



# Antidepressant Activity of Alpha-lactalbumin in Chronic Unpredictable Stress Model in Swiss Albino Mice

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## Abstract

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**BACKGROUND:** Major depressive disorder (MDD) is a leading cause for morbidity and mortality. Whey protein components are recently used as functional foods with health benefits. Alpha-lactalbumin (A-LA) is an important component of whey protein. It is a good source of the essential amino acids.

**OBJECTIVE:** This study aimed to examine the possible antidepressant effect of whey protein against MDD induced by chronic unpredictable stress (CUS) in mice.

**MATERIALS AND METHODS:** CUS model is used to induce depression in mice for 24 consecutive days and aimed to evaluate the ability of A-LA compared to fluoxetine to recover depression symptoms. Thirty minutes before exposure to the physical stressor, mice were given A-LA daily in a dose of 75, 150, and 300 mg/kg bwt.

**RESULTS:** The highest dose of A-LA (300 mg/kg bwt.) lessens depression induced in mice.

**CONCLUSION:** This effect might contribute to elevate the level of glutamate and monoamine neurotransmitters,  $\gamma$ -amino-butyric acid inhibition, and enhancement of glutathione in brain tissue.

## Introduction

Major depressive disorder (MDD) is a determined psychiatric illness which might cause physical impairment. Approximately 350 million people around the world suffer from MDD, whereas about 15 million people at the United States are affected with this mental illness [1].

According to monoamine hypothesis, the deficiency of neurotransmitters directly contributes to the arising of MDDs. The antidepressant drugs act by inhibition of reuptake of neurotransmitters as selective serotonin (SE) reuptake inhibitors of tricyclic antidepressants or inhibiting degradation of monoamine neurotransmitters, for example, monoamine oxidase inhibitors [2].

Genetic factors as well can contribute to the pathogenesis of depression. The synergism between environmental stress and susceptible genes motivates the incidence of MDD [3]. Oxidative stress has also been implicated in the pathophysiology of MDD and that the antidepressant activity can be mediated through the improvement of oxidative stress/antioxidant function [4].

Whey and casein are proteins available in milk. Curds are made from casein while whey remains in an aqueous form [5].

Alpha-lactalbumin (A-LA) is one of the most valuable components of whey protein. A-LA is rich

in cysteine, tryptophan (Trp), and glutamine. These are essential amino acid used by the body to produce glutathione (GSH), SE, and glutamate (Glu), respectively. Trp is an essential amino acid that should be supplied from external source as it is not synthesized in the body. Trp is the precursor of the neurotransmitter SE and the precursor to melatonin which is a hormone involved in the sleep-wake cycle. SE was found to control the appetite, mood, sleep regulation, cognitive performance, and the ability to cope with stress [6].

The chronic unpredictable stress (CUS) procedure mimics the role of chronic stress in precipitating MDD and induces long-term neurochemical, behavioral, neuroendocrine, and physical alterations resembling those noticed in depressed patients [7].

In previous investigations in our lab we had demonstrated an antidepressant effect for whey protein in rats [8]. A-LA represents one of the major constituents of whey protein and is considered one of the most important sources of Trp, the precursor of SE which is a chief neurotransmitter involved in depression. Previously, the effect of administering A-LA has been evaluated as antidepressant acting by increasing the brain content of SE.

Since the three neurotransmitters involved in depression (SE, norepinephrine [NE], and dopamine [DA]) were found to be interconnected, our study aimed to investigate the beneficial effect of A-LA administration

on brain levels of DA and NE as well as its potential effect in ameliorating the depressive symptoms.

## Materials and Methods

### Animals and drugs

Adult male Swiss albino mice, weighing 20–25 g, obtained from the Animal Breeding Unit at National Research Centre (NRC, Giza, Egypt). Mice were kept under standard conditions of natural 12 h light and dark cycle and allowed free access to food and water. Mice were allowed to adapt to the laboratory environment for 1 week before conducting of behavioral experiments. All experimental procedures were approved from the ethical committee of the National Research Centre and according to the recommendations of the proper care and use of laboratory animals and following the recommendations of the National Institutes of Health guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

### Drugs

Fluoxetine hydrochloride (Prozac 20 mg dispersible tablets, Lilly, Spain). The tablets were freshly suspended in distilled water before oral administration.

