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Creatine and alpha-lipoic acid antidepressant-like effect following cyclosporine A administration

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ABSTRACT
Background: Cyclosporine A (CYA), is an immunosuppressant drug used to prevent graft rejection, but it may initiate neuropsychological problems such as depression. The aim was evaluating the antidepressant-like effects of creatine (Crt) a mediator of oxidative phosphorylation and alpha-lipoic acid (ALA) a cofactor for the mitochondrial respiratory chain following CYA administration. **Methods**: Female mice (27±2 g) were used, immobility time during the forced swimming test (FST) was measured, and hippocampal brain-derived neurotrophic factor (BDNF) level was evaluated. CYA 20 mg/kg, ALA 40 mg/kg, fluoxetine 20 mg/kg (IP) and Crt 10 mg/kg (oral) were administered for 6 consecutive days, and the tests were performed on day 7. **Results**: ALA, but not Crt, treatment alone decreased immobility in the FST (i.e., decreases depression-like behavior). CYA administration increased immobility in the FST (175.1±13.16s, vs vehicle 130.9±13.5s, p=0.0364), and this depression-like behavior was prevented by co-administrating, ALA (100±15.9s, p=0.020) or Crt (93.5±16.6, p= 0.009) and the positive control, fluoxetine. Notably, there was a synergistic effect of Crt-ALA co-administration since CYA-induced immobility was lower in this group than the groups pretreated with Crt or ALA. These behavioral changes were observed in the absence of treatment effects on locomotor activity in an open field. CYA treatment increased hippocampal BDNF protein levels that was prevented by co-administration of ALA (with or without Crt) or fluoxetine. **Conclusion:** CYA-induced depression-like behavior might be related to hippocampal mitochondrial dysfunction as ALA and Crt prevented the development of this behavioral phenotype. ALA similar to fluoxetine prevented BDNF alteration and its possible neurological changes.
Keywords: Cyclosporine, depression, creatine, alpha-lipoic acid, brain-derived neurotrophic factor (BDNF)

INTRODUCTION

Calcineurin, a calcium-dependent protein phosphatase enzyme is well known for its effect as a modulator of the immune response. It also participates in neurotransmission, neuronal structure,

and neuronal excitability [1,2]. Calcineurin inhibiting drugs, such as cyclosporine A (CYA) are immunosuppressive drugs that have developed the organ transplantation process by extensively reducing allograft rejection rates in individuals [3,4]. However, some patients that receive these agents suffer from neuropsychological problems such as depression, anxiety, confusion, and tremor [5]. Peripheral administration of high dose (60 mg/kg) CYA has decreased release of serotonin and dopamine and caused prefrontal cortex dysfunction that could be responsible for the increased anxiety and social behavior disturbance [6]. Additionally, CYA may induce neurotoxicity by interaction with brain mitochondria functioning [5]. Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family, it is the most plentiful neurotrophin in the central nervous system (CNS) and is related to neural cell survival, and neural transmission [7]. Reduction of BDNF expression in hippocampal neurons would cause severe stress and could also influence learning, inspiration, and mood [8,9].

Creatine (N-aminoiminomethyl-N-methylglycine; Crt) can be endogenously synthesized by the liver, kidney, pancreas, and to some extent in the brain from the amino acids arginine, glycine, and methionine. Crt is also provided in diets having meat or fish [10]. Adenosine triphosphate (ATP) is the main energy source in the brain that is closely joined to phosphocreatine (PCr). The isoenzymes of Crt kinase are specially localized in high demanding ATP sites like the neurons to efficiently regenerate ATP in situ via PCr[10]. Therefore, oxidative phosphorylation and Crt/phosphocreatine system prepare high energy that is critical for CNS function[11]. Crt has various properties in the CNS including antioxidant, anti-inflammatory, anti-apoptotic, and neuromodulatory activity [10,12] . These effects have provoked further research regarding Crt monohydrate efficacy for treating neurological disorders.

Alpha-lipoic acid (ALA) naturally occurs in vegetables like broccoli, spinach, and tomatoes[13]. ALA is an essential cofactor for mitochondrial respiratory chain enzymes α-keto-dehydrogenase complexes [14]. ALA and its reduced metabolite dihydrolipoic acid have been noticed as antioxidants against hydroxyl radicals, and as inhibitor of lipid and protein oxidation. Interestingly, this free radical quenching antioxidant in contrast to vitamin E (which is fat-soluble), is soluble in both fat and water thus it deactivates free radicals in both fatty and watery areas of cells. ALA can readily spread into CNS and induce protective effects on the nervous system, this has led to its promising therapeutic effects of brain disorders [14].

