DOI: 10.12923/cipms-2026-0011 Curr. Issues Pharm. Med. Sci., Vol. 39, No. 2, Pages 67-72

Current Issues in Pharmacy and Medical Sciences
Formerly ANNALES UNIVERSITATIS MARIAE CURIE-SKLODOWSKA, SECTIO DDD, PHARMACIA

journal homepage: https://czasopisma.umlub.pl/curipms

Evaluation of CREB gene expression to investigate resveratrol's protective effect against sodium valproate-induced oxidative stress in mouse neural tissue

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ARTICLE INFO

Received 31 October 2024
Accepted 20 February 2026

Keywords:
epilepsy;
sodium valproate;
resveratrol;
oxidative stress;
CREB gene.

ABSTRACT

Sodium valproate (SV) is a widely used antiepileptic drug; however, its therapeutic application may be limited by its potential to induce oxidative stress. The present study evaluated the protective effect of resveratrol (REV) on CREB gene expression and malondialdehyde (MDA) levels in mouse neural tissue following prenatal exposure to sodium valproate. Fifteen Balb-C mice were kept in five groups of three per cage (two females and one male) and monitored daily for estrous cycles. After confirming pregnancy, the mice were divided into the following five groups: control, SV (400 mg/kg), SV (400 mg/kg) + REV (600 mg/kg), SV (400 mg/kg) + REV (350 mg/kg), and SV (400 mg/kg) + REV (225 mg/kg). Drug interventions began on days eight to eighteen of pregnancy and continued until delivery. Two to three days before birth, eight fetuses from each group were surgically removed under anesthesia. Thebrain tissue was collected and CREB gene expression was measured using real-time PCR. Lipid peroxidation was assessed using the thiobarbituric acid reactive substances (TBARS) method to measure malondialdehyde (MDA) levels. Sodium valproate significantly reduced CREB gene expression in brain tissue ($p < 0.001$) and increased MDA levels ($p < 0.001$). In contrast, resveratrol significantly upregulated CREB expression ($p < 0.001$) and reduced MDA concentrations, with the most pronounced gene expression effect observed at 350 mg/kg and the strongest reduction in lipid peroxidation at 600 mg/kg. The findings showed that resveratrol can effectively counteract sodium valproate-induced CREB gene downregulation and oxidative stress.

INTRODUCTION

Seizures are transient behavioral and neurological events resulting from the synchronous and excessive discharge of neuronal populations in the central nervous system [1]. When such episodes recur, the condition is defined as epilepsy [2]. Epilepsy comprises a group of chronic neurological disorders with a global prevalence of approximately 0.8%, ranking among the most common neurological diseases worldwide after stroke [3]. Temporal lobe epilepsy represents the most prevalent form of focal epilepsy and often develops months or years after the initial neurological insult [4]. The process through which the brain becomes predisposed to recurrent spontaneous seizures is referred to as epileptogenesis [5]. Pharmacotherapy remains the cornerstone of epilepsy management and is effective in approximately 70% of patients [6].

Sodium valproate (SV), also known as valproic acid (VPA), is one of the most widely prescribed antiepileptic drugs worldwide [7]. It exhibits a broad spectrum of activity and is effective in the treatment of both focal and generalized seizures in adults and children [8]. SV is particularly useful in managing mixed and drug-resistant seizure types. Despite its clinical efficacy, SV is associated with adverse effects, including hepatotoxicity and teratogenicity [9]. Prenatal exposure to valproate significantly increases the risk of neural tube defects, such as spina bifida [10]. Major congenital malformations, including cardiac anomalies, have also been reported following exposure to antiepileptic drugs such as carbamazepine, lamotrigine, valproic acid, and phenobarbital [11].

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VPA possesses antiepileptic mechanisms that include inhibition of voltage-gated sodium channels and enhancement of gamma-aminobutyric acid (GABA)-mediated neurotransmission [12,13]. Additionally, VPA can influence potassium efflux and membrane potential, contributing to its therapeutic effects [14].

Evidence indicates that VPA may induce oxidative stress by impairing mitochondrial function and reducing endogenous antioxidant defenses [15]. VPA-induced neurotoxicity has been linked to increased lipid peroxidation and neuronal injury [16]. Therefore, identifying agents capable of mitigating oxidative stress may improve the safety profile of valproate therapy [17].