A-LA was provided as a generous gift from Davisco Foods International Inc. and it was provided as pure powder for ingestion. The powder was freshly dissolved in distilled water just before oral administration.

### CUS model

Mice were randomly assigned into six groups of 10 individuals: Normal control receiving oral distilled water, depressed control administered oral distilled water plus CUS, A-LA groups receiving orally A-LA at dose levels of 75, 150, and 300 mg/kg plus CUS, and standard drug group orally given fluoxetine 10 mg/kg. Normal control group was housed in a separate room, did not have any contact with the stressed animals, and was undisturbed except for necessary procedures such as routine cage cleaning. All other groups were subjected to CUS procedure for a period of 24 days, where the mice were subjected each day to one of six physical stressors for 30 min after the corresponding drug ingestion. The CUS procedure was performed as described by Mao *et al.* (2009) [7] with a slight modification [9]. Briefly, CUS consisted of a variety of unpredictable stressors, namely, 24-h water deprivation, 6-min cold swimming (at 8°C), 1-min tail pinch (1 cm from the end of the tail), 2-h restraint, 24-h soiled cage (200 mL water in 100 g sawdust bedding), and overnight illumination. One of these stressors (in random order) was given every day for 24 days.

### Behavioral tests

Twenty-four hours after the last stressor behavioral tests were performed; these include open-field test (OFT), tail suspension test (TST), and forced swim test.

#### OFT

The OFT was carried out in a square wooden arena (40 cm × 40 cm × 40cm high) with red walls and white smooth polished floor divided by black lines into 16 equal squares. The test was performed under white light in a quiet room. Each mouse was placed at the same corner square and observed during 5 min [10]. The floor and walls were cleaned after testing each mouse. The following parameters were recorded during 5 min observation period: Latency: Time taken by each animal till it starts moving in the arena, ambulation frequency: Number of squares crossed by the animal, rearing frequency: Number of times the animal stood stretched on its hind limbs with or without forelimb support, and grooming frequency: Number of face scratching and washing with the hind limbs and licking of the forelimbs.

#### TST

The TST is based on the observation that a mouse suspended by the tail shows alternate periods of agitation and immobility. Each mouse was suspended by its tail from a steel bar using an adhesive tape placed approximately 1 cm from the tip of the tail, the distance between floor and tail was about 30 cm. The total duration of immobility during a 6-min test was measured. Mice were considered immobile only when they hung passively in a completely motionless state [11].

#### Forced swimming test (FST)

In this method, each mouse was forced to swim in a restricted space from which it cannot escape according to the method described by. After the initial phase of vigorous activity, swimming attempts cease and the animal adopts a characteristic immobile posture in which it floats in an upright position making only movements necessary to keep its head above water. Each mouse was placed for 6 min in a cylindrical water tank which was transparent, and it had a diameter of 18 cm and height of 25 cm. The water level was about 15 cm and water temperature was maintained at 25 ± 1°C. The total duration of immobility of each animal in the past 4 min was recorded. The tank was emptied and washed with fresh water flush between each mice to remove any traces of urine or feces [12].

#### Biochemical analysis

Twenty-four hours after the completion of behavioral tests, mice were sacrificed by decapitation. Brains were isolated and each brain was washed with

sterile cold physiological saline, blotted between two damp filter papers, and stored at  $-80^{\circ}\text{C}$  until the use for biochemical analysis.

#### Determination of brain amino acids and monoamines

Each brain tissue was weighed and homogenized in 1/10 weight/volume of 75% aqueous high-performance liquid chromatography (HPLC) grade methanol [13]. The homogenate was spun at 4000 r.p.m. for 10 min and the supernatant was divided into two halves; the first was dried using vacuum (70 millipore) at room temperature and used for  $\gamma$ -amino-butyric acid (GABA) and Glu determination, whereas the second half was used for monoamine determination.

