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Protective Effect of Grape Seed Extract against Acrylamide-Induced Neurotoxicity *in Vitro* and *In Vivo*

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Acrylamide (ACR) monomer is an effective neurotoxicant, which damages the central and peripheral nervous systems in humans and animals. It also has broad applications in different industries. Grape seed extract (GSE) possesses antioxidant properties. The present study was designed to evaluate the beneficial effects of GSE against ACR-induced neurotoxicity in both *in vitro* and *in vivo*. In our *in vitro* study, the effect of different concentrations of GSE on ACR toxicity (IC_{50}) in PC12 cells was evaluated using MTT assay. Moreover, in another experiment, the effect of GSE on neural toxicity induced by ACR was evaluated in rats. Pretreatment with GSE (10-100 μM) significantly decreased ACR induced cytotoxicity. In Wistar rats, exposure to ACR (50 mg/kg IP for 11days), significantly induced severe gait abnormalities but treatment with GSE (12.5 mg/kg) reduced ACR induced neurotoxicity in animals. After that, rats were sacrificed and malondialdehyde (MDA) as a marker of lipid peroxidation and GSH content were determined in brain tissue. ACR induced lipid peroxidation and increased level of MDA, while reduced GSH level. Treatment with GSE significantly reduced level of MDA and increased GSH content in cerebral cortex ($p<0.001$). The results suggest that the neuroprotective effect of GSE in this model in part, may be due to its ability to scavenge free radicals and its effective recovery of the antioxidative defense system.

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Introduction

Acrylamide (ACR, H2C = CH–CO–NH2) is a synthetic chemical used to form polyacrylamide which has widely used in electrophoresis and as a part of cosmetics, textiles and paper productions [1]. ACR is a small but highly reactive molecule and is really neurotoxic in human and animals. Humans are exposed to acrylamide through occupational settings, diet and/or lifestyle factors such as cigarette smoking [2]. Long-period ACR exposure causes neurotoxicity characterized by cognitive deficits, gait abnormalities, skeletal muscle weakness, numbness of the hands and feet and ataxia [3].

Additionally, ACR induced carcinogenesis and mutagenesis in rats when orally administrated in high doses, which implies that it is probably carcinogenic to humans [4].

In the cells ACR is metabolized to form glycidamide by cytochrome P450 2E1 (CYP2E1), a reactive substance which may bind to DNA and GSH. The previous studies indicated that diverse toxicity of ACR involves the enhancement of cellular oxidative stress by depleting GSH, formation of reactive oxygen species (ROS) and DNA damage [1].

ACR elevated lipid peroxidation, declined antioxidant capacity in nervous tissue and sciatic nerve and induced apoptosis in cerebral cortex [5]. In recent years, some studies have demonstrated that acrylamide-induced cytotoxicity was relevant to oxidative stress [6, 7]. Other researchers formulated a hypothesis that the acrylamide-induced neurotoxicity is mediated through axonal injury in both central and peripheral nervous system caused by initial distal nerve terminal damage and subsequent retrograde axon degeneration [8]. In numerous cell types, ACR induced cytotoxic effects via raise in different types of oxidative markers such as reactive oxygen species (ROS), 3-nitrotyrosine and activation of COX-2 and NOS [2, 9-11].

Grapes (*Vitis vinifera*) are native to southern Europe and Western Asia cultivated worldwide [12]. Grape Seed Extract (GSE), derived from the seeds of *V. vinifera*, is powerful free radical scavenger. Grape seeds are rich source of flavonoids compounds such as (catechin, epicatechin, procyanidins and anthocyanins) and phenolic acids (gallic acid and ellagic acid) [13]. It has been shown both *in vitro* and *in vivo* models that the antioxidant activity of GSPE is better than that of vitamins C and E and **β**-carotene. It revealed that grape seed extracts are non-toxic to rats [14]. Prior studies have reported various pharmacological properties of GSE such as antioxidant and free radical scavenging, anti-inflammatory, antimicrobial, anticarcinogenic, antidiabetic, cardioprotective, hepatoprotective, antimicrobial activity against Escherichia coli, neurotropic and anti-viral properties [12, 15-18]. Valuable effects of Grape seeds polyphenols in the treatment of metabolic syndrome have been considered [19]. In addition, GSE has neuroprotective properties in the neonatal rat hypoxic- ischemic brain injury model. The results also show that the suppression of free radicals after hypoxic ischemia by grape seed extract is one potential mechanism of this neuroprotection [20].