Resveratrol (REV) is a naturally occurring polyphenolic compound found in grapes and peanuts [18]. It readily crosses the blood-brain barrier and exerts antioxidant and neuroprotective effects [19]. Resveratrol has been shown to attenuate oxidative stress through modulation of intracellular signaling pathways, including activation of the SIRT1/Akt axis [20].

MATERIALS AND METHODS

Animals and experimental design

Balb-C mice weighing between 25 and 30 grams were obtained from the Animal Center of Mazandaran University of Medical Sciences (ethics code IR.IAU.DAMGHAN.REC.1401.018). The animals had access to food and water and were maintained under controlled laboratory conditions. The mice were kept in groups of five, with two females and one male per cage, and monitored daily for estrous cycles. The female mice were examined daily for a vaginal plug, and if a plug was observed, it was considered the first day of pregnancy. After confirming pregnancy, the female mice were randomly assigned to five groups. All subsequent dissections and analyses were performed by investigators who were blinded to the group assignments. Drug interventions were administered from the eighth to the eighteenth day of gestation and continued until delivery. The groups were as follows:

1. control,
2. 400 mg/kg of SV,
3. 400 mg/kg of SV + 600 mg/kg of REV,
4. 400 mg/kg of SV + 350 mg/kg of REV,
5. 400 mg/kg of SV + 225 mg/kg of REV.

SV was administered orally and REV was injected intraperitoneally. On the 18th day, eight embryos from each group were separated [21]. The embryos were anesthetized with ketamine (80 mg/kg), dissected, and their hippocampi were isolated from the brain. We stored the tissue at –80°C for further analysis.

The treatment period from days 8 to 18 of gestation was chosen to cover the critical stages of hippocampal development and neurogenesis. During this period, exposure to valproate can induce neurodevelopmental changes, and REV intervention can have the greatest protective effect. Furthermore, studies investigating the antioxidant effects of REV in various tissues usually employ low doses (typically between 20 and 50 mg/kg), whereas toxicity studies use extremely high doses (greater than 2,500 mg/kg). In the present pilot study, we used doses higher than those commonly reported in the literature: 225, 350, and 600 mg/kg.

All experiments were conducted according to National Institutes of Health guidelines for the care and use of laboratory animals, the European Council Directive of November 24, 1986, on the care and use of laboratory animals (86/609/EEC), and were approved by the Local Ethics Committee.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from hippocampal tissues using the ParsTous RNA isolation kit according to the manufacturer's instructions. In brief, after adding chloroform, the lysate was centrifuged to create a three-phase solution. Then, the aqueous supernatant was separated and contaminants were removed using a silica column. We used a commercial cDNA synthesis kit (ParsTous, Iran) to produce cDNA, following the manufacturer's instructions. Total RNA from each sample was converted into cDNA via reverse transcription using random hexamer primers. The cDNA synthesis protocol was confirmed by universal primers included in the kit and by polymerase chain reaction (PCR) [22].

Quantitative polymerase chain reaction

The Primer3 software was used to design specific primers for the *CREB* gene, considering the GAPDH gene as a reference. First, the complete sequences of these two genes were extracted from the NCBI database (https://blast.ncbi.nlm.nih.gov). Then, appropriate primers were designed for each gene. To verify the specificity and correctness of the primers, they were examined using the BLAST tool to ensure there were no non-specific matches with other sequences. After confirming the primers' specificity, Pishgam Biotechnology Company (Tehran, Iran) synthesized them. The properties of each primer, including their sequences, are presented in Table 1.

Table 1. Sequence and properties of specific primers

Gene name	Accession No.	Primer Sequence (5′ to 3′)	Annealing temperature
CREB	NC_000067.7	F: CAGACAACCAGCAGAGTGGA	60°C
		R: CTGGACTGTCTGCCCATTG	
GAPDH	NC_000072.7	F: AGACAGCCGCATCTTCTTGT	60°C
		R: CCGTTCACACCGACCTTCA	

Quantitative real-time PCR was performed in a final volume of 20 µL containing 10 µL of SYBR Green (2X), 0.5 µL of each forward and reverse primer, and 20 ng of template cDNA. The experiment was performed using an iCycler Thermal Cycler (Bio-Rad iQ5, USA). Melting curve analysis was also used to validate the accuracy of the results. The thermal cycling conditions were as follows: an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was then performed at 72°C for 5 minutes. To analyze gene expression changes, the Ct value of the reference gene was first subtracted from the Ct of the target genes to calculate ΔCt. Then, the ΔCt values of the different groups were subtracted from each other to obtain the ΔΔCt values. The resulting value was then substituted

into equation 2(−ΔΔCt), which indicates the relative expression level of the genes [23,24].

