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. 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. However, some patients that receive these agents suffer from neuropsychological problems such as depression, anxiety, confusion, and tremor. Peripheral administration of high dose (60 mg/kg) CYA has decreased the release of serotonin and dopamine and caused prefrontal cortex dysfunction that could be responsible for the increased anxiety and social behavior disturbance. Additionally, CYA may induce neurotoxicity by interaction with brain mitochondria functioning. 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. Reduction of BDNF expression in hippocampal neurons would cause severe stress and influence learning, inspiration, and mood.

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. Adenosine triphosphate (ATP) is the primary energy source in the brain that is closely joined to phosphocreatine.
inflammatory, anti-apoptotic, and neuromodulatory activity.\(^{10,12}\) Cytosine arabinoside (CYA) 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 an inhibitor of lipid and protein oxidation. Interestingly, this free-radical quenching antioxidant, in contrast to vitamin E (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, leading to its promising therapeutic effects on brain disorders.\(^{16}\)

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

**MATERIALS 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°C on a 12 h light and 12 h dark cycle (lights on at 06:00 AM); standard mice chow and tap water ad libitum. Cages were placed in the behavioral laboratory 24 h prior to the experiments to acclimatize. The experiments were carried out according to the Care and Use of Laboratory Animals Guidelines Issued by The National Ethical Committee of Vice-Chancellor in Research Affairs-Medical University of Isfahan (ethical no: IR.MUI.RESEARCH.REC.1399.200). All the attempts were made to reduce animal distress and the number of animals used in the research.

**Drug administration**

Cytosine arabinoside (Sandimmun, 50 mg/mL; Novartis, Switzerland) 20 mg/kg was injected intraperitoneal (IP) after 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 was 10 mL/kg.

**Experiment design**

Totally 9 groups of animals consisting of 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. The locomotor and forced swimming tests (FST) for measuring animal despair behavior were performed on each animal. After 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 the back legs.

**Forced swimming test**

During FST, mice were forced to swim in 25°C 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 required to keep the animals’ heads 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, was 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 CO\(_2\) 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 -70°C 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% bovine serum albumin, 0.1 mM benzethonium chloride, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride at pH 7.4. According to the manufacturer’s instructions, a mice BDNF ELISA kit (Picokine TM ELISA, catalog no:}

\(\text{PCr}\). The isoenzymes of Crt kinase are specially localized in high-demanding ATP sites like the neurons to regenerate ATP in situ via PCr.\(^{10}\) Therefore, oxidative phosphorylation and Crt/PCr 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.\(^{10,12}\)
EK0309, Boster Biological Technology, Canada) was used. Briefly, the homogenates were centrifuged (10,000×g, 25 min, 4°C), and the supernatant was separated. After dilution, 100 µL of samples were added to each empty well and incubated at 37°C for 90 minutes. After washing the wells, 100 µL of biotinylated anti-mouse BDNF antibody working solution was added and incubated at 37°C for 60 minutes. Then all of the wells were washed three times with polybutylene succinate) and incubated with avidin-biotin-peroxidase complex working solution at 37°C for 30 minutes. Finally, TMB color was added to each well and kept in the dark for 30 minutes. After changing the color to yellow following TMB stop solution addition, the plate optical density absorbance was read at 450 nm 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 4 pg/mL sensitivity. 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.

**Statistical analysis**

Results were expressed as group mean ± standard error of the mean. All behavior results were analyzed by One-Way ANOVA, followed by Tukey’s multiple comparison tests. BDNF results were analyzed by One-Way ANOVA, followed by Tukey’s multiple comparison tests. *P* values less than 0.05 were considered significant. Excel 2010 and GraphPad Prizm (version 8) used the software programs for data analysis and making graphs.

**RESULTS**

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

The locomotor activity results in Figure 1a show no statistically important difference in the total activity between Crt and ALA alone groups compared with the control group (*F*(2, 15) = 3.544, *p* = 1.724). Also, there was no significant difference in the total activity between 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 Figure 1b; Crt insignificantly reduced immobility time in normal animals while ALA significantly reduced immobility time compared to CYA alone (*p* = 0.020). The results of immobility time during FST following CYA injection, the immobility time significantly decreased compared to CYA with ALA (100 ± 15.9 s, *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 ± 2.59 s, *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 ± 18.8 s, *p* = 0.001 vs. CYA group).

According to Table 1, swimming time during the FST following ALA administration was significantly higher than control (141 ± 13.9 s vs. control 76.9 ± 11 s, *p* = 0.002) Crt also slightly increased swimming time. By administrating Crt and CYA, swimming time was significantly higher (*p* = 0.0393) than 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 than CYA alone (*p* < 0.001).