In this study, we verified the protective effect of GSE on ACR-induced neurotoxicity in PC12 cells as a suitable *in vitro* model for evaluation of neurotoxicity and animal model. The results of this study provide insight into the potential GSE mechanisms involved in neuroprotection.

Materials and methods

Reagents

Fetal bovine serum (FBS) and RPMI 1640 were purchased from Gibco (USA). (4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) were obtained from Sigma (Germany). ACR was purchased from Merck (Germany). Malondialdehyde tetrabutylammonium, reduced glutathione (GSH) and DTNB [5, 5 ′ di thiobis-(2-nitrobenzoic acid)] were purchased from Sigma (Germany).

Preparation of Grape Seed Extract

The grapes (*Vitis vinifera*) were collected from the gardens of the Kashmar, city of Khorasan Razavi

and confirmed by Dr. Shoor from Faculty of Agriculture, Ferdowsi University of Mashhad.
Grape seeds were removed from the grapes manually, air-dried in the shade, for one week, and milled to fine powder. The 100 g - grape seed powder was macerated in 400 ml n-hexane for 24 h at room temperature. Then, the mixture was filtered with filter paper, the filtrate was dried at room temperature (about 25°C) to evaporate n-hexane. Then dried powders were macerated for 6 h at 50°C temperature with water bath three times in water/ ethanol (50:50, v/v), each time. The three macerates were combined and concentrated until no ethanol was left using a rotary evaporator under reduced pressure and a water bath temperature <60°C. Finally the aqueous solution was freeze dried and GSE was kept at -20°C [21].

Cell Culture

PC12 cells were obtained from Pasteur Institute (Tehran, Iran). The cells were cultured in RPMI 1640 medium containing 10% (v/v) heat – inactivated fetal bovine serum, 100 units/mL penicillin, and 100 mg/ml streptomycin in a humidified cell incubator with an atmosphere (90%) of 5% CO2 at 37◦C.

Cell Viability Assay

The cell viability was evaluated by the conventional MTT reduction assay. This method is based on reduction of the tetrazolium salt into a crystalline blue formazan product by cellular oxidoreductase [22]. The amount of formazan produced is relative to the number of viable cells. For this test, PC12 cells in a 96-well microtiter plate at a density of 5000 cell/well were cultured. After incubation with GSE (10, 20, 50 and 100 µM) for 12 and 24 h, ACR was added at a final concentration of 5 mM to each well. After 24 h exposure to ACR, cells were treated with MTT solution with final concentration 0.5 mg/mL for 3 h at 37 °C. Finally, the insoluble formazan was solubilized in dimethyl sulfoxide (DMSO). The absorbance was measured at 545 nm (630 nm as a reference) in an ELISA reader (Start Fax-2100, UK).

Experimental Animals

Male Wistar rats weighing 230-250 g were housed at colony rooms 21 ± 2 ° C with a 12-12 h light/dark period and free access to food and water. All animal experiments were performed according to Mashhad University of Medical Sciences, Ethical committee Acts (910553).

Experimental design

To induce neurotoxicity in Wistar rats, the animals were exposed to ACR at a daily dose of 50 mg/kg intraperitoneally (IP) [23, 24]. According to previous studies, this dose and the route of administration can induce significant neurotoxicity in rats.
A total of 48 adult male Wistar rats were randomly divided into 8 groups of 6 animals each including (1) control group (normal saline); (2) ACR treated group (50 mg/kg,); (3) ACR(50 mg/kg,) + GSE 6.25 mg/kg treated group; (4) ACR (50 mg/kg,) + GSE 12.5 mg/kg treated group; (5) ACR (50 mg/kg) + GSE 25 mg/kg treated group [25]; (6) ACR(50 mg/kg,) + vitamin E 200 mg/ kg treated group [26, 27]; (7) GSE 50 mg/kg group and (8) ACR, 50 mg/kg for 11 days + GSE 12.5, 3 days before administration of ACR and continued during treatment with ACR. Agents were administrated intraperitoneally (IP) once a day and vitamin E three times per week for 11 days.