Lipid peroxidation measurement

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) using the thiobarbituric acid (TBA) method. In brief, 0.2 mL of tissue suspension was mixed with 0.1 mL of TBA reagent containing 0.5 N HCl, 15% TCA, and 0.3 g TBA. The mixture was thoroughly homogenized and heated in a water bath at 100°C for 30 minutes. After cooling, 0.2 mL of n-butanol was added and the solution was thoroughly mixed. The mixture was then centrifuged at 3,500 rpm for 10 minutes to separate the n-butanol layer. The absorbance of the n-butanol layer was measured at 532 nm using a spectrophotometer. MDA levels were measured using a standard curve prepared from known MDA concentrations.

Statistical analysis

In this study, the data obtained from the samples were organized. After calculating the mean and standard deviation, different mean values were compared using an ANOVA test (post-test: Tukey) in SPSS software version 22. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Effects of resveratrol on *CREB* gene expression

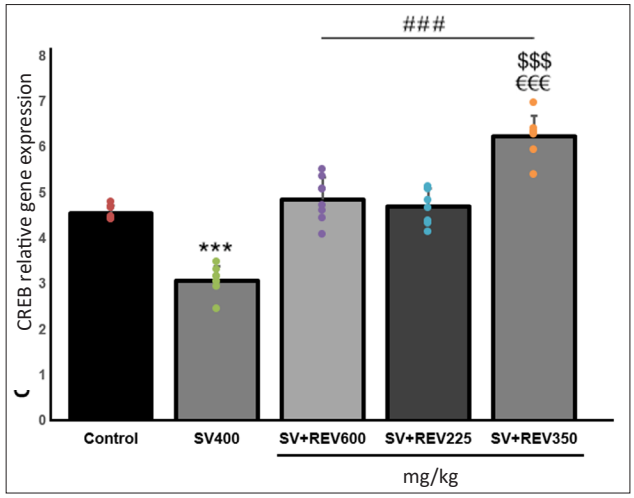
We evaluated the impact of resveratrol administration on the expression of the CREB gene, which regulates antioxidant markers, in brain tissue. Sodium valproate (SV) treatment significantly downregulated CREB expression in brain tissue compared to the control group ($p < 0.001$; $d \approx 6.06$), indicating an oxidative stress-inducing effect of SV. Conversely, REV administration significantly increased CREB expression ($p < 0.001$; SV + REV 600 *vs*. SV 400: $d \approx 4.42$; SV + REV 225 *vs*. SV 400: $d \approx 4.65$; SV + REV 350 *vs*. SV 400: $d \approx 8.28$), suggesting a protective role of REV against SV-induced oxidative stress. The most prominent upregulation of CREB expression was observed at a REV dose of 350 mg/kg, indicating a dose-dependent response. Further analysis revealed statistically significant differences in the effects of different REV dosages. The 350 mg/kg dose had a greater impact than the 225 and 600 mg/kg doses (Figure 1).

These results suggest that REV's neuroprotective properties may be dose-dependent, with the 350 mg/kg dose showing the greatest enhancement of *CREB* gene expression.

The data showed that REV increased the level of this gene at 225 and 600 mg/kg doses, but the greatest enhancing effect on CREB expression was observed at 350 mg/kg. This pattern indicates a dose-dependent effect, with the intermediate dose producing optimal expression, while doses lower or higher than this value are unable to produce a maximal response.

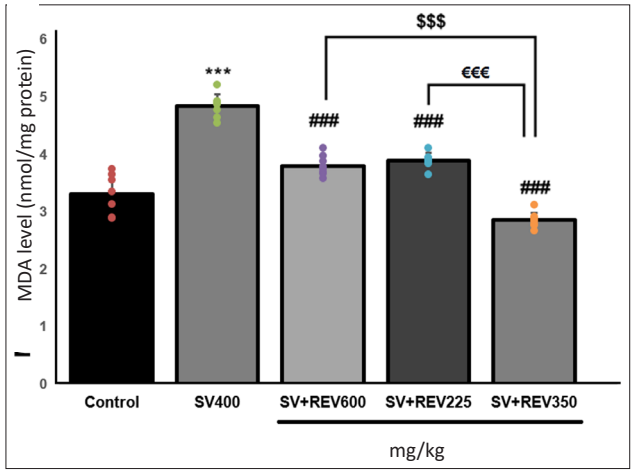
Effects of resveratrol on lipid peroxidation levels