Since CYA might induce depression side effects and administrating prophylactic antidepressant drugs would expose the individual to unnecessary medication and polypharmacy, the aim was introducing an alternative medicine. Therefore, the effect of treatment with two supplements ALA (cofactor for mitochondrial respiratory chain) and Crt (mediator of mitochondria oxidative phosphorylation) were evaluated on CYA induced behavior changes in mice. The study novelty was that, the anti-depressant effects of Crt and ALA were evaluated following CYA administration in mice and finally, the BDNF levels were assessed in mice brain.

MATERIAL AND METHODS

Animals

Female NMRI mice (weighing 27±2 g, 6-8 weeks old) were housed six in each cage and kept at room temperature 21± 2 ºC, on a 12 h light and 12 h dark cycle (lights on at 6 AM), standard mice chow and tap-water ad libitum. Cages were placed in the behavioral laboratory 24 h prior the experiments in order to acclimatize. The experiments were carried out according to the guidelines for the Care and Use of Laboratory Animals Issued by The National Ethical Committee of …. (Ethical No: IR.MUI.RESEARCH.REC.1399.200). All the attempts were made in the research to reduce animal distress and the number of animals used in the research.

Drug administration

CYA (Sandimmun, 50 mg/ml; Novartis, Switzerland) 20 mg/kg was injected IP after it was diluted in 2%v/v EtOH/normal saline [15]. Crt monohydrate (Karen Pharma and Food Supplement, Iran) 10 mg/kg was administered by daily gavage feeding tube [16], ALA (Sigma Aldrich, India) 40 mg/kg was injected IP [17]. A selective serotonin reuptake inhibitors (SSRIs) fluoxetine (Sigma-aldrich, Germany) 20 mg/kg was injected IP [15]. Control groups were injected with normal saline, or received normal saline by gavage feeding tube. In a separate group of mice, Crt and ALA were co-administration with CYA. The volume for all of the injections were 10 ml/kg.

Experiment design

Totally 9 groups of animals consisting 6 mice in each group were studied. Groups included: groups that received each of the drugs (ALA, Crt) alone and the control group that received normal saline (data for IP injection and gavage were similar therefore one group was considered here). The CYA alone group and the vehicle group (2%v/v EtOH/normal saline). Three groups that received ALA, Crt, or fluoxetine (the positive control) concomitantly with CYA; finally, a group that was treated with ALA+Crt together with CYA. All the treatments were administered for 6 consecutive days and the tests were performed on day 7. Initially the locomotor test and then the forced swimming test (FST) for measuring animal despair behavior, were performed on each animal. After the experiments the animals were decapitated the brain was carefully removed on ice and stored in -70 for BDNF evaluation.

Locomotor test

The locomotor activity of mice was assessed in an open arena (45×45 cm) (Borj Sanat, Iran) divided into 15 zones by red beams. Mice were allowed to explore the field for 3 min [18,19], by passing through the beams the number of zone entries (horizontal exploration) was counted automatically while rears on back-legs (vertical exploration) were recorded manually. The total activity for each animal was calculated which was the sum of zone entries and rears on back-legs.

Forced swimming test

During FST mice were forced to swim in 25 ºC water in a 2-liter Pyrex beaker (diameter 12.5 cm, depth 12 cm) for 6 min, the first 2 min was considered for habituation [20]. The animals' despair behavior was evaluated by measuring the immobility time during the last 4 min of the trial. It was considered when animals had no additional activity other than that required to keep the animals' head above the water. Swimming behavior, defined as horizontal movement throughout the beaker which involved at least two limbs; and, climbing behavior, defined as upward movements of the forepaws along the side of the beaker were also recorded. The entire experiment was recorded by a camera and analyzed later. After 6 min, the mice were dried carefully to avoid hypothermia and returned to their home cage.