Brain Glu and GABA were detected by HPLC using the precolumn PTC derivatization technique [14]. Brain monoamines were detected by HPLC [15].

#### Determination of malondialdehyde (MDA) and GSH

Each brain tissue was homogenized in ice-cold saline (20% w/v). The homogenate was divided into two portions for the determination of MDA and GSH levels [16], [17]. MDA is an end product of lipid peroxidation that reacts with thiobarbituric acid producing thiobarbituric acid reactive substance, a pink chromogen is determined spectrophotometrically at 532 nm, an MDA standard was used to make a standard curve. GSH determination is based on the reduction of 5,5-dithiobis (2-nitrobenzoic acid) with reduced GSH to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance can be measured at 405 nm using a commercial kit that was used (Biodiagnostic, Egypt).

#### Statistical analysis

Statistical analysis was performed by one-way analysis of variance ANOVA followed by least significant difference test (LSD test). Data are expressed as means  $\pm$  standard error of mean where any two groups were considered significantly different if the difference between their mean values was more than the corresponding LSD value stated for each experiment at  $p < 0.05$ .

## Results

### Behavioral studies

#### OFT

Group subjected to CUS model showed a significant decrease in the ambulation, rearing, and

grooming frequencies to 57.2%, 34.94%, and 42.86%, respectively, compared to normal control. On the other hand, the latency time increased to 453.6% as compared to normal control. Group given fluoxetine restored the latency time and ambulation frequency to normal level, while the rearing frequency increased to 237.93% as compared to a depressed group, the grooming frequency increased up to 134.01% as compared to normal control. The three-dose levels of A-LA (75, 150, and 300 mg/kg) significantly decreased the latency time to 67.48%, 49.46%, and 34.25%, respectively, compared to the depressed control. Treatment with A-LA in a dose of 75 mg/kg significantly increased the ambulation and rearing frequencies to 148.33% and 217.59%, respectively, compared to the depressed group and normalized the grooming frequency. A-LA (150 mg/kg) elevated the rearing frequency to 225.52% while the ambulation and grooming frequencies are restored to normal levels as compared to normal control group. A-LA (300 mg/kg) normalized the rearing frequency and it increased the ambulation and the grooming frequencies up to 111.67% and 136.05% of the normal group, respectively (Figure 1a and b).

