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AN *IN VITRO* APPROACH TO ASSESS THE NEUROTOXICITY OF VALPROIC ACID-INDUCED OXIDATIVE STRESS IN CEREBELLUM AND CEREBRAL CORTEX OF YOUNG RATS

S. CHAUDHARY AND S. PARVEZ *

Department of Medical Elementology and Toxicology, Jamia Hamdard (Hamdard University), New Delhi 110 062, India

Abstract—Valproic acid (VPA), a branched short-chain fatty acid, is generally used as an antiepileptic drug and a mood stabilizer. VPA is a relatively safe drug, but its use in higher concentrations is associated with idiosyncratic neurotoxicity. Investigations involving cerebral cortex and cerebellum can shed light on whether neurotoxicity induced by branched chain fatty acids like VPA is mediated by oxidative stress. The aim of our investigation was to evaluate the neurotoxic potential of VPA by using preparation of cerebral cortex and cerebellum of young rats as an *in vitro* model. Oxidative stress indexes such as lipid peroxidation (LPO) and protein carbonyl (PC) formation were evaluated to visualize whether the first line of defence was breached. The levels of oxidative stress markers, LPO and PC were significantly elevated. Non-enzymatic antioxidants' effect was also demonstrated as a significant depletion in reduced glutathione (GSH) and non-protein thiol activity (NP-SH), but there was no significant increase or decrease in the concentrations of total thiol (T-SH) and protein thiol (P-SH). VPA also showed significant reduction in the activities of glutathione metabolizing enzymes such as glutathione-*S*-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx) and other antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) in cerebellum and cerebral cortex. A significant elevation was also observed in the activity of xanthine oxidase (XO). Some neurotoxicity biomarkers were investigated in which the activity of acetylcholinesterase (AChE) and sodium–potassium ATPase (Na^+, K^+-ATPase) was decreased and monoamine oxidase (MAO) was increased. These results indicate that VPA induces oxidative stress by compromising the antioxidant status of the neuronal tissue. Further studies are required to decipher the cellular and molecular mechanisms of branched chain fatty acid-induced neurotoxicity.

Key words: cerebellum, cerebral cortex, valproic acid, oxidative stress, neurotoxicity.

INTRODUCTION

Branched chain fatty acids (BCFAs) are generally saturated fatty acids with one or more methyl branches on the carbon chain (Ran-Ressler et al., 2008). In humans, they have been observed in various tissues including skin, brain, blood and cancer cells. Several BCFAs, e.g., phytanic acid, pristanic acid and valproic acid (VPA) have been the focus of research over the years to understand their role in modulation of biochemical and physiological processes (Lheureux et al., 2005). The neurotoxicity of BCFAs was elucidated by several investigators and one of the major mechanisms behind BCFA's toxicity has been attributed to be oxidative stress (Schönfeld et al., 2004). Reports have indicated that BCFAs induce toxic effects on different systems because of their relatively long half life. Besides their effects and implication in several neurodegenerative diseases there is a risk that exposure to BCFAs may lead to damage of the nervous system (Ronicke et al., 2009).

The utility of VPA as an anticonvulsant has been supported by clinicians, which was subsequently challenged due to its side-effects and induced toxicity (Chateauvieux et al., 2010). Although, VPA is a relatively safe drug, but its use in higher concentrations is associated with idiosyncratic neurotoxicity (Gravemann et al., 2008). Numerous studies have also demonstrated that administration of VPA at clinically relevant doses to neonatal rat for anticonvulsant action induced apoptotic neurodegeneration in several regions of the brain, including hippocampus, thalamus, frontal and parietal cortex (Sui and Chen, 2012). Uptake and emission of VPA within the CNS can occur at either the choroid plexus (blood–cerebrospinal fluid barrier) or the blood–brain barrier (brain capillary endothelium). Considering the physiochemical properties of VPA, it is surprising that it is capable of rapidly diffusing into the brain (Auinger et al., 2009).

*Corresponding author. Tel: +91-11-26059688x5573; fax: +91-11-26059663.
E-mail addresses: drsuhelparvez@yahoo.com, sparvez@jamiahamdard.ac.in (S. Parvez).

Abbreviations: ANSA, 1-amino-2-naphthol-4-sulphonic acid; ATC, acetylthiocholine iodide; BAHC, benzylaminehydrochloride; BCFAs, branched chain fatty acids; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; DNPH, 2,4-dinitrophenylhydrazine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylene diamine tetraacetic acid; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase; LPO, lipid peroxidation; NADP(H), reduced nicotinamide adenine dinucleotide phosphate; NP-SH, non-protein thiol; OPA, orthophosphoric acid; PC, protein carbonyl; PCA, perchloric acid; P-SH, protein thiol; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid; T-SH, total thiol; VPA, valproic acid; XDH, xanthine dehydrogenase; XO, xanthine oxidase.

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VPA is metabolized in the liver via cytosolic ω-oxidation which may produce different reactive metabolites, some of which may be biologically active, and some of them may be involved in VPA-induced toxic effects after an acute overdose of VPA (Spiller et al., 2000). Several groups have investigated the mechanisms of VPA toxicity in various cell lines and tissues using different schemes of administration as well as distinct animal species (Tong et al., 2005a; Fu et al., 2010; Gibbons et al., 2011). Despite the broad use of VPA in the therapy of several diseases, it is also associated with toxicity, with the most serious of those being hepatotoxicity (Pourahmad et al., 2012), teratogenicity (Tung and Winn, 2011) and neurotoxicity (Wang et al., 2011) as evidenced in *in vitro* models. VPA toxicity is associated with increased reactive oxygen species (ROS) formation, which in turn constitutes an important risk factor for tissue damage and organ dysfunction (Fourcade et al., 2010). Several studies have reported the implication of an increased generation of free radicals and oxidative stress in the toxicity induction mechanism of VPA (Kiang et al., 2010; Zhang et al., 2010). Different mechanisms have been proposed to explain inhibition of mitochondrial function by VPA (Tong et al., 2005b; Chang and Abbott, 2006). CNS depression is the most common demonstration of toxicity, ranging in severity from mild drowsiness to profound coma and fatal cerebral oedema. Although the developmental neurotoxicity of VPA has been recognized since the past several years but the underlying mechanisms are not well established (Zhou et al., 2011).