Brain BDNF Enzyme-Linked Immunosorbent Assay (ELISA)

Mice were rapidly decapitated after CO2 euthanasia and the whole brain was quickly removed, weighed and the hippocampus was dissected on a cold tile. After that the frozen samples were kept at -70ºc until further assay. Hippocampus of each sample was homogenized for 30 sec with ice-cold extraction buffer containing 50 mM Tris–HCl, 0.6 M NaCl, 0.2% Triton-X 100, 1% BSA, 0.1 mM benzethonium chloride, 1 mM benzamidine, and 0.1 mM PMSF at pH 7.4. A Mice BDNF ELISA kit (Picokine TM ELISA, catalog No: EK0309, Boster Biological Technology, Canada) was used according to the manufacturer's instructions. Briefly, the homogenates were centrifuged (10,000 × g, 25 min, 4 ◦C), and the supernatant was separated [21]. After dilution, 100 µL of samples were added to each empty well and incubate at 37°C for 90 minutes. After

washing the wells 100 µL of biotinylated anti-mice BDNF antibody working solution was added and incubated at 37°C for 60 minutes. Then all of the wells were washed three times with PBS and incubated with ABC working solution at 37°C for 30 minutes. Finally, TMB color was added to each well and kept in dark for 30 minutes. After changing the color into yellow following TMB stop solution addition the plate O.D. absorbance was read at 450nm using a Synergy HTX microplate reader (BioTek, USA). All of the tests were run in duplicate. The standard curves ranged from 9.5 to 380 pg/ml BDNF with a sensitivity of 4 pg/mL. The intra-assay coefficient of variation varied from 2.5 to 5.1% and the inter-assay coefficient of variation varied from 4.8 to 6.3%. BDNF levels were calculated as pg/mg of total protein.

Data processing and statistical analysis

Results were expressed as group mean ± SEM. All behavior results were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. BDNF results were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. P values less than 0.05 were considered significant. The software programs used for data analyzing and making graphs were Excel 2010 and the GraphPad Prizm (version 8).

RESULTS

Effect of Crt, ALA, and CYA on the behavioral tests

The results of locomotor activity depicted in Fig 1a shows that there was no statistically important difference in the total activity between of Crt, ALA alone group compared with the control group ($F(2,15) = 3.544$, $p = 1.724$). Also there was no significant difference in the total activity between of CYA, and the pretreatments with Crt, ALA, or fluoxetine groups compared with the vehicle group ($F(5, 30) = 1.445$, $p = 2.055$). The results of immobility time during FST are presented in Fig 1b, Crt insignificantly reduced immobility time in normal animals while ALA significantly reduced immobility time (81.6±15.9s vs control 142±9.7s, $p = 0.0094$). CYA significantly increased immobility time (175.1±13.16s, vs vehicle 130.9±13.5s, $p = 0.0364$), and by pretreatments with ALA (100±15.9s, $p = 0.020$) or Crt (93.5±16.6, $p = 0.009$) the immobility time significantly decreased compared to CYA alone. By co-administration of ALA-Crt following CYA injection immobility time dropped dramatically (12.2±2.59s, $p < 0.001$ vs CYA). The result was significantly lower than pretreatment with ALA ($p = 0.0022$) or Crt separately ($p = 0.0052$). Fluoxetine as the reference antidepressant drug reduced immobility in CYA treated animals (47.7±18.8s, $p < 0.001$ vs CYA group).

According to Table 1, swimming time during the FST following ALA administration was significantly higher than control (141±13.9s vs control 76.9±11s, $p = 0.002$) Crt also slightly increased swimming time. By administrating Crt along with CYA swimming time was significantly higher ($p = 0.0393$) compared to CYA alone. Similarly, co-administrating ALA-Crt increased swimming time compared to CYA alone ($p = 0.0030$). The Table also shows that treating with ALA-Crt significantly increased the climbing time compared to CYA alone ($p = 0.0298$). Climbing time was also significantly higher in animals treated with fluoxetine compared to CYA alone ($p < 0.001$).

Brain BDNF level

The results of brain BDNF level are depicted in figure 2. BDNF level was not different following administrating ALA or Crt compared to the control group (571±43.1 pg/mg protein). Interestingly CYA significantly increased BDNF level compared with vehicle (1059±81.0 pg/mg protein, $p = 0.0093$). Treatment with Crt did not change the result while treatment with ALA similar to fluoxetine significantly reduced BDNF level compared with CYA alone (684±103

pg/mg protein, p=0.0454). Administrating ALA-Crt also significantly decreased BDNF level compared with CYA alone (p=0.0413). Table 2 shows the percentage of hippocampus to whole brain tissue, in CYA treatments it was significantly higher than the vehicle group, and it was reversed by ALA and Crt, but not fluoxetine.