Figure 2 shows that SV significantly increased lipid peroxidation in fetal brain tissue, as indicated by elevated malondialdehyde (MDA) levels compared to the control group ($p < 0.001$; $d = 5.34$). This increase in lipid peroxidation indicates an intensified oxidative stress response following SV exposure. Resveratrol administration demonstrated a dose-dependent protective effect against lipid peroxidation. Among the tested concentrations, the 600 mg/kg dose produced the most substantial reduction in MDA levels. A significant difference was observed compared to the SV-treated group ($p < 0.001$; SV + REV 600 *vs*. SV 400: $d \approx 5.57$; SV + REV 225 *vs*. SV 400: $d \approx 5.68$; SV + REV 350 *vs*. SV 400: $d \approx 11.84$). These results suggest that resveratrol effectively mitigates oxidative stress induced by SV, with its lipid peroxidation-reducing effect being most evident at the 600 mg/kg concentration.



Group 1 received sodium valproate (SV 400 mg/kg), while Groups 2-4 received SV + resveratrol (SV+REV) at 600, 350, and 225 mg/kg, respectively *** p <0.001 *vs*. control; ### p <0.001 *vs*. SV 400 mg/kg; $$$ p <0.001 *vs*. SV+REV600; €€€ p <0.001 *vs*. SV+REV225

Figure 1. Expression of the CREB gene in brain tissue



Group 1 received SV 400 mg/kg, while Groups 2-4 received SV+REV at 600, 350, and 225 mg/kg, respectively. ***p <0.001 *vs*. control; ### p < 0.001 *vs*. SV 400 mg/kg; $$$ p < 0.001 *vs*. SV+REV225; €€€ p < 0.001 *vs*. SV+REV350

Figure 2. Effect of resveratrol on lipid peroxidation levels in brain tissue induced by sodium valproate

DISCUSSION

The aim of this study was to investigate the protective effects of REV against oxidative stress induced by SV, as well as its impact on *CREB* gene expression in the brain tissue of laboratory mice. The results showed that SV

significantly decreased CREB expression, but REV treatment counteracted this effect, suggesting a potential neuroprotective role for REV.

These results suggest that REV has antioxidant properties that protect against oxidative damage induced by SV in brain tissue. The reduction of CREB expression by SV suggests the induction of the oxidative stress pathway. REV's ability to positively regulate CREB expression aligns with its antioxidant properties. Given these results, further studies should examine investigating the role of natural compounds in reducing the negative effects of SV to better understand effective protective mechanisms. Various studies have used substances such as safranal, melatonin, L-cysteine, and the memantine/aripiprazole drug combination to reduce oxidative stress, prevent apoptosis, and improve tissue function. Comparing these results with the present study's findings, in which REV was used as an antioxidant, suggests that REV may mitigate the adverse effects of valproate.

A study was conducted to investigate the protective effects of Safranal (SAF) on SV-induced hepatotoxicity and the underlying mechanisms. SAF was orally administered at a dose of 25 mg/kg to mice once daily for 14 consecutive days prior to SV treatment (500 mg/kg). SAF treatment alleviated SV-induced liver dysfunction and structural damage and decreased hepatic ATP levels and total antioxidant content. Additionally, SAF reduced the SV-induced increase in hepatic oxidant content and lipid peroxidation. These effects were accompanied by significant upregulation of genes such as carnitine palmitoyltransferase 1A, fibroblast growth factor 21, peroxisome proliferator-activated receptor gamma 1, cytochrome P450 2E1 (CYP2E1), and heme oxygenase 1. The results also showed that SV triggered apoptotic responses, evidenced by increased Bax protein and caspase-3 expression. SAF pretreatment significantly ameliorated these apoptotic signals, indicating its anti-apoptotic actions. The researchers concluded that SAF mitigates SV-induced liver injury by reducing CYP2E1 expression, thereby decreasing oxidative stress and apoptotic signaling and restoring ATP levels [22]. In another study, the potential protective effects of melatonin against SV-induced hepatotoxicity in mice were investigated. The results showed that valproate caused a significant reduction in glutathione (GSH) and an increase in lipid peroxidation products in the liver, as well as elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the serum. These changes were effectively reversed by melatonin. Therefore, due to its potent antioxidant properties, melatonin can effectively counteract valproate-induced hepatotoxicity [23].