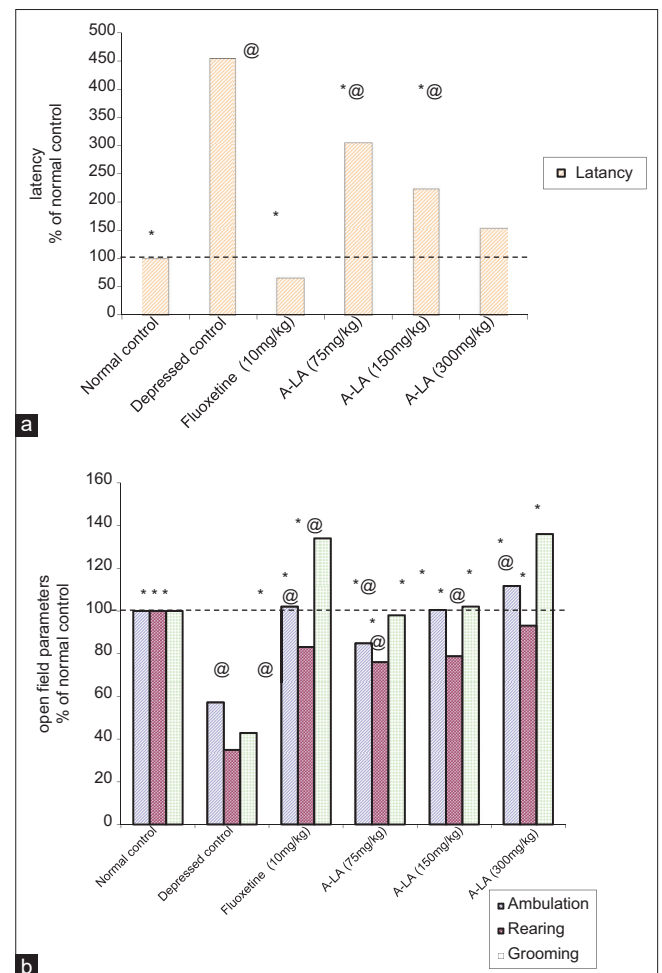


Figure 1:(a) Latency time in open-field test. Values represent the mean%. @ $p < 0.05$ : Statistically significant from normal control, \* $p < 0.05$ : Statistically significant from depressed group, (b) Ambulation, rearing, and grooming frequencies in open-field test values represent the mean%. ( $n = 10$ ). @ $p < 0.05$ : Statistically significant from normal control,\* $p < 0.05$ : Statistically significant from depressed group



### FST

Group subjected to CUS model showed a significant increase in the immobility duration ( $98.5 \pm 8.69$  s) as compared to normal control. Group given fluoxetine restored immobility duration to normal values. Groups given A-LA in a dose of 75 and 150 mg/kg bwt. significantly decreased the immobility duration when compared to depressed group (38.17% and 16.55%, respectively). On the other hand, it was evident that A-LA (300 mg/kg) returned the immobility duration to the normal value (Figure 2).

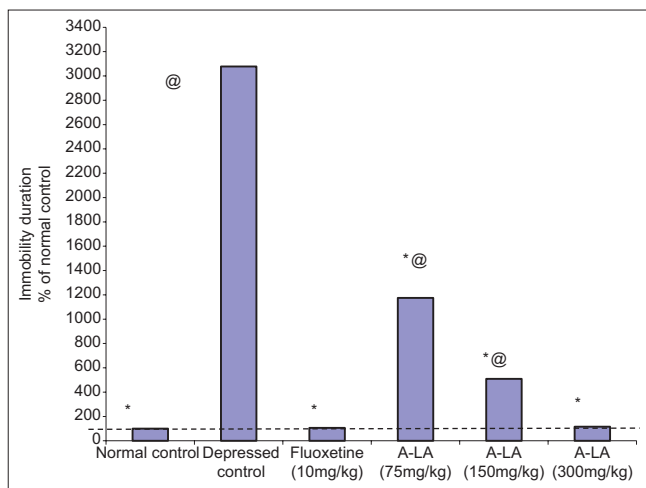


Figure 2: Forced swimming test. Values represent the mean % of five mice for each group ( $n = 10$ ). @ $p < 0.05$ : Statistically significant from normal control, \* $p < 0.05$ : Statistically significant from depressed group

### TST

Group subjected to CUS model showed a significant increase in the immobility duration up to  $140.8 \pm 6.14$  s as compared to normal control. Group given fluoxetine restored immobility duration to normal level. Groups given A-LA in a dose of 75 and 150 mg/kg bwt. showed a significant decrease in the immobility duration when compared to depressed group (54.54% and 46.59%, respectively). A-LA in a dose of 300 mg/kg bwt. restored the immobility duration to normal level (Figure 3).

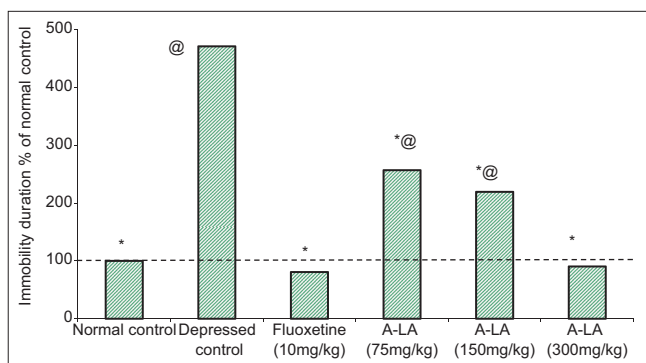


Figure 3: Tail suspension test. Values represent the mean % of five mice for each group ( $n = 10$ ). @ $p < 0.05$ : Statistically significant from normal control, \* $p < 0.05$ : Statistically significant from depressed group

