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Ameliorative effect of trigonelline in restraint stress-induced behavioral alterations in mice

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ABSTRACT

Physical activity and stress are environmental modifiers for various neurodegerative disorders. The consistent psychological restraint stress has impacts the neurobiological changes like brain shrinkage or inhibiting the neurogenesis, mainly affecting the hippocampal CA3 region and exacerbating various neuropathologies. Therefore, stress directly modulates the pathology and neurobiology of human beings. The current research was designed to evaluate the anti-stress ability of trigonelline in behavioral and biochemical changes caused by restraint stress in mice. Various stressful stimuli have affected numerous physiological-based physical processes. There is no particular pharmacotherapy available to date which can count as stress reduction therapy. In the present study, female mice were subjected to restraint stress in flat bottomed restrainers with dimensions of 1.5″ dia × 4″, for 3.5 hours. The behavioral alterations by inducing restraint test were analyzed by using a battery of tests like social interaction, hole board, open field test, and elevated plus maze. The investigation of inducing restraint stress was processed which further resulted in exploratory behavior of grouped mice. Social behavior was measured by exploring the number of head dips and frequency of rearing in the hole board test. Apart from this, biochemical parameters, such as thiobarbituric acid reacting substance (TBARS), catalase (CAT), reduced glutathione (GSH) levels, myeloperoxidase, and superoxide dismutase (SOD), were analyzed. Mice treated with trigonelline (25 and 50 mg/kg, i.p.) significantly ($p < 0.05$) and dose-dependently attenuate stress-induced behavior and oxidative alterations, when compared to the stress control group. The current study confirmed the ameliorating effect of the trigonelline in restraint stress effect by attenuating the increased levels of MPO (Myeloperoxidase) and TBARS as stress parameters and enhancing the levels of protective enzymes such as GSH, SOD, and CAT. Hence, the study proposed a protective mechanism of trigonelline ameliorating stress by modulating the nuclear factor erythroid 2-related factor 2 pathways and oxidative stress.

INTRODUCTION

Stress is a significant rising problem among modern society growing under uncertain disruptive environmental situations affecting human health. Nowadays, stress is growing at a rate and is a comprehensive part of every human life and has become a common problem for every individual, aggravating various pathological conditions. Stress not only attributes a negative impact on human life but also affects other living organisms which are frequently being exposed to variant intrinsic and extrinsic stressful stimuli, and hence contributing to the distortion of physiological

processes. Stress causes specific chemical changes within the human body that affect the normal physiological processes of the human body, and as a result, the homeostasis is threatened. Although the human biological system comprises various adaptive mechanisms in order to maintain the homeostasis in response to external and internal stressful stimuli, the various adaptive processes like immune system, hormone release, nervous system or many enzymatic reactions work together to control stress . The activation of such adaptive mechanisms in response to the stress further initiates to trigger many pathological conditions related to gastrointestinal tract (Brzozowski *et al*., 2000), cardiovascular system (Munhoz *et al*., 2008), and central nervous system (CNS) (García-Bueno *et al*., 2008). Whenever there are neurochemical changes during stressful events, they directly affect the physiology of various organ systems such as CNS (Chrousos *et al*., 1992), cardiovascular, and gastrointestinal system; thus, the driving stress

leads to major depression (Munhoz *et al.*, 2008), schizophrenia (Bueno *et al.*, 2008), and neurodegenerative diseases (Liu *et al.*, 2005).

Undoubtedly, stress is an inevitable evil, yet no specific medicine is available in modern medicine which can qualify as therapy for stress management. Therefore, people have been trying to find an effective way in which they can enhance the resistance of the body toward stress and stress-induced changes in the body. When the body is exposed to stress stimuli, it persuades a variety of alterations such as changes in exudation of hormones, adrenocorticotropic hormone (ACTH), autonomic function of the body, and behavior (Kar *et al.*, 1999). Immobilization stress leads to the secretion of stress hormones like ACTH and corticosterone. The deleterious effects of restraint stress are well documented with impairments and alterations in cognitive, affective, and neuroendocrine functions (Gonzalo *et al.*, 2003). Besides, prolonged contact with stress has been observed to prompt depression (Willner *et al.*, 1998) and behavioral changes in animal models (Swiergiel *et al.*, 2007) and human beings (Van Praag, 2004).

