when compared to the control group. Pre-exposure of melatonin, quercetin, and piperine significantly attenuated ($^{##}p < 0.01$, $^{##}p < 0.01$, $^{###}p < 0.001$) the activity of MAO in the cerebral cortex and cerebellum, while pre-treatment with piperine contributed no significant changes in the activity of MAO in the cerebellum when compared to VPA.

3. DISCUSSION

In the present study, we evaluated the neurotoxic effects of VPA in the supernatant of the cerebral cortex and cerebellum region of rat brains and its attenuation by the different types of antioxidants. The perfect antioxidant properties of melatonin, quercetin, and piperine and VPA-induced oxidative stress reminds us of the toxic insult of VPA to the cerebral cortex and cerebellum could be challenged by the intervention of these antioxidants. Free radical-induced damage to macromolecules like lipid, protein, and nucleic acids is considered as an important factor in the acceleration of neurodegeneration.[37] In our study, we examined the level of LPO and found that VPA elevated LPO levels in the cerebral cortex as well as the

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cerebellum region. Pre-treatment of antioxidants significantly reversed the elevated level of LPO and altered the status of antioxidants. It has been documented that antioxidants such as melatonin, quercetin, and piperine have been able to modulate oxidative stress marker LPO by scavenging ROS, and these antioxidants could reinforce a constructive action against LPO, which may act as an added compensation mechanism to retain cell integrity and protection against free radical dmages.[38−41] Our preliminary results of oxidative stress biomarkers prompted us to investigate whether VPA provokes protein oxidation, as detected by a marker elevation in the formation of PC contents. Thereafter, we have observed that administration of VPA increased the level of PC in the cerebral cortex and cerebellum region of the brain. Oxidative damage usually contributes to loss in distinct protein function, and the observed enhancement in the level of PC content in the cerebral cortex and cerebellum region may be due to raised production of ROS or altered membrane fluidity.[42] Treatment with melatonin and quercetin prior to VPA exposure, however, prevented the enhanced level of PC content and restored the neuronal cells to their normal physiological state. Piperine could not restore VPA-induced protein oxidation in the cerebral cortex and cerebellum. To reduce the cellular oxidative damage, cells produce endogenous antioxidant molecules which buffer free radicals from cells and provide protection to the cells by metabolic conversion. The antioxidant system like GSH which resist free radicals play a major role in the cellular defense system by scavenging ROS.[43] Subsequently, we examined the level of GSH and found that VPA caused a significant reduction in the level of GSH in our study. The inhibition in the level of GSH as seen in our results leads to enhancement in the ROS production such as H_2O_2, and $OH^{\bullet}$ and causes damage consistent with oxidative stress. This corroborates with preliminary observation that VPA induces oxidative stress and alterations in the cellular defense status may result in adverse functional consequences.[34] The present study also confirmed that pre-treatment of melatonin, quercetin, and piperine totally restored the level of GSH, thereby protecting against VPA-induced oxidative damage in the supernatant of the cerebral cortex and cerebellum regions of rat's brains. The neurotoxicity of VPA was also demonstrated by measuring the activities of brain-specific enzymes like AChE, Na^+, K^+ -ATPase, and monoamine oxidase (MAO) in our study. Alterations in the activity of these enzymes such as AChE, Na^+, K^+ -ATPase, and MAO are essential in observing the neurotoxic effects caused by VPA. AChE is an enzyme that catalyzes acetylcholine, a neurotransmitter associated with learning and memory.[44] AChE plays an important role in the cholinergic transmission and neurodegenerative disease. In the present study, exposure to VPA significantly inhibited the activity of AChE in the supernatant of the cerebral cortex and cerebellum regions of rat brains. The inhibited activity of AChE in the brains may not lead to acetylcholine degradation and subsequent reduction in the stimulation of acetylcholine receptors which causes suppression of cholinergic transmission and progressive impairment of cognition.[45] Melatonin and quercetin supplementation significantly prevented the altered activity of AChE in the cerebral cortex and cerebellum samples, probably attributed to their antioxidant effects, but piperine could not prevent the diminished activity of AChE in the cerebellum sample. We have also examined the effects of VPA on the activity of Na^+, K^+ -ATPase enzyme. Na^+, K^+ -ATPase plays an important role in neurotransmission, ion transport, and maintenance of the membrane potential, and this enzyme is highly prone to free radical attack.[46] The activity of Na^+, K^+ -ATPase was altered by the exposure of VPA in the cerebral cortex and cerebellum. The inhibited activity of Na^+, K^+ -ATPase in both the region of the brain may lead to diverse toxic effects such as partial membrane depolarization in neurons, cell swelling, increased influx of Ca^{2+}, and disruption in the membrane potential which is further associated with neurodegeneration.[47] Melatonin, quercetin, and piperine supplementation significantly counteracted changes in the activity of Na^+, K^+ -ATPase.