### HPLC determination of SE, NE, and DA in brain tissue

Group subjected to CUS model reported a significant decrease in SE, NE, and DA levels as compared to normal control (44.8%, 36.1%, and 63.3%, respectively). Group given fluoxetine showed significant increase in SE, NE, and DA (117%, 94.5%, and 101.3%, respectively) as compared to normal control. Group given A-LA in a dose of 75 mg/kg bwt. increased SE significantly (180.8%) when compared to depressed groups while NE and DA were restored to normal levels. Meanwhile, A-LA (150 mg/kg) normalized the SE and NE contents and showed a significant increase in the DA level (126.67%) compared to the normal control. On the other hand, treatment with A-LA (300 mg/kg) restored the normal SE level and significantly increased the NE and DA contents to 158.64% and 212.67% of normal control, respectively (Figure 4).

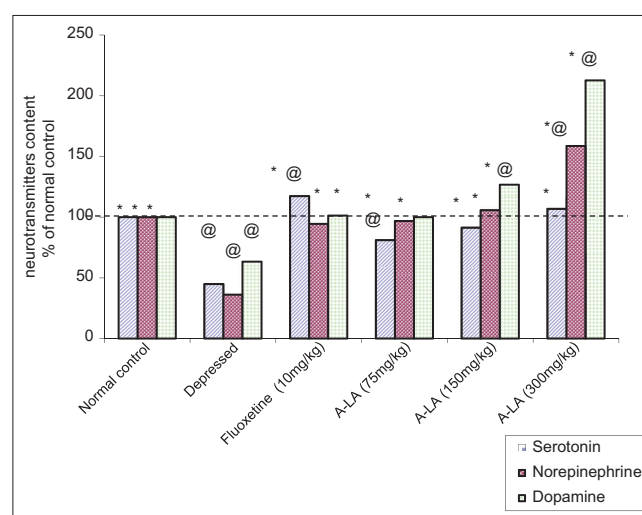


Figure 4: Determination of serotonin, norepinephrine, and dopamine. Values represent the mean % of five mice for each group ( $n = 10$ ). @ $p < 0.05$ : Statistically significant from normal control, \* $p < 0.05$ : Statistically significant from depressed group

### HPLC determination of GABA and Glu in brain tissue

Depressed mice showed a significant elevation in GABA level (202.9%) while Glu level was significantly decreased (27.9%) when compared to control normal. Group given fluoxetine on the contrary showed a significant decrease in GABA and a significant increase in Glu level (71% and 193.7%, respectively) as compared to control normal. Oral administration of all the three doses of A-LA repossessed the normal level of GABA and regarding the Glu level, although the dose level (75 mg/kg) did not have any effect on Glu compared to the depressed group, A-LA (150 mg/kg) reinstated its normal level, while A-LA (300 mg/kg) significantly elevated the Glu level up to 175.05% of the normal control (Figure 5).

