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Research Article
Investigating the Effects of Trolox on Behaviour and Biochemical Parameters in Mice Exposed to Immobilization Stress

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Abstract

Background and Objective: Stress is known to play a causal role in several neuropsychiatric disorders. Trolox has been reported to show potent antioxidant activity against reactive oxygen species. The aim of this study was to investigate the effect of trolox on the behaviour and biochemical parameters of mice exposed to immobilization stress. **Materials and Methods:** The mice were subjected to 6 h of immobilization stress daily for 7 consecutive days. The 48 mice were randomly divided into 6 groups with 8 mice each: (I) Control, (II) Immobilization (IM), (III) Immobilization+Trolox (IM+T), (IV) Immobilization+Fluoxetine (IM+F), (V) Immobilization+Trolox+Fluoxetine (IM+T+F) and (VI) Trolox (T). At the end of day 7, open field test (OFT) and forced swimming test (FST) were performed to assess the locomotor activity and anxiety-like behaviour in mice. After the completion of these tests, brain tissue samples were removed for biochemical analysis. **Results:** The OFT and FST results indicated that the incidences of anxiety-like and depression-like behaviour were significantly lower in the IM+T group compared to the IM group. A significant improvement was observed in the groups treated with trolox for the catalase, total oxidant status, total antioxidant status and oxidative stress index values deteriorated by immobilization. **Conclusion:** The results implicated that antioxidant molecules such as trolox can lead to favourable outcomes in the treatment of oxidative damage either in isolation or in combination with classic treatment methods.

Key words: Stress, trolox, antioxidant, oxidative stress index, open field test

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Stress is considered a leading health problem in the modern world and is essentially a physiological response that is a normal part of daily life. As in all physiological processes, exposure to stress above a certain dose and duration can lead to serious health problems including hormonal imbalances, cardiovascular diseases and neuropsychiatric syndromes[1]. Among these neuropsychiatric syndromes, memory loss, anxiety and depression have been shown to be associated with stress[2,3]. Anxiety is defined as a response to an internal or unpredictable threat and aids in dealing with external threats. Depression, on the other hand, is a common disorder that has been diagnosed in patients that have presented to psychiatry clinics over the last decades[1,4].

Stress mechanisms leading to neuropsychiatric disorders have recently become a major concern among researchers. The studies have converged to suggest that stress results in hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, thereby leading to a profound effect on neurotransmission and synaptic morphology in brain areas associated with behavioural responses and mental states[1,3]. Additionally, experimental stress studies have also shown that stress promotes generation of reactive oxygen species (ROS) and lipid peroxidation products, thereby leading to oxidative stress damage[5-7].

Stress also results in additional nutritional burden for biological systems, mainly because the organism accelerates the protein, lipid and carbohydrate metabolisms and activates the antioxidant defence mechanism to deal with the biochemical changes induced by stress[8]. The antioxidant defence mechanism involves not only enzymatic systems such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) but also nonenzymatic antioxidant systems such as vitamin E[4,9].

Trolox, chemically known as 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid ($C_{14}H_{18}O_4$) with a molecular weight of 250.29 g mol^{-1}, is a synthetic derivative of vitamin E[10]. However, as it is a water-soluble analogue, trolox has advantages over vitamin E[11]. Trolox has been reported to show potent antioxidant activity against ROS including superoxide anion, hydroxyl radicals and hydrogen peroxide as well as against reactive nitrogen species such as peroxynitrite[10,12].

Based on the antioxidant effects of trolox, the present study hypothesized that trolox may prevent anxiety and biochemical alterations following stress. With this background, the present study was designed to examine the effects of trolox on immobilization-induced stress in mice. To determine this effect, the mice were subjected to an open field test (OFT) and a forced swimming test (FST). Additionally, biochemical analysis of some oxidative markers was performed in the brain tissues of mice that were collected after the completion of the testing period.

MATERIALS AND METHODS

Animals: The study was conducted at Dicle University Health Sciences Application and Research Centre between September and December 2018. The study included a total of 48 male BALB/c mice (7-8 weeks old, weighing 30-40 g). The animals were housed at room temperature (23±2°C) with a 12/12 h light/dark cycle (light onset at 8.00 PM) with 60% humidity. The study was initiated after obtaining an approval from the Experimental Animals Ethics Committee (2018-17) and care was taken to follow the Guidelines for the Care and Use of Laboratory Animals proposed by the National Institutes of Health.