The Behavioral Index (gait scores) Examination

After 11 days treatment, the gait scores were examined in each animal of all the groups, according to previously described method [23]. Rats were placed individually in a clear plexiglass box and were observed for three minutes to assign subjective gait scores (1–4 levels) level 1 = normal (unaffected gait); level 2 = a slightly affected gait (foot splay, slight hindlimb weakness and spread); level 3 = a moderately affected gait (foot splay, moderate hindlimb weakness, moderate limb spread during ambulation); and level 4 = a severely affected gait (foot splay, severe hindlimb weakness, dragging hindlimb, inability to rear).

Tissue sampling

After determination of gait score, rats were sacrificed and the cerebral cortex was dissected [11, 28, 29], then samples were snap-frozen in liquid nitrogen and stored at −80 °C until use.

Determination of lipid peroxidation

Lipid peroxidation was assayed by measuring levels of malondialdehyde (MDA) in the cerebral cortex. The main principle of the analysis is based on the fact that MDA in the medium reacts when heated with thiobarbituric acid and creates a pink chromogen which has maximum absorbance at 532 nm. The intensity of pink color is in direct proportion to MDA concentration. For this test, 3 ml phosphoric acid (1%) and 1 ml TBA (0.6%) were added to brain tissue homogenate 10% in KCl, and then the mixture was heated for 45 min in a boiling water bath. After cooling the mixture, 4 ml of n-butanol was added and vortex-mixed for 1 min followed by centrifugation at 3000 g for 10 min. The organic layers were removed, transferred to other tubes and the absorbance was measured at 532 nm [30]. A calibration curve was designed using malondialdehyde tetrabutylammonium. MDA levels were expressed as nmol/g tissue.

Reduced Glutathione (GSH) content

GSH was evaluated in brain tissue by the method of Moron et al [31]. The basis of the test was on formation of yellow colour after adding DTNB [5,5′ di thiobis-(2-nitrobenzoic acid)] to compounds containing sulfhydryl groups. For this purpose, 300 µl of tissue homogenates were mixed with 300µl of 10% tricolor acetic acid (TCA) and vortexed. After centrifugation at 2500 g for 10 min, the upper layers were removed and blended with reaction mixtures containing 2 ml phosphate buffer (pH: 8) and 500 µl DTNB. Within 10 min, the absorbance of yellow colour was read at 412 nm using spectrophotometer (Jenway 6105 uv/vis, UK). At the end, the amount of GSH was determined based on a standard curve drawn with commercially available GSH. Levels of GSH were expressed as nmol/g tissue.

Statistical Analysis

The mean ± SD were determined for each study group. The statistical comparisons among groups in each experiment were done with one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons. The P values less than 0.05 were considered significant.

For gait score test, data were shown as median with interquartile range. Non-parametric Kruskal Wallis test was used for statistical analysis of this test.

Results

Effect of GSE on the gait abnormalities induced by ACR in Wistar rat

ACR-administered rat for 11 days exhibited hind limb splay, ataxia, and muscle weakness, with a progressive increase in gait scores as shown in Fig. 1. Treatment of rats with GSE (12.5 mg/kg), 3 days after exposure to ACR could not reduce severe gait abnormalities. Exposure to GSE (50 mg/kg) alone didn't change gait score in animals as compared to control (P>0.05). According to obtained results administration of GSE (12.5 mg/kg) simultaneous with ACR didn't change movement abnormality as compared to ACR treated animals (P >0.05).