DISCUSSION

This research proved that the complementary drugs, ALA and Crt, prevent depressive-like behavior initiation by CYA in mice, and ALA-Crt showed synergistic antidepressant effect. While CYA increased the brain BDNF level treatment with Crt did not change BDNF level and ALA reduced it. This study was carried out on female mice, since it was shown previously that Crt effects on depressive behavior in FST is sex-dependent, and female rats had shown better antidepressant-like response [22].

FST is the most commonly used behavior test used for antidepressant screening [23]. Following initial escape attempts movements in the inescapable water filled beaker, rodents perform a typical immobile posture. Immobility time during FST was measured as a certain depressive-like phenotype that is despair behavior. The locomotor activity was evaluated prior the FST, since no noticeable difference was observed between different therapies and normal animals thus changes in the immobility time in FST could be interpreted as animal depressive–like behavior. CYA administration increased immobility time that indicated animal despair behavior. It was also reported previously that following CYA single dose injection immobility time that was measured after 4h and 24h in separate groups of animals increased in FST [15]. After nephrotoxicity neurotoxicity is the most serious CYA related side effect that was proved to be related to drug interface with brain mitochondria [5].

In the following experiment Crt prevented CYA induced despair behavior, as the immobility time was significantly lower than CYA group. It has been proven that Crt could protect neurons against neurotoxic substances such as harmful levels of excitatory amino acid, glutamate, by buffering ATP levels [24]. The mitochondrion and Crt and PCr establish a system that is critical in energy homeostasis in high energy demanding organs, such as the brain. Crt buffers against ATP depletion since as a substrate for Crt kinase it increases PCr that converts adenosine diphosphate to ATP and therefore exert its neuroprotection effect [25]. Therefore, Crt by stimulating the rate of ATP synthesis and producing high amounts of PCr may have prevented the CYA induced neurotoxicity and depressive-like behavior in mice. Pretreatment with Crt also increased the swimming time, although on the downside of the study neurotransmitters were not measured but according to previous studies serotonergic system may be involved in the antidepressant-like effects. The SSRI drugs increase the swimming time while the catecholamine related antidepressants increase the climbing time [26]. Although the pretreatment of fluoxetine with CYA showed unexpected results as the climbing time was significantly higher than control that may be because of the interaction between CYA and fluoxetine. It was shown previously that high dose CYA decreases serotonin release [6].

Pretreatment with ALA also prevented CYA induced depression-like behavior, as immobility time reduced significantly. ALA has been recognized as a potent antioxidant that is naturally found in diets, indeed when given as a supplement there would be increased functional capacity [27]. ALA antioxidant capacity could be the reason for its protective effects against CYA induced depression. In support of our study animal studies have shown that ALA along with reducing neurodegeneration in the hippocampus reduced peripheral oxidative damage by increasing total anti-oxidative potential [28]. In addition, supplemented ALA in old rats have increased mitochondrial membrane capacity and declined oxidative damage [29]. Therefore ALA has