Experimental groups and drug administration: The 48 mice were randomly divided into 6 groups with 8 mice each: (I) Control group (CONT) received intraperitoneal (ip) injection of 0.1 mL normal saline for 7 days, (II) Immobilization (IM) group underwent immobilization and received ip injection of 0.1 mL normal saline for 7 days, (III) Immobilization+Trolox (IM+T) group underwent immobilization and received ip injection of trolox for 7 days, (IV) Immobilization+Fluoxetine (IM+F) group underwent immobilization and received ip injection of fluoxetine for 7 days, (V) Immobilization+Trolox+Fluoxetine (IM+T+F) group underwent immobilization and received ip injection of trolox and fluoxetine for 7 days and (VI) Trolox (T) group received ip injection of trolox for 7 days and underwent no additional procedure.

The mice were subjected to immobilization stress as described in a previous study[2]. In brief, mice were subjected to 6 hours of immobilization stress daily for 7 consecutive days, with the mice placed in cages with a diameter of 4 cm and a height adjustable to the height of mice. Injections were performed 30 min before the administration of immobilization. Both trolox and fluoxetine (Sigma Chemical Co, St. Louis, MO, USA) as well as saline were administered intraperitoneally at a dose of 20 mg kg^{-1} diluted to 0.1 mL. On the 8th experimental day, the mice underwent OFT and FST. After the completion of these tests, the mice were sacrificed by cervical decapitation under ether anaesthesia and the prefrontal cortex and hippocampus tissue samples were removed for biochemical analysis and were stored at -80°C until analysis.

Open field test: The OFT was performed to assess the locomotor activity and anxiety-like behaviour in mice. The use of OFT for the assessment of anxiety-like behaviour was first described by Prut and Belzung[13]. The OFT consists of a 40×40×20 cm black painted arena illuminated by a light source. Each mouse was placed in the centre of the arena and the spontaneous ambulatory locomotion of each mouse was video recorded for 5 min. Video recordings were analysed using Ethovision XT 11.0 (Noldus Inf. Tech. Netherlands) which also allowed calculation of the total movement distance and mean velocity for each mouse. To assess the anxiety level of the mice, the area in the middle of the arena (20×20 cm) was accepted as the central zone. The time spent in the centre was calculated using the same program and considered as the index of anxiety.

Forced swimming test: The FST was performed using a plexiglass cylinder (50 cm in height and 20 cm in diameter) containing 30 cm deep water. The mouse was placed in a cylinder with no escape and was left there for 6 min. To avoid additional stress for mice, the temperature of the water in the cylinder was kept at 24-26°C throughout the test period. The movements of the mice in the cylinder were video recorded. The duration of immobility in the last 4 min of the experiment was calculated for each mouse using Ethovision XT 11.0 and considered as the index of depression.

Biochemical analysis: Brain tissue samples were homogenized with 9 (w/v) volumes of ice-cold phosphate buffered saline (PBS; 0.01M, pH: 7.4) solution. The homogenate was centrifuged at 5,000 rpm for 5 min and the supernatant was separated for analysis.

Catalase (CAT) levels were measured spectrophotometrically (UV-1205 Shimadzu) at 405 nm using a commercially available kit (Elabscience, Wuhan, China). The CAT activity was expressed as U mg^{-1} protein. Total oxidant status (TOS), total antioxidant status (TAS) and Paraoxonase-1 (PON-1) levels were measured in the supernatant fraction of homogenates and serum samples using a commercially available kit (Rel Assay Diagnostic) with an auto analyser (Architect c16000). The PON-1 results were expressed as U mg^{-1} protein, TOS results were expressed in terms of micromolar hydrogen peroxide equivalent g^{-1} protein and TAS results were expressed as mmol Trolox equivalent g^{-1} protein. The ratio percentage of the TOS to the TAS potential gave the oxidative stress index (OSI), which is an indicator of the degree of oxidative stress[14].

Statistical analysis: Data were analysed using SPSS for Windows version 16.0 (SPSS Co., Chicago, IL, USA). Descriptives were expressed as mean±standard deviation (SD). Variables were compared using one-way ANOVA followed by a *post hoc* Tukey test. A $p<0.05$ was considered significant.

RESULTS

Weight change: The mean weight of mice increased by 0.1 g in the CONT and T groups and decreased by 2.2, 2.0, 0.9 and 0.3 g in the IM, IM+T, IM+F, IM+T+F groups between the first and final day of the experiment (i.e., day 0-7), respectively (Table 1).

Open field test: Table 2 presents the OFT results for each group. Total movement distance and mean velocity were significantly lower in the IM group compared to the CONT group ($p<0.05$) and were significantly higher in the IM+F and IM+T+F groups compared to the IM group ($p<0.05$ for both). Although total movement distance and mean velocity were higher in the IM+T group compared to the IM group, no significant difference was found between the two groups ($p>0.05$).