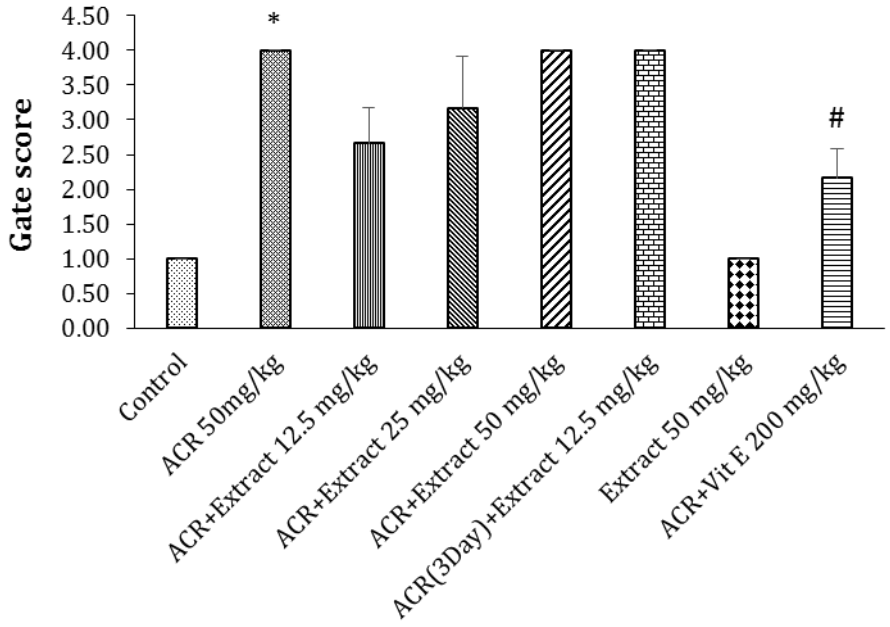

Fig. 1. Effects of GSE on movement disorders caused by ACR. GSE 12.5, 25 and 50 mg/kg and vitamin E 200 mg/kg were injected in animals for 11 days.Data are median with interquartile range (n=6). Non-parametric Kruskal Wallis test was used for statistical analysis. # P <0.05 compared with ACR and * P <0.05 compared to control group.

Effect of GSE on brain lipid peroxidation induced by ACR

The oxidative stress induced in rats by ACR is associated with an increase in the cerebral cortex lipid peroxidation levels (measured as MDA). As shown in Fig. 2, exposure to ACR significantly increased level of MDA as compared to control (P< 0.001). Treatment with doses of GSE (12.5 and 25 mg/kg) reduced oxidative stress and the best effect was obtained in dose of 12.5 mg/kg. Administration of GSE 3 days after exposure to ACR was effective in reduction of lipid peroxidation. Exposure to GSE (50 mg/kg) alone didn't increase levels of MDA in brain tissue when compared to control group (P>0.05).

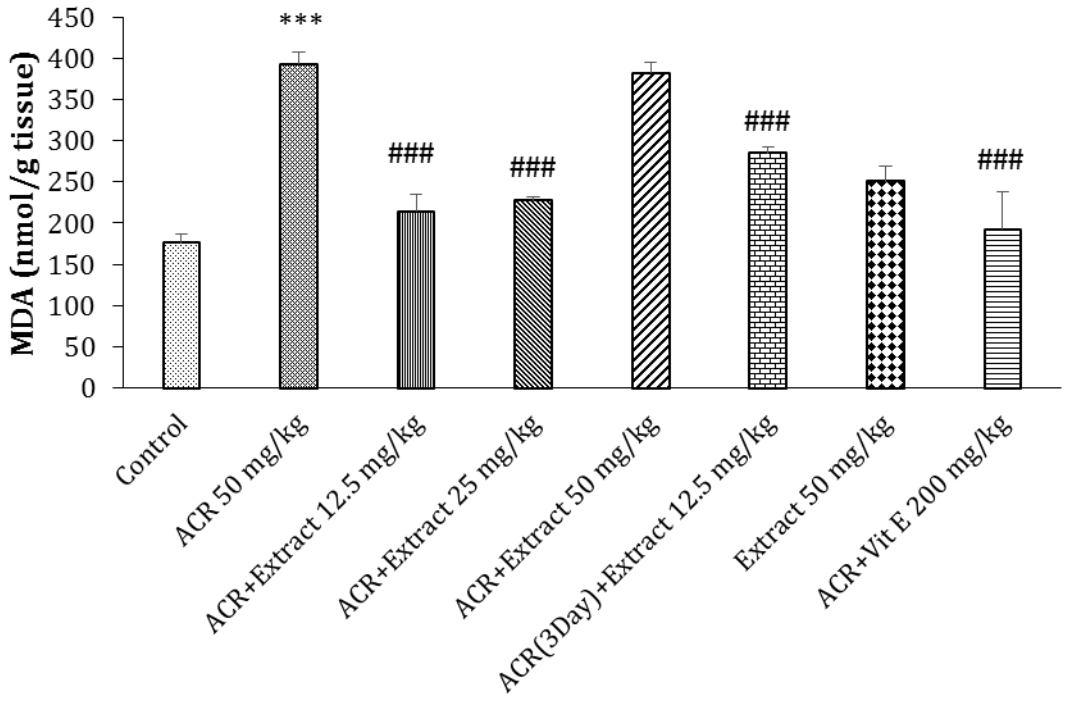

Fig. 2. Effects of GSE on lipid oxidation in cerebral cortex induced by ACR. MDA denotes the amount of lipid peroxidation in cerebral cortex. Data are expressed as Mean ± SD (n=6). # # # P <0.001 compared with ACR, *** P <0.001 compared to control group.