Cerebellum and cerebral cortex are important regions of the brain and play a prominent role in the coordination of motor movements as well as basic facets of memory and learning (Pederzolli et al., 2007). There are several investigators who have reported that these parts of the brain are highly vulnerable to oxidative stress because cerebellum and cerebral cortex have low capacity of antioxidants as compared with other tissues. These regions of the brain have also a high content of iron, which facilitates the Fenton reaction and leads to hydroxyl radical generation (Fernandes et al., 2010). Role of oxidative damage in both regions induced by numerous xenobiotics has been investigated in detail (Esparza et al., 2005). Oxidative stress is an important consequence related to the pathophysiology of many neurodegenerative diseases. As the cerebral cortex is responsible for cognitive functions and the cerebellum is critical for posture and motor coordination, oxidative damage caused by VPA in these parts of the brain may explain the impairments of the CNS functions.

The paucity of literature was striking enough for us to design the present study where we focused for the first time our attention on the potential neurotoxic effects of VPA in cerebellum and cerebral cortex of rat brain. Based on the previous studies in relation to oxidative stress induced by different xenobiotics in cerebellum and cerebral cortex region of the brain (Leipnitz et al., 2010), we have used cerebellum and cerebral cortex preparation of rat brain as an *in vitro* model to evaluate the effect of VPA-induced neurotoxicity. In the present investigation, the neurotoxic effects of VPA were evaluated through oxidative stress indexes, antioxidant defence profile and neurotoxicity biomarkers.

EXPERIMENTAL PROCEDURES

Chemicals

Acetylthiocholine iodide (ATC), benzylaminehydrochloride (BAHC), bovine serum albumin (BSA), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), oxidized glutathione (GSSG), reduced glutathione (GSH), reduced NADP(H), thiobarbituric acid (TBA) and xanthine were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). 1-amino-2-naphthol-4-sulphonic acid (ANSA), butylated hydroxyl taulene (BHT), 1-chloro-2,4-dinitrobenzene (CDNB), 2,4-dinitrophenylhydrazine (DNPH), epinephrine, EDTA, orthophosphoric acid (OPA), perchloric acid (PCA), sulphosalicylic acid, sodium azide and trichloroacetic acid (TCA) were purchased from Merck Limited (Mumbai, India). Sodium salt of VPA was obtained from ROAQ Chemicals Pvt. Ltd. (Gujarat, India).

Animals

Male Wistar rats (3–4 weeks old) weighing 100–120 g, were used for current experiments and procured from the Central Animal House of Jamia Hamdard (Hamdard University), New Delhi, India. Rats were kept at temperature 22 ± 1 °C with relative humidity at 65 ± 10% and at a photoperiod of 12-h light/dark cycle. Food and water were supplied *ad libitum* prior to the start of the experiment. All the experiments were carried out according to the standard guidelines of Institutional Animal Ethics Committee (IAEC).

Cerebellum and cerebral cortex preparation

Cerebellum and cerebral cortex preparation was done by differential centrifugation method with slight modifications (Leipnitz et al., 2010). After the rats were sacrificed by cervical dislocation without anaesthesia and the brain was rapidly excised on a petri dish placed on ice, the blood and external vessels were carefully removed. The olfactory bulbs, pons, medulla and striatum were discarded, and the cerebellum and cerebral cortex regions of the brain were dissected, weighed and separately homogenized in 10 volumes (1:10, w/v) of 0.1 M sodium phosphate buffer, pH 7.4 with a Potter-Elvehjen homogenizer. The homogenates of brain regions were centrifuged at 750*g* for 10 min at 4 °C to discard nuclei and cell debris. The pellet was discarded and cerebellum and cerebral cortex supernatant was separated and used for determination of oxidative stress biomarkers, biochemical parameters and neurotoxicity biomarkers.

Experimental design

To investigate the neurotoxicity of VPA using the salt of sodium valproate under *in vitro* conditions, the cerebellum and cerebral cortex preparation was incubated with different concentrations of sodium valproate (0.1–10 mg) for 3 h at 37 °C in a temperature controlled water bath. The concentrations and incubation time used for sodium valproate were based on an *in vitro* study with hepatocytes (Tong et al., 2005a). Sodium phosphate buffer was added to controls instead of sodium valproate at the same volume. The stock and working solutions were prepared in such a way that the same volume was added in the supernatant of cerebellum and cerebral cortex for incubation.

Determination of lipid peroxidation (LPO)

LPO was measured using the procedure of Uchiyama and Mihara (1978) as modified by Tabassum et al. (2007). Briefly, 0.25 mL of homogenate prepared from cerebellum and cerebral cortex was mixed with 10 mM BHT, OPA (1%) and TBA (0.67%) were added and mixture was incubated at 90 °C for 45 min. The absorbance was measured at 535 nm. The rate of LPO was expressed as μmol TBARS formed/h/g tissue based on the molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Determination of protein carbonyl (PC) content

PC content was quantified by the procedure of (Floor and Wetzel, 1998). The most convenient procedure for PC estimation is the reaction between DNPH and protein carbonyl contents. DNPH reacts with protein carbonyls to produce the corresponding hydrazone. The supernatant of cerebellum and cerebral cortex (0.5 mL) was reacted with 10 mM DNPH in 2 M HCl for 1 h at room temperature and precipitated with 40% TCA. The pelleted protein was washed thrice by resuspension in ethanol/ethyl acetate (1:1) mixture. Proteins were then solubilized in 6 M guanidine hydrochloride, 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 nmol DNPH incorporated/mg protein based on the molar extinction coefficient of $2.1 \times 10^4\ M^{-1}\ cm^{-1}$.

Analysis of thiols

Total thiol (T-SH), protein-bound thiol (P-SH) and non-protein bound thiol (NP-SH) were determined in the tissue samples by using the method of (Sedlak and Lindsay, 1968) as adopted by Govil et al. (2012). For the determination of NP-SH 0.5 mL of the supernatant of cerebellum and cerebral cortex was precipitated with 0.5 mL of 40% TCA and then centrifuged at 3000*g* for 15 min. Then the supernatant was used for the measurement by adding 0.4 M Tris buffer (pH 8.9), and 10 mM DTNB. In T-SH estimation the reaction mixture contained 0.2 M Tris buffer (pH 8.2), absolute methanol, 10 mM DTNB and 0.1 mL cerebellum and cerebral cortex supernatant. The P-SH was calculated by subtracting the T-SH and the NP-SH levels. The molar extinction coefficient of $13{,}100\ M^{-1}\ cm^{-1}$ at 412 nm was used for the determination of thiol content. The values were expressed μmol/g tissue.