prevented CYA neurotoxicity through interaction with brain mitochondria functioning [5]. The most interesting finding was that co-administrating ALA-Crt had synergistic antidepressant-like effect as immobility time during FST reduced dramatically. Meanwhile climbing and particularly swimming time significantly increased. It was supposed that serotonin and mitochondria have a close interconnection, since by improving mitochondrial functioning swimming time increased. Variable mitochondrial activity equals energy demand to energy supply throughout the neurons and controls replacing of mitochondria in the periphery [30]. Researchers have found that serotonin and 5-HT1A receptor in hippocampal neurons, are involved in mitochondrial trafficking [31].
In the present study brain BDNF level almost doubled following CYA administration, and AIA similar to fluoxetine prevented this effect. BDNF, and its receptor tyrosine kinase receptor B (TrkB) play essential roles in cell survival, neurogenesis, synaptic plasticity and neuron survival during the course of life [32]. Previous studies have shown that chronic administration of CYA in rats for 30 days reduced brain BDNF level that could be responsible for the depressant effect of cyclosporine [8]. It was observed that following 14 days of treatment with fluoxetine, BDNF expression was decreased [33]. However, following 21 days of fluoxetine administration, BDNF expression was upregulated [34]. It was also reported that BDNF level increased in an animal model of schizophrenia and it was suggested that this elevation is in response to toxic materials as a defensive mechanism [35,36]. After 7 days of BDNF infusions into the ventral tegmental area latency to immobility in the FST declined, suggesting a depressive-like behavior that is against the BDNF role that is proposed in the hippocampus [37]. In addition, some studies have shown that increased BDNF level is related to depression [38]. According to these studies brain BDNF level could be influenced by different therapies and the exposure time. In our study after depressive-like behavior initiated by CYA, BDNF level increased that might be related to CYA neurotoxic effect. However although Crt pretreatment had anti-immobility effect in FST but it did not alter the rise in BDNF level. Only ALA pretreatment reduced BDNF level similar to fluoxetine, probably ALA is more effective in preventing CYA neurotoxic initiation, this warrants further investigation. In addition, the higher hippocampus to whole brain ratio and elevation of BDNF level in CYA treated groups are related to the increased immobility during FST.
In conclusion, the present study showed that CYA induced depressive-like behavior was prevented by mitochondrial antioxidant ALA, and Crt. Therefore, it was postulated that at least part of CYA induced depressive effect is mediated by mitochondrial dysfunction neurotoxicity. Also, we observed that ALA could hinder BDNF alteration and its possible neurological changes as effective as fluoxetine.

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Conflict of Interest: The authors confirm there is no conflict of interest in relation to this article.

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Table 1. Swimming and climbing time during the FST.

Groups (n=6)	Swimming time (s)	Climbing time (s)
Control	76.9±11	21.14±12.01
ALA	141±13.9^	17.50±10.16
Crt	113±9.65	8.50±3.26
Vehicle	76.3±17.4	1.66±0.91
CYA	69.7±18.3	3.16±1.68
CYA+ALA	117±10.1	22.50±9.60
CYA+Crt	132±18.2*	20.17±8.64
CYA+ALA+Crt	174±18.1**,VV	54.17±18.67*
CYA+Fluoxetine	84.2±21.2	91.50±15.51***

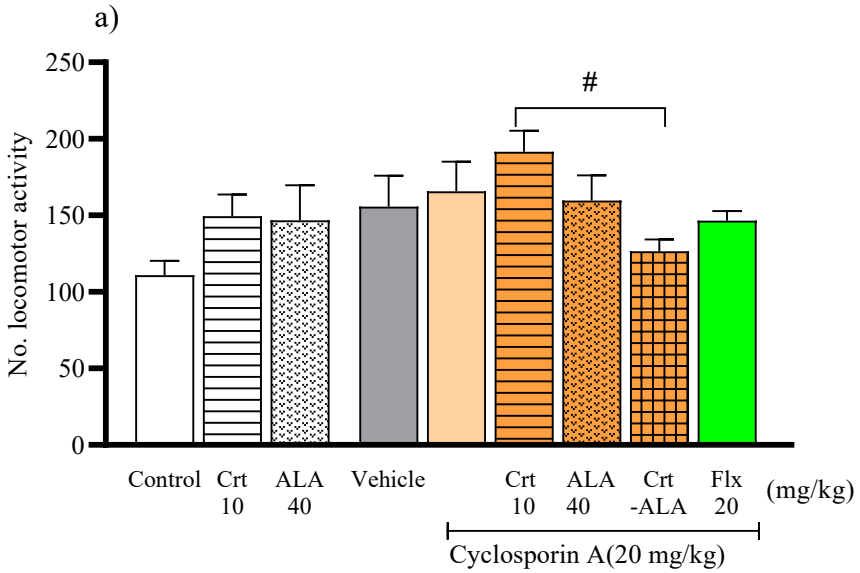
The control and vehicle group received normal saline and 2%v/v EtOH/normal saline, respectively. Results are expressed as group mean ± SEM and analyzed by ANOVA followed by Tukey's comparison test (n=6). ^*P<0.05*, compared with the control; vv *P<0.01*, compared with vehicle. **P<0.05*, ***P <0.01*, compared with CYA group.

Table 2. Hippocampus to whole brain percentage.