Effect of GSE on GSH content in brain tissue following exposure to ACR

In the ACR treated group, GSH levels decreased in cerebral cortex (P<0.001) (Fig 3). Treatment with GSE in doses (12.5 and 25 mg/kg) significantly increased GSH level compared to ACR treated rats (P <0.001). GSE (12.5 mg/kg) in both administration ways simultaneously or 3 days after exposure to ACR could protect cerebral cortex against ACR toxicity. Treatment with GSE (50 mg/kg) alone had no effect on the GSH level (P>0.05 vs control group).

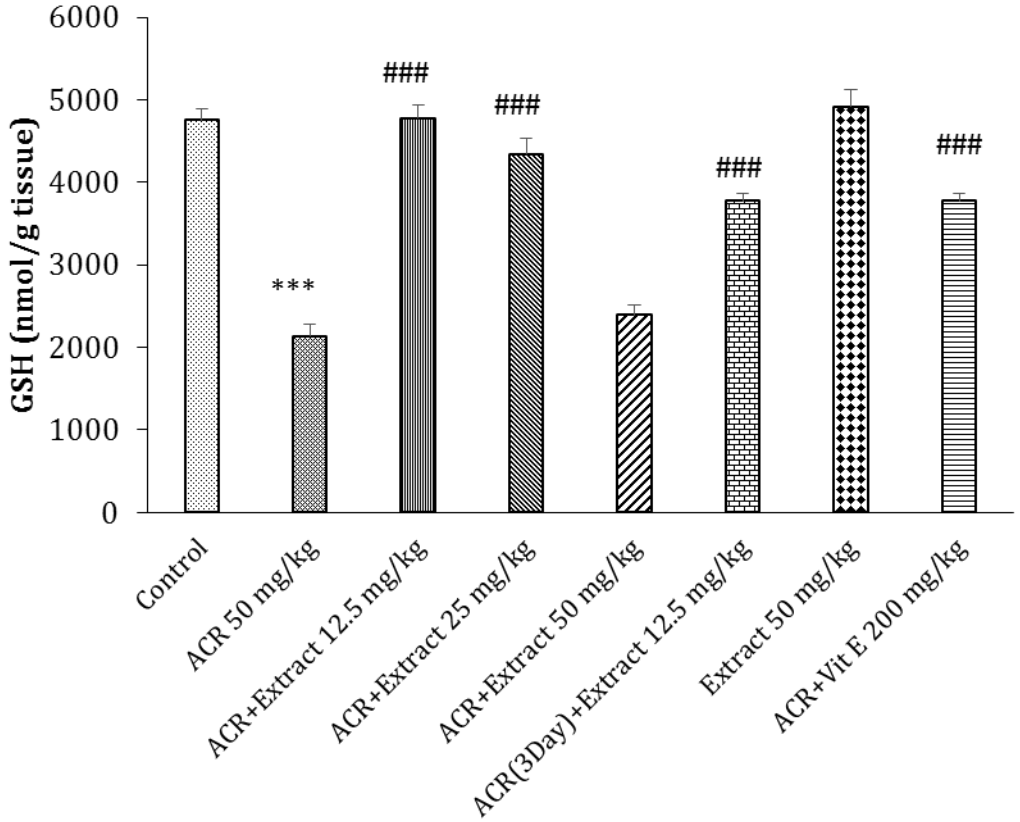

Fig. 3. Effects of GSE on GSH content in cerebral cortex following exposure to ACR . Data are expressed as Mean ± SD (n =6). # # # P <0.001 compared with ACR and *** P <0.001 compared to control group.

Effect of ACR in PC12 cells

In this study, we used PC 12 cells to determine the effect of GSE on ACR-induced cytotoxicity. In order to determine the concentrations at which cell toxicity was induced by ACR, PC12 cells were exposed to different concentrations of ACR (1-20 mM) for 24 h. As shown in figure 1, treatment of cells with ACR decreased cell viability in the dose dependent manner. The IC_{50} (50% inhibitory concentration) value of ACR for 24 h was 5 mM (Fig 4).