Reduced glutathione (GSH) content

GSH content was estimated according to the method of (Jollow et al., 1974). The reaction is based on the fact that the thiol group of GSH reacts with the –SH reagent (DTNB) to form thionitrobenzoic acid. The supernatant of cerebellum and cerebral cortex (10%) was mixed with 4% sulphosalicylic acid. It was then incubated at 4 °C for a minimum time period of 1 h and then centrifuged at 4 °C at 1200*g* for 15 min. The reaction mixture contained 0.1 M phosphate buffer (pH 7.4), 10 mM DTNB and 0.4 mL supernatant prepared from 10% homogenate of cerebellum and cerebral cortex of rat brain. The yellow colour developed was read immediately at 412 nm on spectrophotometer. The GSH concentration was calculated as μmol GSH/g tissue using a molar extinction coefficient of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$.

Glutathione-*S*-transferase activity

The method of Habig et al. (1974) with some modification was used to measure the glutathione-*S*-transferase (GST) activity. This reaction is measured by observing the conjugation of CDNB with GSH forming a coloured conjugate glutathione 2,4-dinitrobenzene. For GST activity measurement, the reaction mixture contained 0.1 M sodium phosphate buffer (pH 7.4), 10 mM GSH, 10 mM CDNB, and 0.2 mL cerebellum and cerebral cortex supernatant. The enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of $9.6 \times 10^3\ M^{-1}\ cm^{-1}$ at 340 nm.

Glutathione peroxidase (GPx) activity

GPx activity was assayed according to the method of (Haque et al., 2003). The assay mixture consisted of 0.1 M phosphate buffer (pH 7.4), 1 mM EDTA, 1 mM sodium azide, 1 mM GSH, 0.2 mM NADP(H), 0.25 mM H_2O_2 and 0.1 mL supernatant of cerebellum and cerebral cortex. Oxidation of NADP(H) was recorded spectrophotometrically at 340 nm. The enzyme activity was calculated as nmol NADP(H) oxidized/min/mg of protein, using a molar extinction coefficient of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$.

Glutathione reductase (GR) activity

GR activity was assayed by the method of Mohandas et al. (1984). The assay system consisted 0.1 M phosphate buffer (pH 7.4), 0.5 mM EDTA, 1 mM GSSG, 0.1 mM NADP(H) and 0.3 mL supernatant of cerebellum and cerebral cortex in a total volume of 2.0 mL. The enzyme activity was quantitated at 25 °C by measuring the disappearance of NADP(H) at 340 nm, and was calculated as nmol NADP(H) oxidized/min/mg protein using a molar extinction coefficient of $6.223 \times 10^3\ M^{-1}\ cm^{-1}$.

Catalase (CAT) activity

CAT activity was assayed by the method of Clairborne (1985). This method is based on the disappearance of H_2O_2. The reaction volume contained 0.1 M sodium phosphate buffer (pH 7.4), 0.05 M H_2O_2, and 0.05 mL supernatant prepared from 10% homogenate of cerebellum and cerebral cortex. Change in absorbance was recorded kinetically at 240 nm. CAT activity was calculated in terms of μmol H_2O_2 consumed/min/mg protein using a molar extinction coefficient of $39.6\ M^{-1}\ cm^{-1}$.

Superoxide dismutase (SOD) activity

SOD activity was assayed by the method of (Misra and Fridovich, 1972). The assay was based on the ability of SOD to inhibit the auto-oxidation of epinephrine at alkaline pH. The assay mixture contained 50 mM glycine buffer (pH 10.4), supernatant of cerebellum and cerebral cortex (prepared in glycine buffer), and epinephrine. SOD activity was measured kinetically at 480 nm. The activity was measured indirectly by the oxidized product of epinephrine, i.e., adrenochrome. SOD activity was expressed as nmol of (−) epinephrine protected from oxidation/min/mg protein by the sample compared with the corresponding reading in the blank using a molar extinction coefficient of $4020\ M^{-1}\ cm^{-1}$.

Xanthine oxidase (XO) activity

The activity of XO was assayed by the method of Stripe and Della Corte (1969). The reaction mixture consisted of 0.2 mL supernatant of cerebral cortex and cerebellum, which was incubated for 5 min at 37 °C with 0.1 M phosphate buffer (pH 7.4). Then 0.15 mM xanthine was added to the reaction mixture and kept at 37 °C for 20 min, which was followed by the

addition of 10% PCA and double-distilled water in a total volume of 4 mL. The mixture was then centrifuged at 1500*g* for 10 min and the OD was taken at 290 nm. The enzyme activity was calculated as nmol uric acid formed/min/mg protein, using a molar extinction coefficient of 12,200 M^{-1} cm^{-1}.

Acetylcholinesterase (AchE) activity

AchE was estimated by using the method developed by Ellman et al. (1961). The artificial substrate ATC is broken down in the presence of AChE to release thiocholine, which reacts with 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 cerebellum and cerebral cortex supernatant. The absorbance was measured at 412 nm. The enzyme activity was calculated as nmol ATC hydrolysed/min/mg protein using a molar extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}.

Na^+, K^+-ATPase activity

Na^+, K^+-ATPase activity is measured as the release of inorganic phosphate (P_i) by the method of Saleem et al. (2006). The supernatant of cerebellum and cerebral cortex was prepared in 0.2 M Tris–HCl buffer (pH 7.4). 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 supernatant 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 1500*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 room temperature for 30 min, and the OD was taken at 660 nm. The activity was measured as µg P_i liberated/min/mg protein.

Monoamine oxidase (MAO) activity

MAO was measured by using the method developed by Holt et al. (1997), based on oxidation of BAHC to benzaldehyde. The reaction mixture consisted 0.1 M phosphate buffer (pH 7.4), distilled water, 0.1 M BAHC, 0.2 mL supernatant of cerebral cortex and cerebellum, which was incubated for 30 min at room temperature. 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 nmol BAHC hydrolysed/min/mg protein using a molar extinction coefficient of 7.6925 M^{-1} cm^{-1}.

Protein estimation

The protein content was determined in cerebral and cerebellum supernatant by the method of Lowry et al. (1951) using BSA as a standard.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). All data were analysed using analysis of variance (ANOVA) followed by Tukey's test. Values of $P < 0.05$ were considered as significant. All the statistical analyses were performed using graph pad prism 5 software (Graph Pad Software Inc., San Diego, CA, USA).