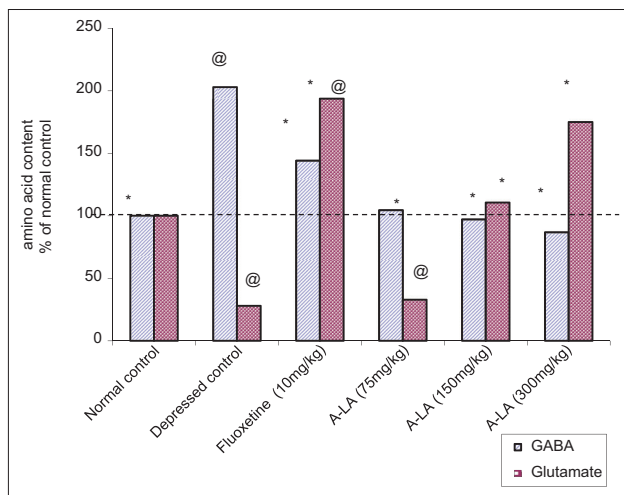


Figure 5: Determination of  $\gamma$ -amino-butyric acid and glutamate. Values represent the mean% of five mice for each group ( $n = 10$ ). @ $p < 0.05$ : Statistically significant from normal control, \* $p < 0.05$ : Statistically significant from depressed group

### Effect of A-LA on brain MDA and reduced GSH in CUS-depressed mice

GSH level was significantly decreased in depressed mice to 47.5% of the normal control. On the other hand, MDA level was significantly elevated up to 124.5% when compared to control group. On the contrary, mice given fluoxetine restored the level of GSH and MDA to normal levels. Treatment with A-LA (75 mg/kg and 150 mg/kg) significantly increased the GSH level up to 146.45% and 189.83% as compared to depressed mice, administration of A-LA in a dose of 300 mg/kg restored the normal GSH level. Moreover, treatment with A-LA (75 mg/kg) retrieved the MDA level while A-LA (150 mg/kg and 300 mg/kg) significantly decreased the MDA level to 62.29% and 56.72%, respectively, as compared to the depressed group (less than the normal level by 22.41% and 29.35%, respectively), as shown in Figure 6.

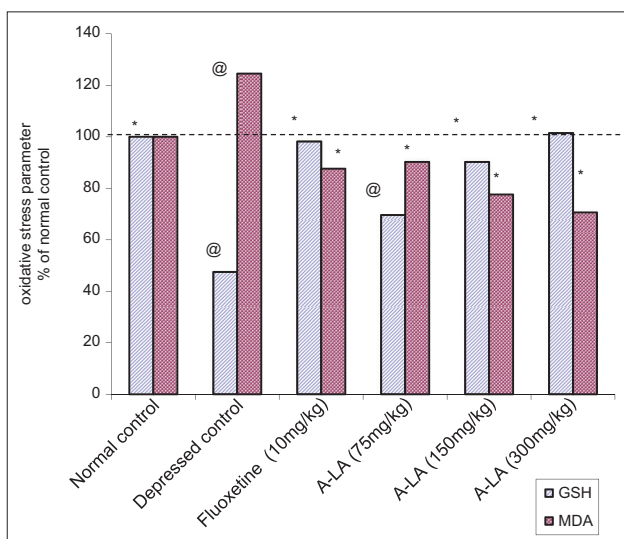


Figure 6: Determination of glutathione and malondialdehyde. Values represent the mean % of five mice for each group ( $n = 10$ ). @ $p < 0.05$ : Statistically significant from normal control, \* $p < 0.05$ : Statistically significant from depressed group

## Discussion

Chronic stress is believed to be a compelling factor involved in depression onset and relapse. CUS was found to induce long-term neurochemical, behavioral, neuroendocrine, and physical alterations resembling those noticed in depressed patients. OPT, FST, and TST are the most impeccable tests to evaluate the efficacy of new antidepressant drugs [7], [18].

The present data reported that depression by CUS model reduced ambulation, rearing, and grooming frequencies and increased the latency time in the OFT, indicating the prevalence of depression. These results are in agreement with a previous study [19] where a decrease in ambulation, rearing, and grooming frequencies was recorded in OPT after induction of depression using CUS model in mice and rats. In the current study, the depressed group showed an increase in the immobility duration in FST and TST. Similar results were obtained by Ran *et al.* [20]. In the present study, treatment with fluoxetine improved the depressed condition induced by the CUS where it stored the ambulation, grooming frequencies, and latency time and increased the rearing frequency significantly as compared to CUS-induced group. Furthermore, the immobility duration was normalized in both FST and TST. These observations are consistent with the results obtained from a previous study [21]. A-LA given in a dose of 300 mg/kg bwt. decreased the latency time significantly as compared to CUS-induced group and normalized the ambulation, rearing, and grooming frequencies. In the FST and TST, A-LA normalized the immobility duration. The overall results were comparable to those of fluoxetine. These results might be due to A-LAs content of Trp. In a previous Trp, potential antidepressant effect was investigated in mice using FST and OPT. Results showed that Trp in revealed an antidepressant effect and this effect increased by increase the dose of Trp till 125 mg/kg bwt. Authors explained these results that Trp is a precursor of SE so by increasing Trp, the SE, in turn, will increase and though elevating the animal's mood [22].