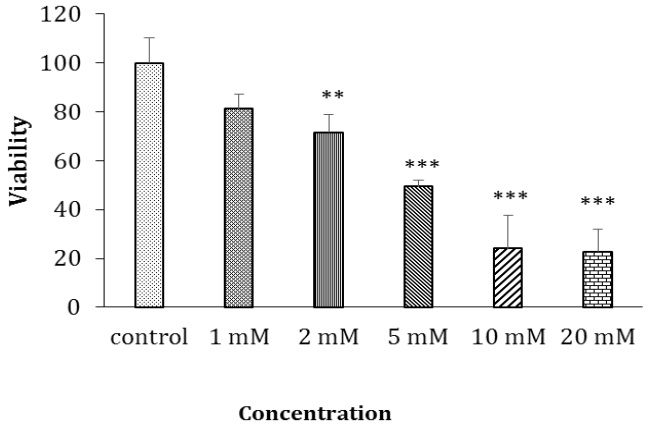

Fig. 4. Effect of ACR on PC12 cell viability. Cells were exposed to various concentrations of ACR for 24 hours. Data are expressed as Mean ± SD (n =4). P <0.001 ***, P <0.01 ** compared to control group.

Effect of GSE on ACR induced cytotoxicity in PC12 cells

For determination of the nontoxic concentrations of GSE in PC12 cells, the effects of different concentrations of GSE were analyzed after 48 h exposure using MTT assay. Results showed that exposure to GSE (1, 2.5, 5, 10, 20, 50,100 µg/ml) for 48 h did not change cell viability as compared to control (Fig 5).

For evaluation the protective effect of GSE, cells were treated with different concentrations of GSE (10, 20,50 and 100 µM) for 12 and 24 h .Then, the cells were treated with final concentration (5 mM) of ACR. After 24 h exposure, cell viability was evaluated using MTT test. Pretreatment of cells with GSE at different times had different results. Following exposure for 12 h with GSE (10, 20, 50 and 100 µg/ml) cell viability was meaningfully higher than ACR (P<0.001) (Fig 6A), also exposure to GSE (10, 20, 50 and 100 µg/ml) for 24 h significantly decreased the ACR-induced cytotoxicity in comparison to ACR (P<0.001) (Fig. 6B).

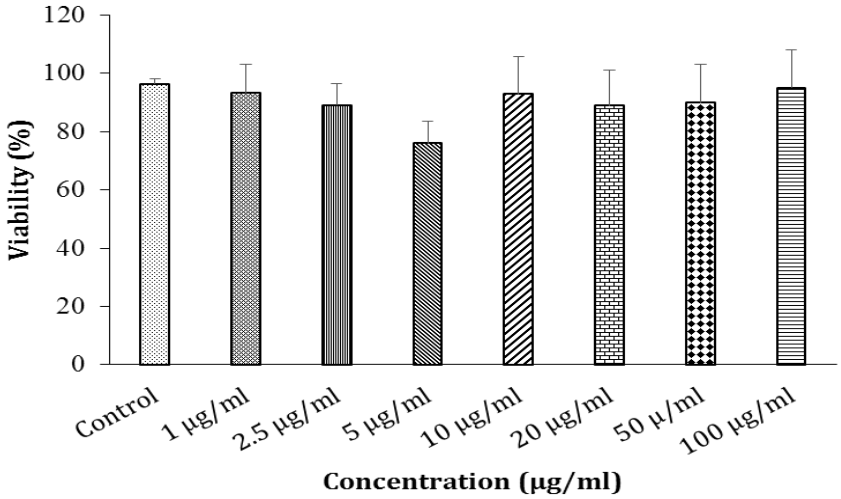

Fig. 5. Effect of GSE on PC12 cell viability. Cells were exposed to various concentrations of GSE for 48 hours. Data are expressed as Mean ± SD (n =4).