RESULTS

VPA induces LPO

The effect of VPA on LPO was investigated by assessing TBARS level in cerebellum and cerebral cortex homogenates of rat brain. TBARS levels were significantly increased in the 0.5-mg VPA-exposed group ($P < 0.05$) and in 1- and 10-mg VPA-exposed groups ($P < 0.001$) in cerebellum and cerebral cortex of rat brain when compared to the control (Fig. 1A). Exposure with 0.1-mg VPA showed no significant change in LPO level when compared with control.

VPA provokes protein oxidative damage

Fig. 1B represents the effect of VPA on PC in cerebellum and cerebral cortex prepared from rat brain. VPA

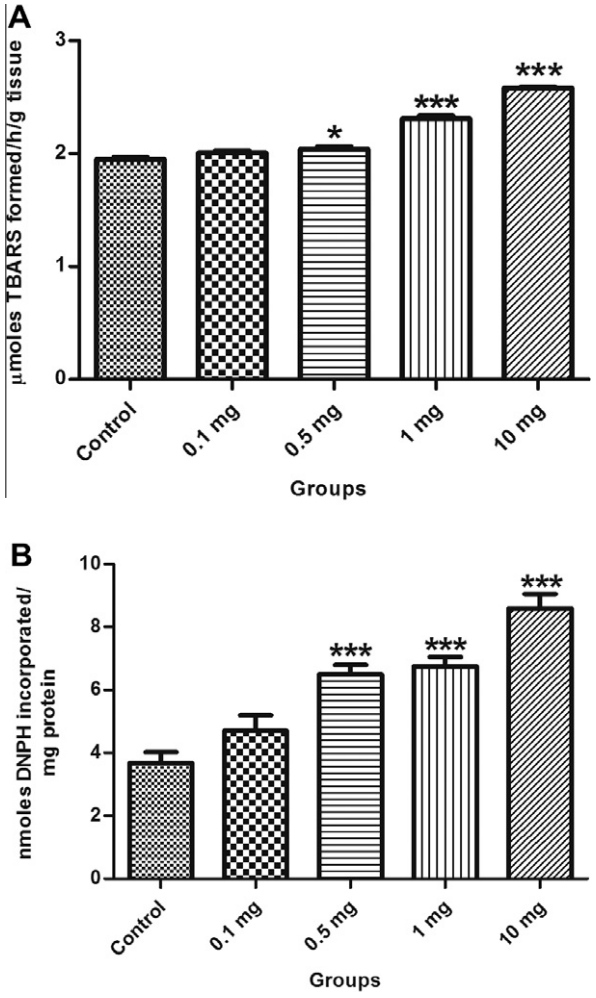

Fig. 1. Effect of different concentrations of VPA on (A) LPO and (B) PC in cerebellum and cerebral cortex preparation of rat brain. Values were expressed as mean ± SEM ($n = 6$). LPO and PC were measured as µmol TBARS formed/h/g tissue and nmol DNPH incorporated/mg protein respectively. Significant differences were indicated by ($^*P < 0.05$ and $^{***}P < 0.001$) when compared with control.

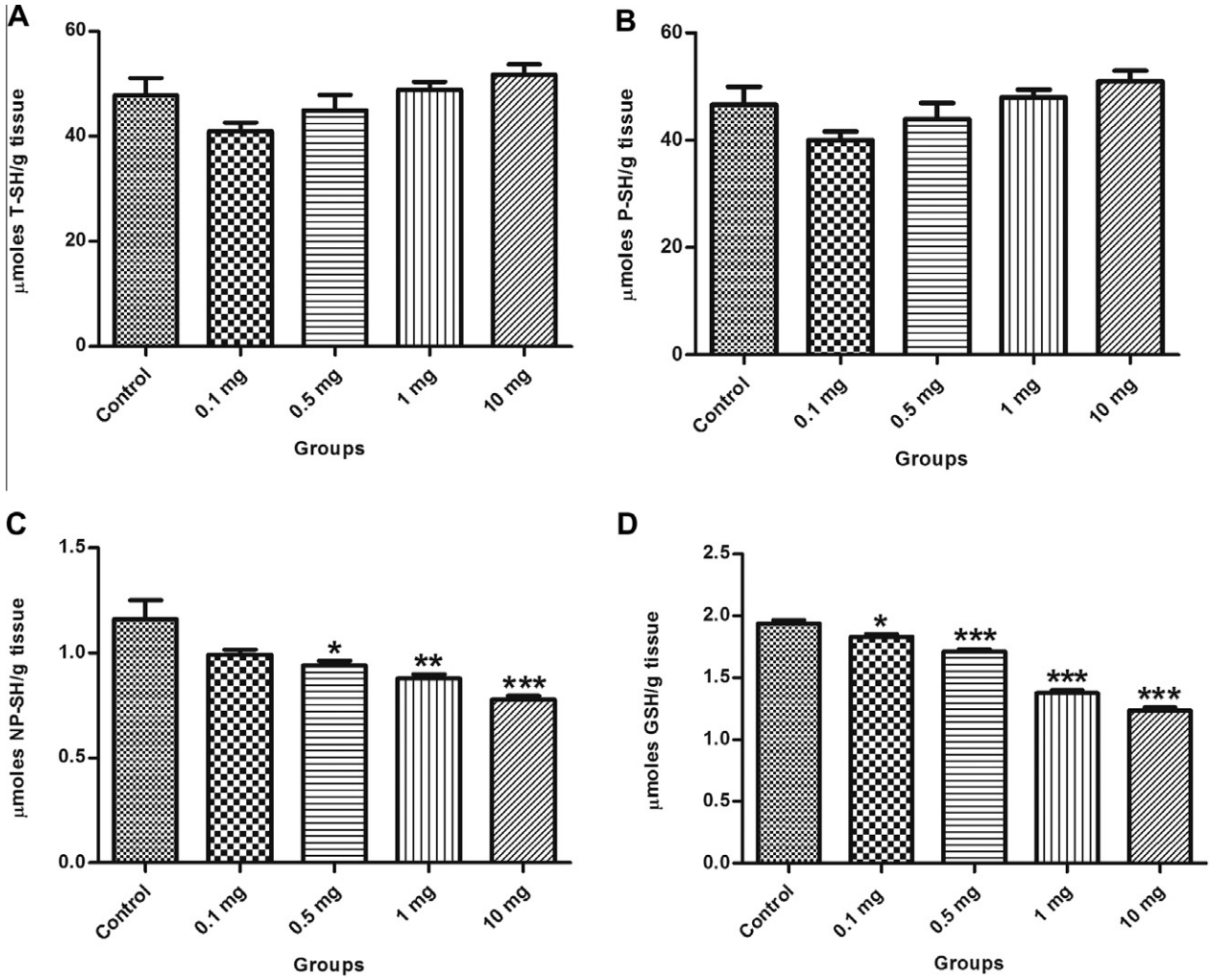

Fig. 2. Effect of different concentrations of VPA on (A) T-SH, (B) P-SH, (C) NP-SH and (D) GSH in cerebellum and cerebral cortex preparation of rat brain. Values were expressed as mean ± SEM ($n = 6$). T-SH, P-SH, NP-SH and GSH were measured as µmol T-SH/g tissue, µmol P-SH/g tissue, µmol NP-SH/g tissue and µmol GSH/g tissue respectively. Significant differences were indicated by ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$) when compared with control.

significantly ($P < 0.001$) raised PC contents in a dose-dependent manner when compared to the control group.