Time spent in the central zone was analysed to assess the anxiety levels of the mice. The results indicated that the time spent in the central zone was significantly lower in the IM group compared to the CONT group ($p<0.05$). In the IM+T, IM+F and IM+T+F groups, however, time spent in the central zone was significantly higher compared to the IM group ($p<0.05$) (Table 2).

Table 1: First day and last day weights and weight changes of subjects

Parameters	First day (g)	Last day (g)	Weight changes
CONT	33.8±1.62	33.9±0.68	0.1
IM	32.6±1.94	30.4±1.75[a]	-2.2
IM+T	33.7±1.36	31.7±1.01	-2.0
IM+F	32.1±2.56	31.2±2.32	-0.9
IM+T+F	33.6±3.60	33.3±3.82	-0.3
T	33.6±3.60	33.7±3.82	0.1

Each row represents the mean±standard deviations of the mice, CONT: Control, IM: Immobilization, T: Trolox, F: Fluoxetine

Table 2: Open field test results of the subjects

Parameters	Distance (cm)	Velocity (cm sec^{-1})	Central time (sec)
CONT	1482.1±408.2	7.42±2.05	96.1±32.4
IM	1007.6±123.5[a]	4.97±0.68[a]	16.8±13.5[d]
IM+T	1285.4±322.1	6.43±1.62	67.1±21.2[c]
IM+F	1556.6±187.0[b]	7.80±0.94[b]	82.1±34.7[b]
IM+T+F	1549.7±165.5[b]	7.89±0.86[b]	81.1±38.6[b]
T	1449.7±374.1[c]	7.24±1.87[c]	98.2±47.5[b]

Each row represents the mean±standard deviations of the mice, one-way ANOVA followed by a *post hoc* Tukey test was used for statistical analysis, CONT: Control, IM: Immobilization, T: Trolox, F: Fluoxetine. [a]$p<0.05$ vs. CONT, [b]$p<0.01$ vs. IM, [c]$p<0.05$ vs. IM, [d]$p<0.01$ vs. CONT

Forced swimming test: The duration of immobility increased in the IM group and established a significant difference compared to that of CONT group ($p<0.05$). In the IM+T, IM+F and IM+T+F groups, however, the duration of immobility was significantly lower compared to the IM group ($p<0.05$ for both) (Table 3).

Biochemical analysis: Figure 1 illustrates the results of the biochemical analysis on the brain tissue samples. The CAT levels decreased significantly in the IM group compared to the CONT group ($p<0.05$) (Fig. 1a). The initially decreased CAT levels in the IM group subsequently increased significantly in the IM+T group ($p<0.05$), whereas, no such increase was observed in the IM+F group ($p>0.05$). On the other hand, although the CAT levels increased in the IM+T+F group compared to the IM group, no significant difference was observed between the two groups ($p>0.05$).

Paraoxonase-1 (PON-1) enzyme levels decreased significantly in the IM group ($p<0.05$) (Fig. 1b). Although the PON-1 levels increased in the IM+T, IM+F and IM+T+F groups compared to the IM group, no significant difference was observed between these groups and the IM group ($p>0.05$).

In the IM group, the TAS levels (Fig. 1d) decreased significantly and the TOS (Fig. 1c) and OSI levels (Fig. 1e) increased significantly compared to the CONT group ($p<0.05$ for all). The IM-induced deterioration was reversed in the IM+T and IM+T+F groups compared to the CONT group ($p<0.05$). However, no significant difference was observed in TOS levels between the IM+F group and the IM group ($p>0.05$).

Table 3: Immobile times of the subjects in the forced swimming test

Parameters	Immobile times (sec)
CONT	86.3±15.0
IM	154.2±24.9[a]
IM+T	108.5±32.3[b]
IM+F	96.8±29.2[c]
IM+T+F	92.8±18.2[c]
T	88.3±34.2[c]

Each row represents the mean±standard deviations of the mice, one-way ANOVA followed by a *post hoc* Tukey test was used for statistical analysis, CONT: Control, IM: Immobilization, T: Trolox, F: Fluoxetine, [a]$p<0.01$ vs. CONT, [b]$p<0.05$ vs. IM, [c]$p<0.01$ vs. IM