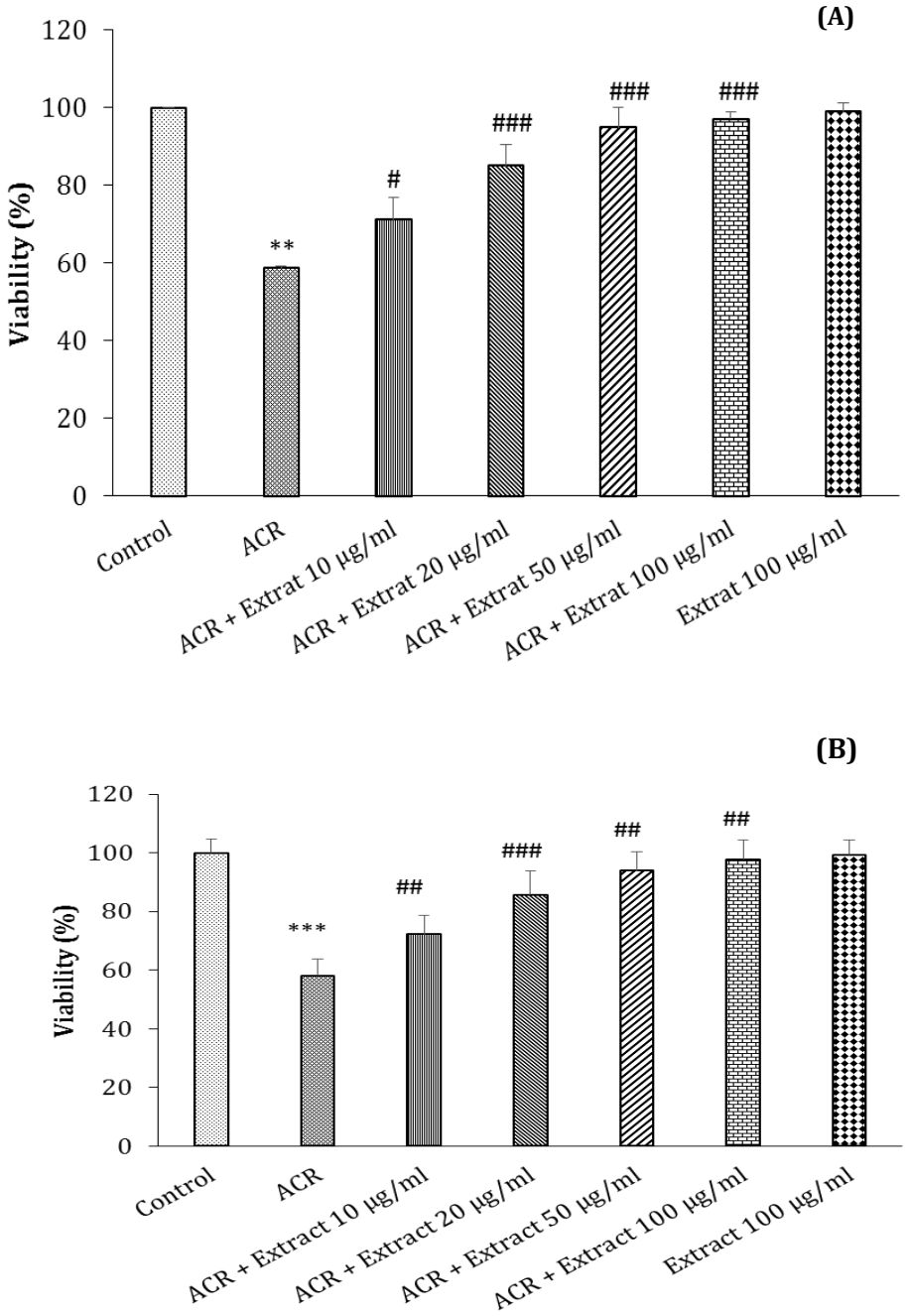

Fig. 6. A) Effects of GSE on cytotoxicity in PC12 cells induced by ACR. Cells were exposed to different concentrations of GSE (10, 20, 50 and 100 µg/ml) for 12 h. Then ACR (5 mM) was added to the cells. After 24 h exposure cell viability was assessed using MTT test. Data are expressed as Mean ±SD (n =4). # # # P <0.001 compared with ACR and *** P <0.001 as compared to control group. **B)** Effects of GSE on cytotoxicity in PC12 cells induced by ACR. Cells were exposed to different concentrations of GSE (10, 20, 50 and 100 µg/ml) for 24 h. Then (5 mM) ACR was added to the cells. After 24h exposure cell viability was assessed by MTT. Data are expressed as Mean ±SD (n =4). Tukey-Kramer test was used for statistical analysis. # # # P <0.001 compared with ACR and p <0.001 *** as compared to control group.