VPA diminishes non-enzymatic antioxidant defences

There were no significant alterations observed on T-SH level in the cerebellum and cerebral cortex of brain tissue between control and other VPA-exposed groups (Fig. 2A).

Fig. 2B shows the concentration of P-SH in the cerebellum and cerebral cortex supernatants of rat brain. There was no significant increase or decrease in the concentrations of P-SH in different exposed groups of VPA when compared with the control. For NP-SH (Fig. 2C) level in both cerebellum and cerebral cortex, each exposed group of VPA has markedly depleted ($P < 0.05$, $P < 0.01$ and $P < 0.001$) the contents of NP-SH when compared with control. Fig. 2D indicates the contents of GSH in the cerebellum and cerebral cortex prepared from rat brain. There was a significant decrease ($P < 0.05$ and $P < 0.001$) in the 0.5–10 mg concentrations in GSH level as compared to the control.

VPA inhibits glutathione metabolizing enzymes activity

VPA exposure with the dose from 0.5 to 10 mg has shown a significant decrease in the activity of GST ($P < 0.05$ and $P < 0.001$) in the cerebellum and cerebral cortex of brain tissue when compared with control. Minimal dose of VPA (0.1 mg) has shown no significant change in the brain when compared with control (Fig. 3A). VPA caused a significant ($P < 0.01$ and $P < 0.001$) depletion in the activity of GR in a dose-dependent manner in comparison to control group (Fig. 3B). The GPx activity was markedly depleted ($P < 0.001$) on exposure with the dose from 0.5 to 10 mg of VPA (Fig. 3C). No significant difference was observed with a dose of 0.1 mg of VPA in cerebellum and cerebral cortex when compared with control.

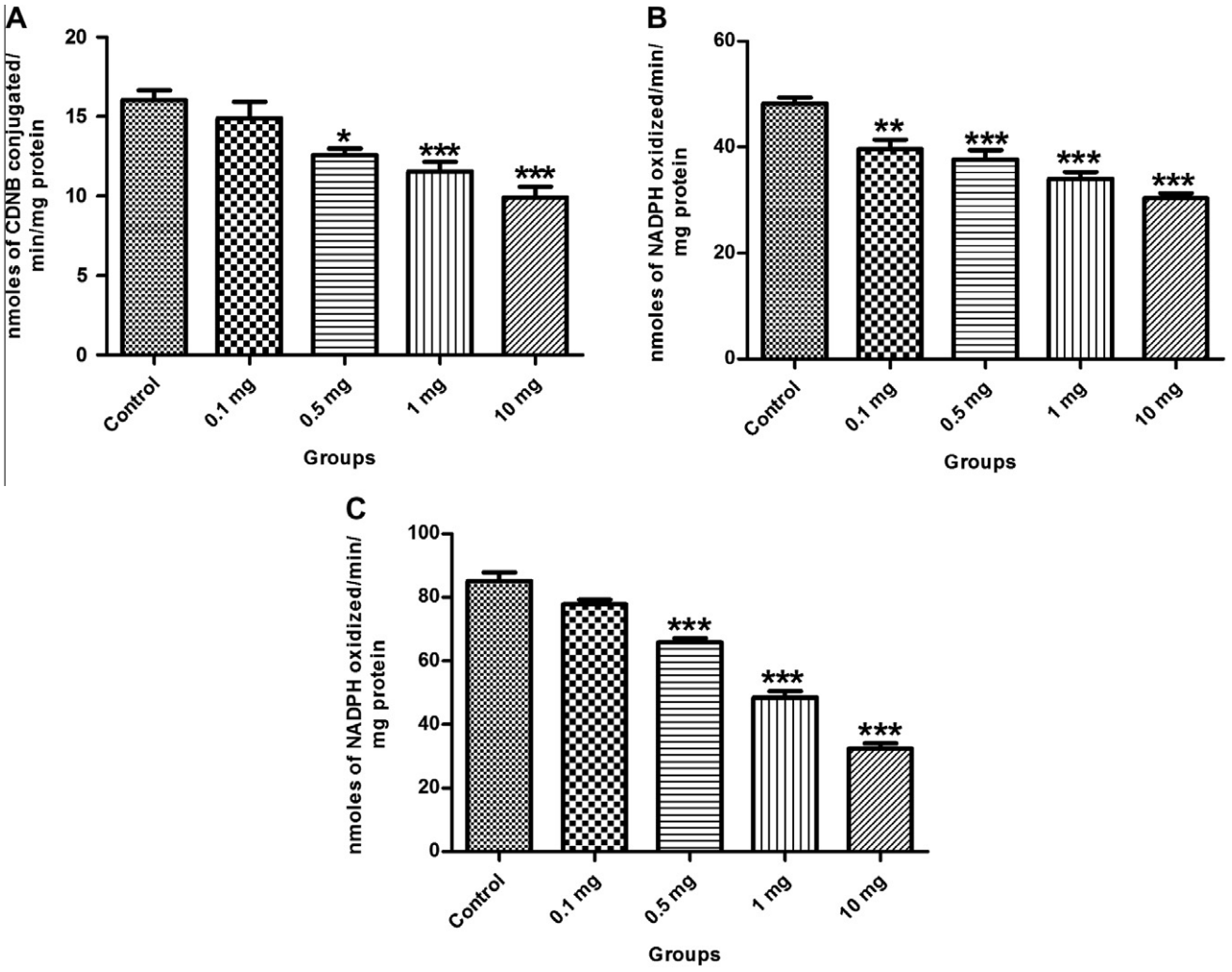

Fig. 3. Effect of different concentrations of VPA on (A) GST, (B) GR and (C) GPx activity in cerebellum and cerebral cortex preparation of rat brain. Values were expressed as mean ± SEM (n = 6). GST activity was measured as nmol CDNB conjugate formed/min/mg protein. GR and GPx activities were measured as nmol NADP(H) oxidized/min/mg protein respectively. Significant differences were indicated by ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$) when compared with control.