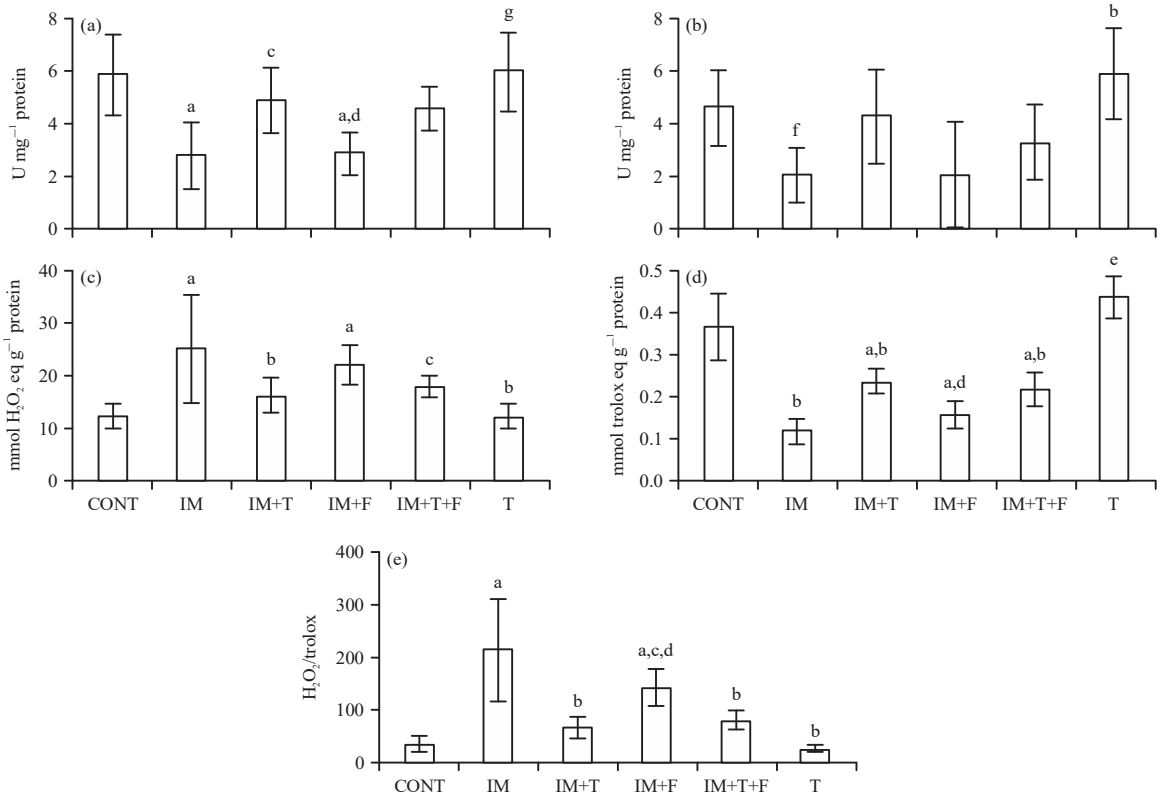

Fig. 1(a-e): Groups' brain (a) CAT, (b) PON-1, (c) TOS, (d) TAS and (e) OSI results

Each column represents the mean±standard deviations of the mice, one-way ANOVA followed by a *post hoc* Tukey test was used for statistical analysis, CONT: Control, IM: Immobilization, T: Trolox, F: Fluoxetine, CAT: Catalase, PON-1: Paraoxonase-1, TAS: Total antioxidant status, TOS: Total oxidant status, OSI: Oxidative stress index, [a]$p<0.01$ vs. control, [b]$p<0.01$ vs. IM, [c]$p<0.05$ vs. IM, [d]$p<0.05$ vs. IM+T, [e]$p<0.01$ vs. IM, IM+T, IM+F and IM+T+F, [f]$p<0.05$ vs. CONT, [g]$p<0.01$ vs. IM and IM+F

DISCUSSION

Severe and long-term stress exposure have been implicated in numerous physical and mental disorders and these disorders are thus termed stress-related disorders, mainly including cardiovascular, metabolic and neuropsychiatric disorders[1]. Among neuropsychiatric disorders, memory loss, anxiety and depression have been extensively attributed to stress[1-3]. It is commonly known that stress leads to neurochemical and neurohormonal imbalances in numerous systems, particularly in the sympathetic nervous system and the HPA axis[15,16]. In addition, stress has also been shown to cause oxidative damage and inflammation, thereby leading to adverse outcomes[17]. Accordingly, antioxidant agents and pharmacological agents such as classic anxiolytic and antidepressant drugs have been studied for the treatment of stress-related disorders[16].