Discussion

The results of our study demonstrated that ACR increased the oxidative stress in the cerebral cortex tissue through reduction of GSH content and elevation of MDA level.

Lipid peroxidation which is expressed as thiobarbituric acid reactive substance reflects a condition of oxidative stress. MDA as a marker of lipid peroxidation activity was associated with a significant reduction in the level of reduced GSH, an important antioxidant defense mechanism. The results of the current study are in agreement with previous reports which exhibited treatment with ACR (50 mg/kg) for 11 days elevated MDA level, decreased GSH content in cerebral cortex and induced behavioral defects [8, 11]. It is reported that ACR can inhibit glutathione S-transferase activity and deplete GSH content [32]. Additionally, ACR is capable of interacting with vital cellular nucleophiles possessing –SH, –NH2 or –OH, so, it reacts with GSH in a similar manner and forms GSH S-conjugates which is the initial step in the biotransformation of electrophiles into mercapturic acid. Consequently, oxidant/antioxidant balance is spoiled and the initiation of oxidative stress induced cellular damage. Previous studies also showed that conjugation with GSH is an important mechanism for the detoxification of ACR [33]. Regarding to important role of oxidative stress in ACR-induced neurotoxicity, it seems that antioxidants can effectively recover ACR toxicity. It has been shown that chrysin [29], thymoquinone [28] and crocin [22] as powerful antioxidant agents exhibited neuroprotective effects against ACR-induced neurotoxicity models.

GSE, which is rich in catechin, epicatechin and procyanidin, showed greater antioxidant activity than vitamins C and E, as well as β-carotene [34], and is considered to be a safe and effective antioxidant compound. The antioxidant and anti-inflammatory capacity of GSE has been previously demonstrated in other tissues [35]. GSE, as a potent antioxidant exhibited neuroprotective properties protects damaged neurons [34]. Also, in another study it has been reported that resveratrol (a potent antioxidant in grape) reduced Parkinson disease induced by 6-OHDA in Wister rats [36].

Furthermore, neuropharmacological studies based on cellular and animal models demonstrated that resveratrol has significant effects on cognitive and memory function [37].

ACR is considered to be the typical chemical among the many toxicants that causes a central-peripheral distal axonopathy. The initial target of ACR appears to be nerve terminals in both the CNS and PNS, resulting in autonomic, behavioral, sensory and motor disturbances [38]. We found that the rats receiving ACR (50 mg/kg, IP) for 11 days suffer from hind-limb paralysis and walking difficulties.

Effect of GSE in ACR-induced cytotoxicity in PC12 cells was evaluated in our study. According to obtained data, exposure to ACR significantly reduced cell viability in the concentration-dependent manner. These results are agreement with previous studies which showed ACR activated caspase-3 and increased sub-G1 population in SH-SY5Y cells [39]. Furthermore, ACR increased ROS production and induced apoptosis in PC12 cells by increasing the bax/bcl2 ratio and activation of caspase 3 [22]. Induction of apoptosis and production of ROS have been considered as main mechanisms which involved in ACR cytotoxicity.It has been reported grape seed polyphenolic extract reduced cytotoxicity in PC12 cell that induced by Aβ40 and Aβ42 [40]. It has been shown consumption of grape seed extract prevents amyloid-β deposition and decreases inflammation in brain of an Alzheimer's disease mouse [41]. Also, the findings of another study demonstrated that grape seed extract enhanced the antioxidant status and decreased the incidence of free radical induced protein oxidation in aged rats thereby protecting the central nervous system from the reactive oxygen species [42]. Furthermore in recent study, it has been shown that resveratrol may have potential clinical applications in selected cases of PD-affected patients by regulates energy homeostasis through activation of AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1) and raise of mRNA expression of a number of PGC-1α's target genes resulting in enhanced mitochondrial oxidative function [43].

In our research, pretreatment of cells with GSE for 12 or 24 h markedly inhibited ACR-induced cytotoxicity in PC12 cells. It is concluded that protective effects of GSE in this model in part can be due to potent antioxidant effect of this extract.

Conclusion

According to the findings of peresent study, grape seed extract reduced ACR-induced toxicity in Wistar rat and PC12 cells. The effect of grape seed extract can be mediated in part through significant antioxidant properties. However, to clarify the neuroprotective effects of GSE it is suggested to evaluate the cellular and molecular mechanisms of this extract in further studies

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Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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