VPA alters enzymatic antioxidant defences and elicits the activity of XO

In SOD determination, there was a significant decrease in SOD activity from 0.5-mg ($P < 0.05$), 1-mg ($P < 0.01$) and 10-mg VPA-exposed groups ($P < 0.001$) as compared to the control (Fig. 4A). There was a significant decrease in CAT activity ($P < 0.001$) with increasing concentrations of VPA as compared to the control in the cerebellum and cerebral cortex prepared from brain homogenate of rat (Fig. 4B). Fig. 4C represents XO activity was significantly increased from 0.1 mg ($P < 0.05$) and 0.5–10 mg ($P < 0.001$) as compared to the control.

VPA modulates neurotoxicity biomarkers

Fig. 5A shows the activity of AChE in the cerebellum and cerebral cortex. There was a significant decrease in AChE activity for the 0.1-mg VPA group ($P < 0.01$) and 0.5–10 mg VPA-exposed group ($P < 0.001$) as compared to the control.

A significant decrease was also observed in a dose-dependent manner ($P < 0.001$) in the activity of the enzyme in exposed groups of VPA when compared with the control. Fig. 5B represents the activity of Na^+, K^+-ATPase in the cerebellum and cerebral cortex prepared from brain tissue of rat. In MAO determination, there was a significant increase in MAO activity for the 0.5-mg group ($P < 0.05$), 1- and 10-mg VPA-exposed groups ($P < 0.001$) as compared to the control (Fig. 5C).

DISCUSSION

In the present study, we investigated the possible role of oxidative stress in VPA-induced neurotoxicity in order to evaluate its participation in the brain damage mechanisms responsible for the neurological impairment. Membrane lipids in the brain contain high levels of polyunsaturated fatty acids and are therefore particularly sensitive to oxidation. LPO and PC are

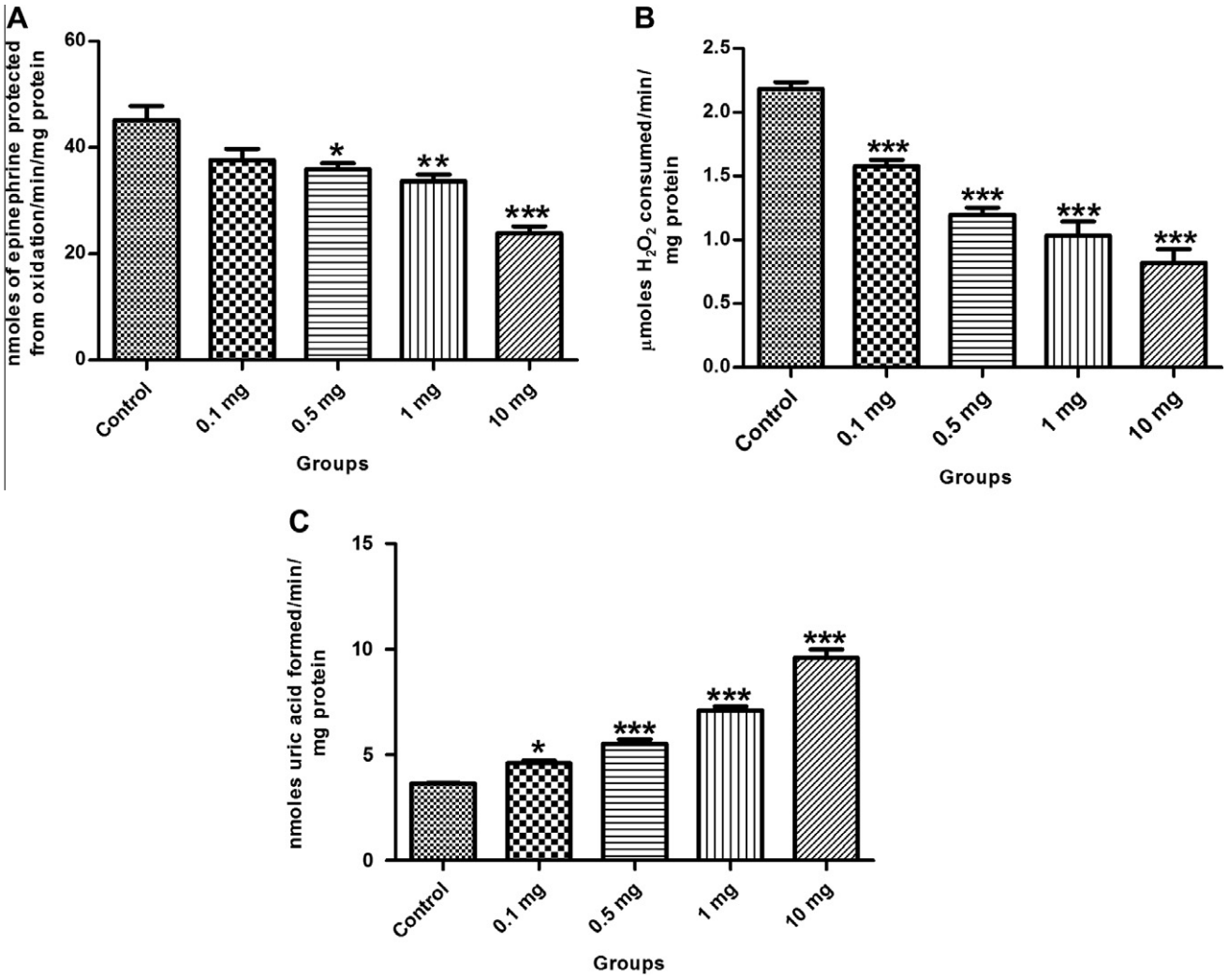

Fig. 4. Effect of different concentrations of VPA on (A) SOD (B) CAT (C) XO activity in cerebellum and cerebral cortex preparation of rat brain. Values were expressed as mean ± SEM (n = 6). SOD, CAT and XO activities were measured as nmol (−) epinephrine protected from oxidation/min/mg protein, µmol H_2O_2 consumed/min/mg protein, and nmol uric acid formed/min/mg protein respectively. Significant differences were indicated by ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$) when compared with control.

plausibly the most extensively investigated process induced by free radical and hence regarded as excellent indexes of oxidative stress. Free radical generation or oxidative stress develops when there is an imbalance between pro-oxidants and antioxidants ratio, leading to the generation of ROS. LPO is also one important cause of neuronal damage and the major consequence of enhanced LPO is attributed to the oxidative deterioration of the cellular membranes (Pederzolli et al., 2010). LPO produces major disturbances in cell function by damaging cellular macromolecules. We first observed that VPA significantly elevated the level of LPO dose dependently. LPO has been hypothesized to be a major mechanism of cell damage by free radicals. The obtained data revealed that the significant increase in the level of LPO may be due to its poor antioxidant defence or the inactivation of antioxidant enzymes due to oxidative stress. A study by Leipnitz et al. (2010) also found that BCFAs like phytanic acid caused a significant induction of both LPO and PC contents in cerebellum as well as in cerebral cortex of rat brain.