In the present study, experimental stress was induced by immobilization (6 h/day for 7 days). Immobilization has been commonly performed in animal studies as it causes both physical and psychological stress[2,16,18]. Moreover, immobilization has also been shown to cause neurobehavioural alterations[16,19,20] and to increase ROS generation and decrease antioxidant enzyme activity[20]. In turn, the increased ROS production primarily targets the central nervous system that contains a large amount of lipids and consumes high amounts of oxygen[16,20,21].

In this study, total movement distance and mean velocity over the 5 min period on OFT were significantly lower in the IM group compared to the CONT group. The OFT is successfully performed in the assessment of anxiety-like behaviour[12]. Moreover, the significant decrease in time spent in the central zone in the IM group compared to the CONT group suggests that immobilization leads to motor function impairment in addition to anxiety-like behaviour. The FST was first described by Porsolt *et al.*[22,23] for the evaluation of antidepressant therapy and is based upon the response of the animals against an inescapable stressor and development of learned helplessness. In this study, the duration of immobility was significantly higher on FST in the IM group compared to the CONT group. Additionally, the TAS, PON-1 and CAT levels decreased significantly and the TOS and OSI levels increased significantly in the IM group compared to the CONT group. Taken together, these findings implicate that the immobilization model used in this study led to severe oxidative damage and motor function impairment as well as depression and anxiety-like behaviour in mice.

Trolox is a water-soluble derivative of vitamin E[10]. However, trolox has been shown to have advantages over vitamin E, the latter being only lipid-soluble due to the presence of a carboxyl group instead of a phytol chain which imparts trolox with water solubility[24]. Wattamvar *et al.*[10] reported that trolox has little or no cytotoxic effects and can reduce cellular oxidative stress by half. Prut and Belzung[13] evaluated the effects of trolox on the oxidative stress in mouse thymocytes induced by hydrogen peroxide (H_2O_2) and reported that trolox prevented the apoptosis induced by oxidative stress. Besides, there are some studies that demonstrated that trolox is a potent radical scavenger[12,25].

In the present study, the OFT and FST results indicated that the incidence of depression and anxiety-like behaviour was lower in the groups that received either trolox or fluoxetine in isolation or in combination following immobilization. In particular, this effect was even higher in the groups that received fluoxetine either in isolation or in combination. However, in terms of motor function, no significant improvement was observed in the IM+T group with regard to total movement distance and mean velocity. Considering all these results, it is safe to assert that although trolox yielded similar outcomes to fluoxetine, the combined use of these two molecules yielded no additional benefit.

Additionally, the CAT, PON-1, TOS and TAS levels were also measured in the brain tissue samples and OSI was calculated in this study. Catalase (CAT) is an antioxidant enzyme with a critical role in the detoxification of H_2O_2 into molecular oxygen and water[26]. The PON-1 enzyme is a member of the paraoxonase gene family which has a glycoprotein structure and is an esterase that is tightly bound to high-density lipoproteins[27]. The PON-1 has been reported to show peroxidase activity and to have a significant role in the prevention of neurodegenerative and psychiatric diseases[27,28]. In this study, in lieu of measuring the levels of each oxidant and antioxidant molecule in the tissue samples separately, TOS and TAS levels were measured using the method proposed by Erel *et al.*[29,30]. These two parameters have been extensively shown to be reliable indicators of oxidative status. Lately, it has also been widely documented that OSI may reveal the oxidative status more correctly than TOS or TAS level alone[31,32].

In the present study, the use of trolox in isolation or in combination with fluoxetine following immobilization led to a significant improvement in all the biochemical parameters except for PON-1. However, this improvement was not observed in the IM+F group. These findings suggest that trolox is highly effective in the prevention of oxidative damage in immobilization stress models.

CONCLUSION

Stress is a ubiquitous health problem around the world which is responsible for numerous neuropsychiatric problems and severe oxidative damage. Classic pharmacological agents are successfully used in the treatment of neuropsychiatric disorders. However, these treatment options remain inadequate in the prevention of oxidative stress induced by stress. In the present study, trolox was found to be an effective agent in the treatment of stress-related disorders such as depression and anxiety, though not as effective as fluoxetine. Nevertheless, trolox was found to be superior to fluoxetine due to its therapeutic effect on oxidative damage. Based on these results, it can be assumed that antioxidant molecules such as trolox can lead to favourable outcomes in the treatment of oxidative damage either in isolation or in combination with classic treatment methods.

SIGNIFICANCE STATEMENT

The present study discovered the beneficial effects of trolox against immobilization stress via antioxidant activity. The study will help future researchers to uncover the critical areas of stress, behaviour changes and antioxidant molecules that many researchers have not been able to explore to date.

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