Another study was designed to investigate the protective effect of L-cysteine on antioxidants in brain tissue of rats. The study revealed that the cerebral cortex of animals treated with SV exhibited increased lipid peroxidation (LPO) and decreased enzymatic antioxidant activity and total antioxidant levels. After four weeks of daily combined treatment with SV and LC, significant improvements were observed in the activity of marker enzymes and cathepsins, as well as in brain structure. LC ameliorated the oxidative stress deficit observed in SV-treated rats. LC reduced LPO levels and restored the activity of antioxidant enzymes and the structural deficits observed in the brains of SV-treated animals. The researchers concluded that LC's protective effect in SV-treated rats is achieved by reducing oxidative stress, which highlights LC's therapeutic role in individuals undergoing SV treatment [24]. Another study investigated the role of chronic combined memantine and aripiprazole treatment in managing prenatal valproic acid (VPA)-induced autistic/cognitive deficits in male Wistar rats. The memantine/aripiprazole combination increased p-CREB, BDNF, and Glt-1 protein expression levels by restoring the GABA/glutamate balance. This reduced VPA-induced neurobehavioral changes, autism-like symptoms, and improved cognitive function. The study revealed that the memantine/aripiprazole combination has favorable cognitive effects in individuals with autism. These effects may be due to enhanced CREB/BDNF signaling, increased astrocytic Glt-1 expression, and restoration of the GABA/glutamate balance. This ultimately inhibits the formation of neurofibrillary tangles (NFTs) and hippocampal neuronal apoptosis [25].

CREB is a transcription factor that increases gene expression and activates antioxidant genes such as SOD, catalase, and HO-1 by binding to CRE in the promoter of genes [26]. CREB also increases the antioxidant response by activating the Nrf2 pathway. The phosphorylation of CREB at serine 133 and its binding to CBP enhances the transcription of protective genes [28]. SV induces oxidative stress and decreases the activity of antioxidant enzymes [29]. CREB activation can increase the expression of antioxidant genes and protect cells from SV-induced damage [30]. CREB also activates survival and neurotrophic genes, such as BDNF, in neurons. Its inhibition increases cells' sensitivity to SV toxicity [25, ~~31~~].

The aforementioned studies demonstrate the effects of various natural substances on SV toxicity in different tissues. In our study, we used the active ingredient REV. These findings support the use of REV as an antioxidant to overcome the adverse effects of valproate. Studies investigating the antioxidant effects of REV in various tissues commonly use low doses (typically between 20 and 50 mg/kg). However, in toxicity studies, very high doses (*e.g.*, above 2,500 mg/kg) are employed [31]. In the present study, we used doses higher than those typically reported in the literature. Through a pilot experiment, we determined the specific doses used in this study and administered them accordingly.

Resveratrol has been shown to reduce oxidative stress markers and enhance antioxidant enzyme activity [32]. It affects various signaling pathways, including the PI3K/AKT pathway, which plays a crucial role in cell survival and protection against oxidative damage. Additionally, resveratrol (REV) can regulate gene expression in oxidative stress responses [33].

Studies have demonstrated that resveratrol protects against oxidative stress-induced damage in neural tissue. It increases the expression of SIRT1, a key regulator of cellular responses to stress [34]. SIRT1 activation leads to downstream effects, including the upregulation of antioxidant enzymes and anti-apoptotic genes [35]. Furthermore, REV modulates transcription factors such

as cAMP-response-element-binding protein and promotes the expression of neuroprotective genes such as Bcl-2 [36].

While this study provides important results on the protective effects of REV against valproate-induced oxidative stress, it has some limitations. First, the study only examined fetal mouse brain tissue and did not investigate other tissues or postnatal behavioral effects. This limits the generalizability of the results. Second, the limited doses of REV used and the lack of long-term evaluation reduce the possibility of fully examining the dose-response relationship and stability of the effects. Furthermore, the study examined only *CREB* gene expression and lipid peroxidation and did not assess protein expression or other antioxidant parameters. The precise molecular mechanisms by which REV affects CREB and lipid peroxidation have not yet been elucidated. Finally, the study examined only one animal model; further research in different models and clinical settings is needed to confirm the results.

CONCLUSIONS

The findings of this study highlight REV's significant protective effects against SV-induced oxidative stress and gene expression changes in the brain. By reducing oxidative damage and restoring normal cellular functions, REV shows promise as a therapeutic agent for conditions related to oxidative stress. Further research is needed to explore the underlying mechanisms of these effects and translate them into clinical applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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