This prompted us to investigate possible VPA-provoked protein oxidation, as detected by a marked increase of PC formation. It should be emphasized that oxidation of sulfhydryl groups from specific cysteine residues to form disulphide potentially alters the redox state of protein and leads to their inactivation. Sulfhydryl groups of proteins are oxidized by reactive free radicals giving rise to carbonyl levels. It is therefore presumed that attack of reactive species which are induced by this short branched chain fatty acid leads to the oxidative damage to proteins (Leipnitz et al., 2011). Our results corroborate this observation as PC content increased dose dependently on being exposed to VPA in cerebral cortex and cerebellum.

Subsequently, we examined the level of thiol profile in which we have observed that there was no significant alteration in the T-SH and P-SH contents in both regions of the brain. T-SH includes free amino thiols (–SH) such as P-SH, GSH and NP-SH which are natural reservoirs of the reductive capacity of the cell. It has been widely recognized that –SH plays an integral part

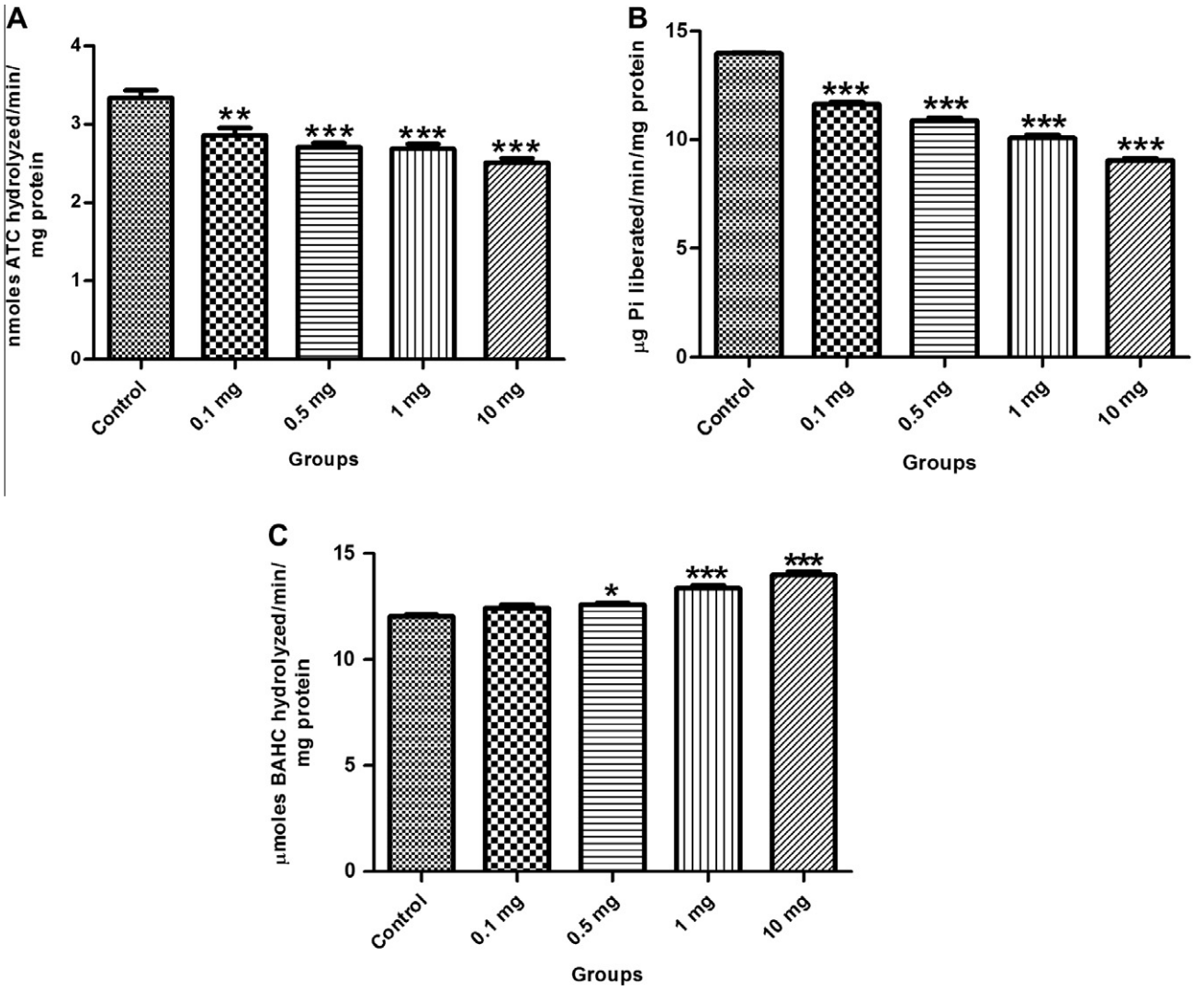

Fig. 5. Effect of different concentrations of VPA on (A) AChE (B) Na^+, K^+-ATPase and (C) MAO activity in cerebellum and cerebral cortex preparation of rat brain. Values were expressed as mean ± SEM (n = 6). AChE, Na^+, K^+-ATPase and MAO activities were measured as nmol ATC hydrolysed/min/mg protein, µg P_i liberated/min/mg protein, and µmol BAHC hydrolysed/min/mg/protein respectively. Significant differences were indicated by ($^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$) when compared with control.

in homoeostasis and has an extensive role in oxidative physiology (Malgorzata et al., 2004). NP-SH level was significantly decreased in cerebellum and cerebral cortex, which supports the finding of a significant decrease in GSH level in the brain tissue as it constitutes more than 90% of NP-SH pool. GSH is the major antioxidant which buffers free radicals in brain tissue and provides protection to the cells from oxidative damage by reducing disulphide groups of cellular molecules or by scavenging free radicals and ROS (Khan et al., 2010). VPA significantly reduced the total content of GSH. Thus, GSH inhibition could increase the susceptibility of plasma membrane towards peroxide attacks (Kiang et al., 2011).