This occurs often due to free radical scavenging and neuroprotective properties of these natural compounds. A significant elevation in the activity of MAO enzyme was also observed in the supernatant of the cerebral cortex and cerebellum regions of the brain by the exposure to VPA. MAO is an enzyme that plays an important role in the metabolism of monoamines. Fang et al. in 2012 and other studies have also demonstrated that MAO activity was increased in aging and dementia, which affected the transmission of information and the metabolism of the monoamine transmitter and was involved in memory shortages.[48,49] In our study, pre-treatment of melatonin, quercetin, and piperine in the cerebral cortex and cerebellum supernatant has shown the replenished enhanced activity of MAO. A pre-treatment study of natural compounds including melatonin, quercetin, and piperine has been advocated to be beneficial for the brain tissues and mitigate the induced toxic response for MAO activity in the brain. Under normal physiological conditions, a dedicated balance exists between the rate of ROS formation and rate of their neutralization. Biological systems are equipped with a cellular enzymatic and non-enzymatic antioxidant defense system to buffer the generation of ROS. The enzyme system plays an important role in neutralizing oxidative damages.[30] Deficiency of the antioxidant system in the brain implicates a deficient antioxidant defense and therefore decreased capability of attenuating the production of ROS in neuronal cells.[50]

4. CONCLUSIONS

To conclude, the result of investigations states that alterations in the activates of AChE, Na^+, K^+- ATPase, and MAO enzymes by VPA intoxication can lead to disruptions in the brain metabolism and can also contribute to the neurotoxic effects induced by VPA. Therefore, protection against the VPA-induced neurotoxicity can be attained through the supplementation of antioxidants. Although melatonin was found to be more effective in ameliorating oxidative stress induced by VPA in comparison to quercetin and piperine, the degree of protection imparted by melatonin was more effective. Thus, sufficient dietary intake of these antioxidants by individuals at high risk of VPA exposure could prove beneficial in combating the adverse effects of VPA. However, some more detailed and in vivo studies are needed to show the effect of the antioxidants like melatonin, quercetin, and piperine on the rat brain specifically on the cerebral cortex and cerebellum region against VPA exposure to confirm the same effect.

5. MATERIAL AND METHODS

5.1. Chemicals. ATC, 1-amino-2-naphthol, 4-sulphonic acid (ANSA), benzyl amine hydrochloride (BAHC), bovine

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serum albumin (BSA), butylated hydroxyl toluene (BHT), DNPH, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), ortho-phosphoric acid (OPA), perchoric acid (PCA), reduced GSH, sodium azide, sulfosalicylic acid, thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St Louis, MO, USA). Other routine chemicals used in this study were obtained from Hi-Media Labs (Mumbai, India). Melatonin, quercetin, piperine, and VPA were also purchased from Sigma-Aldrich (St Louis, MO, USA).

5.2. Animals. In this study, male Wistar rats (4−5 weeks old) weighing 150−180 g were maintained under standard conditions in the Central Animal House of Jamia Hamdard (Hamdard University), New Delhi, India. Rats were kept at a temperature 22 ± 2 °C with relative humidity at 65 ± 10% and a photoperiod of 12 h light/dark cycle. Standard rodent food and water were supplied to the animals *ad libitum* prior to the start of the experiments.

5.3. Cerebral Cortex and Cerebellum Preparations. The supernatant of the cerebral cortex and cerebellum regions of the rat brain was prepared by the differential centrifugation method, as described by Leipnitz et al. (2010).[25] After sacrificing the rats by cervical dislocation, the brain was taken out and placed in a Petri dish on ice, and the blood and external vessels were carefully removed. The cerebral cortex and cerebellum regions of the brain were dissected, weighted, and separately homogenized in 10 volumes (1:10 w/v) of 0.1 M sodium Phosphate buffer, pH 7.4 with a Teflon-fitted Potter-Elvehjen homogenizer. The homogenates of the cerebral cortex and cerebellum regions were centrifuged at 800*g* for 10 min at 4 °C to separate nuclei and cell debris. The pellet was disposed of, and the supernatant of the cerebral cortex and cerebellum was separated and used for parameter evaluation.

5.4. Experimental Design. To investigate the neuro-toxicity of VPA in the rat brain homogenate preparation, the supernatant of the cerebral cortex and cerebellum was pre-incubated with different antioxidants such as melatonin (10 μm), quercetin (10 μm), and piperine (10 μm) for 1 h prior from the VPA treatment. After that, VPA (10 mg) was added and further incubated for 3 h at 37 °C in a temperature-controlled water bath. The concentration of VPA and antioxidants used in this study were based on previously published studies.[26−29] Sodium phosphate buffer was taken as control. The stock and working solutions were prepared in such a way that the same volume was added to the supernatant of the cerebral cortex and cerebellum for incubation.