**Brain BDNF level**

The results of brain BDNF levels are depicted in Figure 2. BDNF level was not different following administrating ALA or Crt compared to the control group (571 ± 431 pg/mg protein). Interestingly CYA significantly increased BDNF levels 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 levels compared with CYA alone (684 ± 103 pg/mg protein, *p* = 0.0454). Administering ALA-Crt also significantly decreased BDNF levels compared with CYA alone (*p* = 0.0413). Table 2 shows BDNF levels were not different following administrating ALA-Crt compared to the control group (571 ± 431 pg/mg protein). Interestingly CYA significantly increased BDNF levels compared with vehicle (1059 ± 81.0 pg/mg protein, *p* = 0.0093).
DISCUSSION

This research proved that the complementary drugs, ALA and Crt, prevent depressive-like behavior initiation by CYA in mice, and ALA-Crt showed a synergistic antidepressant effect. While CYA increased the brain BDNF level, treatment with Crt did not change the 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 are 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 Rodents perform a typical immobile posture following initial escape attempts movements in the inescapable water-filled beaker. Immobility time during FST was measured as a certain depressive-like phenotype that is despair behavior. The locomotor activity was evaluated prior to 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, indicating animal despair behavior. It was also reported previously that following CYA single-dose injection, immobility time measured after 4 h and 24 h in separate groups of animals increased in FST.15 After nephrotoxicity, neurotoxicity is the most severe 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 the CYA group. It has been proven that Crt could protect neurons against neurotoxic substances such as harmful excitatory amino acid glutamate levels by buffering ATP levels. 24 The mitochondrion and Crt and PCr establish a critical system in energy homeostasis in high energy demanding organs, such as the brain. Crt buffers against ATP depletion since it increases PCr as a substrate for Crt kinase, which converts adenosine diphosphate to ATP and, therefore, exerts its neuroprotection effect.25 Therefore, by stimulating the rate of ATP synthesis and producing high amounts of PCr, Crt may have prevented the CYA-induced neurotoxicity and depressive-like behavior in mice. Pretreatment with Crt also increased the swimming time, although neurotransmitters were not measured on the downside of the study; according to previous studies, the

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*      | 54.17 ± 18.67*   |
| CYA+fluoxetine | 84.2 ± 21.2   | 91.50 ± 15.51*** |

The control and vehicle groups received normal saline and 2% v/v EtOH/normal saline. 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;*: p<0.01, compared with the vehicle; *: p<0.05, **: p<0.01, compared with CYA group. FST: Forced swimming test, ALA: Alpha-lipoic acid, Crt: Creatine, CYA: Cyclosporine A, SEM: Standard error of the mean

Table 2. Hippocampus to whole-brain percentage

| Groups (n= 5) | % Hippocampus to the 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^                   |
| CYA+ALA      | 7.42 ± 0.96*                    |
| CYA+Crt      | 8.58 ± 0.51                     |
| CYA+fluoxetine | 8.22 ± 0.76                  |

The control and vehicle groups received normal saline and 2% v/v EtOH/normal saline. 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 the vehicle. *: p<0.05, **: p<0.01, compared with CYA group. FST: Forced swimming test, ALA: Alpha-lipoic acid, Crt: Creatine, CYA: Cyclosporine A, SEM: Standard error of the mean
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. However, the pretreatment of fluoxetine with CYA showed unexpected results as the climbing time was significantly higher than control because of the interaction between CYA and fluoxetine. It was shown previously that high-dose CYA decreases serotonin release.

Pretreatment with ALA also reduced CYA-induced depression-like behavior, as immobility time was reduced significantly. ALA has been recognized as a potent antioxidant naturally found in diets; indeed, there would be increased functional capacity when given as a supplement. 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. In addition, supplemented ALA in old rats has increased mitochondrial membrane capacity and declined oxidative damage. Therefore ALA has prevented CYA neurotoxicity through interaction with brain mitochondria functioning. The most exciting finding was that co-administrating ALA-Crt had a 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 mitochondria in the periphery. Researchers have found that serotonin and 5-HT1A receptors in hippocampal neurons are involved in mitochondrial trafficking.

In the present study, brain BDNF levels almost doubled following CYA administration, and ALA similar to fluoxetine prevented this effect. BDNF and its receptor tyrosine kinase receptor B play essential roles in cell survival, neurogenesis, synaptic plasticity, and neuron survival during life. Previous studies have shown that chronic administration of CYA in rats for 30 days reduced brain BDNF levels that could be responsible for the depressive effect of CYA. It was observed that following 14 days of treatment with fluoxetine, BDNF expression was decreased. However, following 21 days of fluoxetine administration, BDNF expression was upregulated. 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. After 7 days of BDNF infusions into the ventral tegmental area, latency to immobility in the FST declined, suggesting a depressive-like behavior against the BDNF role proposed in the hippocampus. In addition, some studies have shown that increased BDNF level is related to depression. According to these studies, different therapies and exposure time could influence brain BDNF levels. In our study, after depressive-like behavior initiated by CYA, BDNF levels increased, that might be related to CYA neurotoxic effect. However, although Crt pretreatment had an anti-immobility effect in FST, it did not alter the rise in BDNF levels. Only ALA pretreatment reduced BDNF levels similar to fluoxetine, and probably, ALA is more effective in preventing CYA neurotoxicity 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.

**CONCLUSION**

In conclusion, the present study showed that mitochondrial antioxidant ALA and Crt prevented CYA-induced depressive-like behavior. 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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**Ethics**

**Ethics Committee Approval:** Care and Use of Laboratory Animals Guidelines Issued by The National Ethical Committee of Vice-Chancellor in Research Affairs-Medical University of Isfahan (ethical no: IR.MUI.RESEARCH.REC.1399.200).

**Informed Consent:** Not applicable.

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**Authorship Contributions**

Concept: M.A., A.M., Design: A.M., M.A., Data Collection or Processing: A.S.M., A.M., Analysis or Interpretation: A.M., M.A., Literature Search: A.M., M.A., A.S.M., Writing: A.M., M.A.

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