Groups (n=5)	% Hippocampus to whole brain

Control	9.02±0.44
ALA	7.15±1.22
Crt	6.31±0.52^
Vehicle	8.06±0.32
CYA	10.34±0.54^{V}
CYA+ALA	7.42±0.98 *
CYA+Crt	8.58±0.51
CYA+ALA+Crt	8.22±0.76
CYA+Fluoxetine	11.31±0.31^{V}

The control and vehicle group received normal saline and 2%v/v EtOH/normal saline, respectively. Results are expressed as group mean ± SEM and analyzed by ANOVA followed by Tukey's comparison test (n=6). ^*P<0.05*, compared with the control; v *P<0.05*, compared with vehicle; **P<0.05*, compared with CYA group.



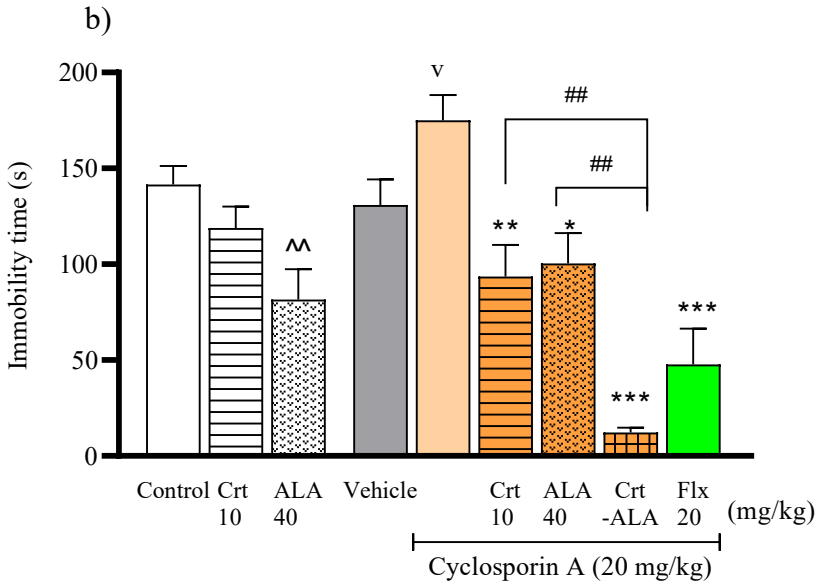


Figure 1. Effect of Crt, and ALA, on mice behavior following CYA administration. Total activity during locomotor test= (horizontal +vertical) exploration (a), and immobility time during FST (b). The control and vehicle group received normal saline and 2%v/v EtOH/normal saline, respectively. Results are expressed as group mean ± SEM and analyzed by ANOVA followed by Tukey's comparison test (n=6). ^P<0.05, compared with the control; v P<0.05, compared with vehicle. *P<0.05, **p <0.01, and ***p<0.001 compared with CYA group. ##P<0.01 as shown.

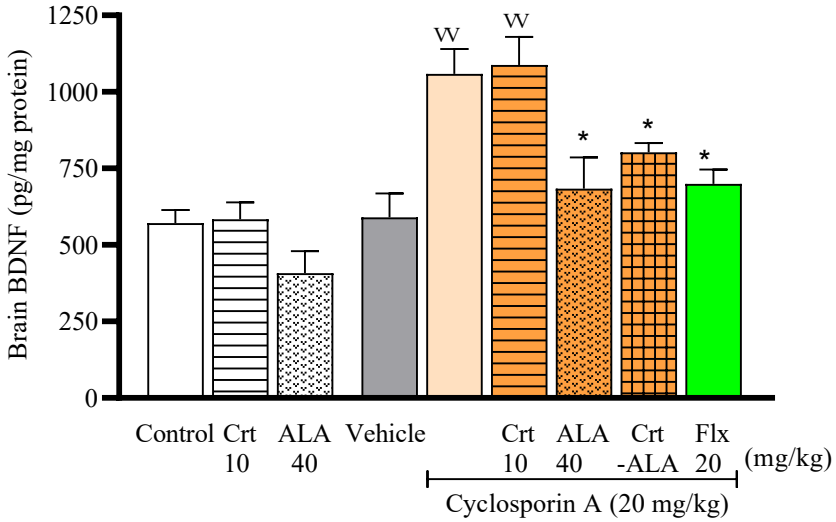


Figure 2. Normalized brain derived BDNF level. The control and vehicle group received normal saline and 2%v/v EtOH/normal saline, respectively. Results are expressed as group mean ± SEM and analyzed by ANOVA followed by Tukey's comparison test (n=6). vv P<0.01, compared with vehicle; *P<0.05, compared with CYA group.