In the present study, depressed animals showed a significant decrease in brain SE, NE, DA, and Glu. On the contrary, GABA was increased. Similar findings revealed that the application of CUS on mice resulted in significant reduction of NE and DA in the hippocampus and prefrontal cortex in rat brains. Moreover, findings showed that CUS reduced levels of glutamine and Glu in the prefrontal cortex and the ratio of Glu and Glu/GABA was also reduced in the hippocampus [8]. Administration of fluoxetine in our study restored the levels of SE, NE, and DA, and moreover, it increased the level of Glu while the level of GABA was significantly reduced as compared to depressed group. These results agreed with those of Bymaster *et al.* [23] and Lazarevic *et al.* [24] who reported that in rat prefrontal cortex, acute systemic

administration of fluoxetine produced a continuous elevation in NE and DA levels. In another study, it was reported that fluoxetine increased levels of both SE and NE levels [25]. Several studies showed that 5-HT<sub>2C</sub> receptors localized on GABA cells in the raphe nucleus facilitate GABA [26]. A-LA (300 mg/kg) highly elevated the monoamines concentrations as compared to the control group. Moreover, A-LA treatment was more potent than fluoxetine regarding NE and DA levels. GABA level was normalized while Glu level was significantly elevated compared to the normal control. The current results agreed with results obtained from a previous study which reported that the administration of A-LA enhanced SE release and rewarding effect, though improving the mood [27]. Moreover, besides being rich in Trp, A-LA also is rich in glutamine, phenylalanine, and tyrosine [6]. Glutamine is abundantly found in CNS and plays a major role in the synthesis of Glu, aspartate, and GABA [28].

In the current study, the stressed group showed that MDA level was significantly increased while the reduced GSH was significantly decreased indicating that causal relation between oxidative stress and depression. Our findings agreed with those of Duda *et al.* [29] where depression was induced using CUS model for 21 days showed an increase in MDA and reduction in GSH concentrations in brain tissue. On the contrary, group given fluoxetine restored the GSH and MDA levels when compared to stress group representing an antioxidant effect. This finding is in rhyme with the previous results, indicating that SE exerts a protective effect against oxidative damage in nerve cells [30]. It has been reported in a previous study on mice that long-term treatment for 28 days with fluoxetine (10 mg/kg, p.o.) increased the GSH levels in the hippocampus [31]. In another study, mice subjected to CUS when treated with fluoxetine for 24 days showed markedly reduced MDA levels [32]. GSH level was normalized and MDA level was also significantly decreased by the administration of A-LA in a dose of 300 mg/kg bwt. as compared to the control group. In a previous study, it was found that A-LA is rich in the essential amino acids cysteine and glycine which are the precursor of GSH [6]. Further studies including its effects on the specific transport of A-LA into brain of Wistar rats or in other strains, also the potential use of A-LA in clinical depression is recommended.

Further investigations are recommended to study the mechanism of action of A-LA on brain neurotransmitters in terms of release, uptake, and metabolism. In addition, it is important to evaluate its applicability to be used as an antidepressant drug.

## Conclusion

A-LA administered at a dose level of 300 mg/kg showed significant effects against the depressive symptoms

induced in mice by applying the CUS model and its effect was comparable to that of fluoxetine. The antioxidant potential of A-LA could contribute, in part, in ameliorating the depressive symptoms.

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