5.5. Determination of LPO. LPO was measured using the procedure reported by Tabassum et al. (2010).[30] Briefly, 0.25 mL of the biological sample was mixed with 10 mM BHT, OPA (1%), and TBA (0.67%), and the mixture was incubated at 90 °C for 45 min. The absorbance was measured at 535 nm. The rate of LPO was expressed as μmoles TBARS formed/h/g tissue based on the molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

5.6. Determination of PC Contents. PC content was quantified by the procedure reported by Chaudhary et al. (2015).[31] The most convenient procedure for PC estimation is the reaction between DNPH and PC content. DNPH reacts with PC content to produce the corresponding hydrazone. The tissue supernatant (0.5 mL) was reacted with 10 nM DNPH in 2 HCL for 1 h at room temperature and precipitation with 40% TCA. The pelleted protein was washed thrice by resuspension in the ethanol/ethyl acetate (1:1) mixture. Proteins were then solubilized in 6 M guanidine hydrochloride and formic acid (50%) and centrifuged at 1600*g* for 5 min to remove any trace of insoluble material. The carbonyl content was measured spectrophotometrically at 340 nm. The results were expressed as n moles DNPH incorporated/mg protein based on the molar extinction coefficient of 2.1 car^4 M^{-1} cm^{-1}.

5.7. Reduced GSH Contents. GSH contents were estimated according to the method reported by Parvez et al. (2008).[32] The thiol group of GSH reacts with the −SH reagent (DTNB) to form thionitrobenzoic acid. The tissue supernatant was mixed with 4% SSA. It was then incubated at 4 °C for a minimum time of 1 h and then centrifuged at 4 °C for 15 min at 1,200 g. The reaction mixture contained 0.1 M phosphate buffer (pH 7.4), 10 mM DTNB, and 0.4 mL supernatant. The yellow color developed was read at 412 nm by a spectrophotometer. The GSH concentration was calculated as μ moles GSH/g tissue using a molar extinction coefficient of 1.36 $oben^4$ M^{-1} cm^{-1}.

5.8. AChE Activity. The activity of AChE was estimated by using the method reported by Govil et al. in 2012.[33] In the artificial substrate provided, ATC is broken down in the presence of AChE to release thiocholine, which reacts with −SH reagent DTNB to form thionitrobenzoic acid. The reaction volume contained 0.1 M sodium phosphate buffer (pH 7.4), 10 mM DTNB, ATC, and 0.4 mL biological samples. The absorbance was measured at 412 nm. The enzyme activity was calculated as n moles ATC hydrolyzed/min/mg protein using a molar extinction coefficient of 1.36 $roly^4$ M^{-1} cm^{-1}.

5.9. Na^+, K^+- ATPase Activity. Na^+, K^+- ATPase activity was measured as the release of inorganic phosphate (Pi) by the method reported by Chaudhary and Parvez (2012).[34] The reaction mixture for the Na^+, K^+- ATPase assay contained 0.1 M $MgCl_2$, 1 M NaCl, 0.2 M KCl, and 0.2 M Tris−HCl buffer (pH 7.4). The mixture was incubated at room temperature for 5 min, and then 0.025 M ATP was added to the biological samples to start the reaction. The mixture was again incubated at 37 °C for 15 min, and 10% TCA was added to both the reaction mixtures to stop the reaction. Centrifugation was carried out at 1,500 g for 10 min. The pellet was discarded, and the supernatant, distilled water, ammonium molybdate, and ANSA were taken to make a final volume of 5 mL. The mixture was incubated at 37 °C for 30 min, and the OD was taken at 660 nm. The activity was measured as rePi liberated/min/mg protein.

5.10. MAO Activity. MAO was measured by using the method reported by Vishnoi et al. in 2015, based on oxidation of BAHC to benzaldehyde.[35] The reaction mixture consisted of 0.1 M phosphate buffer (pH 7.4), distilled water, 0.1 M BAHC, and 0.2 mL of tissue homogenate, which was incubated for 30 min at 37 °C. Then 10% PCA was added to the reaction mixture and then centrifuged at 1500*g* for 10 min, and the OD was taken at 280 nm. The enzyme activity was calculated as n moles BAHC hydrolyzed/min/mg protein using a molar extinction coefficient of 7.6925 M^{-1} cm^{-1}.

5.11. Protein Estimation. The protein contents were determined by the method reported by Bradford[36] using BSA as a standard with a range of 10−100 μg protein. Briefly, 20 μL of biological samples was mixed with 3 mL of Bradford reagent, 1.5 mL of ethanol (95%), and 3 mL of OPA (85%), which were added to each tube and mixed well, and the

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mixture was incubated at room temperature for at least 5 min. The absorbance was measured at 595 nm.

5.12. Statistical Analysis. Data were expressed as mean ± SEM for absolute values of all experiments. Assays were performed in duplicate or triplicate, and all data were analyzed using analysis of variance followed by Tukey's test. All the statistical analyses were performed using Prism program version 5 (Graph Pad Software, Inc. San Diego, CA, USA).

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Author Contributions

S.P. designed the study. S.C. conducted the experiments. S.V., P., and S.P. analyzed the data. S.C. and S.P. wrote the manuscript. All authors have read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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