Conflicting results also exist with respect to the effect of VPA on the levels of glutathione metabolizing enzymes. The neurotoxic effect of VPA was demonstrated by a significant decrease in the activity of GST in cerebellum and cerebral cortex of rat brain. GST is a metabolizing enzyme which plays a prominent role in the detoxification of oxidized metabolites and may serve as antioxidant (Shagirtha et al., 2011). A marked suppression of GST activity of VPA-exposed cerebral cortex and cerebellum indicates insufficient conjugation of electrophiles and detoxification of the reactive species. The compensatory biotransformation activity can also be attributed to the decreased activity of GST. GR is another important enzyme for maintenance of intracellular concentration of GSH (Ahmad et al., 2006). The activity of GR was significantly reduced in cerebral cortex and cerebellum. GR activity contributes directly to the protection and repair of –SH protein under oxidative stress. Therefore, the relative decrease of GR could be an indicator of cellular response to the decreased modification of –SH containing protein in the brain (Kaur et al., 2003). GPx is an enzyme that plays an important role in removing excess free radicals and hydroxides and catalyses the conversion of H_2O_2 into water in the

presence of GSH. In the present investigation decreased GPx activity leads to H_2O_2 accumulation in the brain which in turns inactivates SOD.

SOD and CAT are the most important defence mechanisms against toxic effects of oxygen metabolism. These antioxidant enzymes can therefore, alleviate the toxic effects of ROS (Jena et al., 2012). SOD is an important antioxidant enzyme in the body. It catalyses the conversion of superoxide radicals into H_2O_2 and water whereas, CAT detoxifies H_2O_2 into water. In the present investigation the activity of both enzymes was diminished in cerebellum and cerebral cortex. The superoxide radical is the most well known oxygen-derived free radical and can lead to the formation of additional reactive species. H_2O_2, because of its non-ionized state, is able to diffuse through hydrophobic membranes and can form hydroxyl radicals that react with organic lipids to act like highly reactive free radicals (Freitas et al., 2004). These can cause cellular damage and cell death. The decreased activity of SOD can also lead to the formation of superoxide radicals and H_2O_2, which in turn can form hydroxyl radical. The diminished CAT activity induced by VPA may be due to the flux of superoxide radicals. In our study, the activity of XO, a superoxide initiating enzyme was found to be significantly enhanced after exposure of VPA consecutive dose. XO, a highly versatile enzyme that is worldwide distributed from bacteria to human, exists predominantly as NAD^+-dependent xanthine dehydrogenase (XDH), that itself has no role in the initiation or potentiation of oxidative damage in cells/tissue. However, in many pathological conditions XDH is converted into XO. XO catalyses the oxidation of hypoxanthine/xanthine to uric acid and generates superoxide radical. H_2O_2 formed from superoxide radicals could be converted into highly reactive hydroxyl radical ($^{\cdot}OH$) leading to high oxidative stress as a result of oxidation of biological molecules. In our result, a significant increase in XO activity in a concentration-dependent manner of VPA could produce a burst of free radicals. Once $O_2^{\cdot -}$ radical is produced, H_2O_2 and $^{\cdot}OH$ are continuously produced by Haber–Weiss reaction and/or Fenton type reaction (Yalfani et al., 2011).

Alterations in the activity or concentration of neurotoxicity biomarkers such as AChE, Na^+, K^+-ATPase and MAO can also provide for a measurement of the degree of neurotoxicity. The study of brain enzyme activities, such as AChE is essential in observing the neurotoxic effects caused by VPA. AChE is a cholinergic enzyme, which is very important in the synthesis and metabolism of ACh (Santos et al., 2012). In our study we observed a significant decrease of the expression of AChE activity. The inhibition of AChE activity by VPA in cerebellum and cerebral cortex, results in the accumulation of ACh at cholinergic synapses which leads to the over stimulation of muscarinic and nicotinic receptors, and decreases the cellular metabolism, and also induces deformities of cell membrane and disturbs metabolic and nervous activities (Roy and Chaudhuri, 2006). We therefore examined that VPA-inhibited Na^+, K^+-ATPase activity in a dose-dependent manner. Na^+, K^+-ATPase is responsible for the active transport of sodium and potassium ions in the nervous system to maintain neuronal excitability (Ribeiro et al., 2007). In fact, –SH groups of this enzyme are highly susceptible to oxidative stress and oxidizing agents. The marked inhibition of Na^+, K^+-ATPase activity may compromise neurotransmission and lead to partial membrane depolarization allowing excessive Ca^{2+} entry inside neurons with resultant toxic events or production of ROS (Stefanello et al., 2005). Recent studies have also highlighted that the activity of Na^+, K^+-ATPase inhibited by *in vitro* effect of BCFAs in rat cerebral cortex (Busanello et al., 2010).

The present study evidenced that VPA caused a significant elevation of the expression of MAO in cerebral cortex and cerebellum. MAO is also a brain specific enzyme which plays an important role in the metabolism of monoamine substance. In our assessment, the activity of MAO was enhanced. In several studies it has been manifested that MAO activity was raised in aging and dementia which affected the transmission of information and the metabolism of monoamine transmitter and was involved in memory shortages (Fang et al., 2012).

ROS have been associated with many pathophysiological conditions such as cancer and in various neurodegenerative diseases (Leipnitz et al., 2011). Under normal physiological conditions, a delicate balance exists between the rate of ROS formation and the rate of their neutralization. Biological systems are equipped with cellular enzymatic and non-enzymatic defence mechanism to undermine such ROS. Enzyme system plays an important role in neutralizing the oxidative damage (Tabassum et al., 2010). Deficiency of the antioxidant system in the brain implicates a deficient antioxidant system and therefore decreases the capability of attenuating ROS production in brain cells (Pederzolli et al., 2007). The activities of such enzymes can be altered by VPA intoxication which can lead to the disruptions in the brain metabolism and can also contribute to the neurotoxic effect induced by VPA.

CONCLUSION

In the light of results, these investigations provide us with initial impetus that VPA induces oxidative stress by compromising the antioxidant status of the neuronal tissue and subsequently may cause cell death in the brain cells. This is the first study to explore the neurotoxic effect of VPA on specific regions of rat brain. These new findings thus provide a new perspective to further our understanding of the molecular mechanisms underlying the neurotoxicity of VPA on neuronal tissue.

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