The transcription factor, i.e., nuclear factor erythroid 2-related factor 2 (Nrf2), is encoded by the *NFE2L2* gene in humans (Moi *et al.*, 1994). It is the rudimentary leucine zipper (bZIP) protein that has the function of regulating antioxidant proteins that have a mechanism of protection against oxidative damage that can be caused due to inflammation or injury. Nowadays, management of diseases occurring due to oxidative stress are characterized under a variety of drugs stimulating the *NFE2L2* pathway (Zhang *et al.*, 2018). It is evident that the transcription factor (Nrf2) has an essential role in regulating antioxidant proteins (Jaiswal, 2004), which help in the prevention of neuro-inflammation. Dysfunctioning of the Nrf2 factor leads to neuroinflammation and generation of free radicals. Furthermore, it leads to neurodegeneration and reinforces a level of oxidative stress (Arlt *et al.*, 2013). However, no anti-stress drug has been discovered which can alter the deleterious effect of Nrf2 transcription factor in stress.

Trigonella foenum-graecum L. (fenugreek), a traditional herb from China, has components that are favorable in the inhibition and management of metabolic and neuronal disorders (Abdel-Daim *et al.*, 2014). The activities of pharmacological significance in trigonelline, which is a chief alkaloid constituent of fenugreek, has hypolipidemic, hypoglycemic, antimigraine, neuroprotective, memory-improving, sedative, antibacterial, anti-tumor, and antiviral activities (Zhou *et al.*, 2011). It has been found to show platelet aggregation and reduce diabetic auditory neuropathy (Bailey & Day, 1989). Trigonelline is a component that has a variety of medicinal advantages and is a strong antioxidant and is an Nrf2 transcription factor inhibitor (Arlt *et al.*, 2013). The present study was carried out to estimate its anti-stress upshot in animals under experimentation and modulate restraint-induced stress in mice.

MATERIALS AND METHODS

Animals

Swiss albino female mice, weighing 22–30 g, were used for the current study. Animals were subjected to a typical laboratory diet and water *ad libitum*. For 12 hours they were retained in the animal house of the department and were exposed to ordinary cycles of light and dark for 12 hours each. The investigational procedure was permitted by the Departmental Institutional Animal Ethics Committee. The animals were preserved under standard conditions laid down by the Committee for the Purpose of Control and Supervision of Experimental Animals.

Drugs and chemicals

Trigonelline were purchased from Sigma-Aldrich (St. Louis, MO) and other chemicals of analytical grade were procured from SD-Fine Chemical Limited, Mumbai.

Experimental procedure

Induction of restraint stress

Mice were subjected to restraint stress in flat bottomed restrainers with dimensions of $1.5'' \times 4''$ (diameter) for 3.5 hours (Madhyastha *et al.*, 2008). This type of restrainer only restricts the movement of animals.

Behavioral measurements

Hole board test

This test was carried out in a box made of wood that measured 68×68 cm to observe the exploratory animal behavior for about 10 minutes in which head dips frequency and their rearing were recorded for the behavioral patterns (Brown *et al.*, 2008).

Social interaction test

Social interaction test was conducted for 10 minutes. The behavior patterns like following the partner (social behavior) and avoiding the partner (non-social behavior) were observed. Social interaction behavior of mice includes sniffing, nipping, and grooming (Rex *et al.*, 2004).

Open field test

The open field test was expressed as an unconditional measure of anxiety based on the conception of a conflict between the mice's investigative drive and its fear of disclosure to an open area. The activity level of the mice was represented by the total line crossings and rearings (Angrini *et al.*, 1998).

Elevated plus maze (EPM) test

The EPM model was used to test anti-anxiety behavior in Swiss female albino mice by accessing the average time spent and number of entries in the open and closed arm for 5 minutes.

Biochemical estimations

Thiobarbituric acid reactive substances (TBARS) were estimated using brain tissue by a procedure given by Ohkawa *et al.* (1979), superoxide dismutase (SOD) by Willcox *et al.* (2004), catalase (CAT) by Benzie (1996), reduced glutathione (GSH) by Ellman *et al.* (1965), and MPO in a method described by Beutler and Kelly (1963).

EXPERIMENTAL PROTOCOL

The present study involved six groups comprising female mice ($n = 6$).

Group I: vehicle control group

Female mice were not exposed to any type of stressor. Animals were lacerated instantly after behavioral calculation and the tissue homogenates were prepared for analyzing various biochemical parameters.

Group II: Stress control group

Mice were exposed to restraint stress for 3.5 hours and consequently the different behavioral examinations were executed. Animals were sacrificed immediately after behavioral assessment and the tissue homogenates were prepared for analyzing various biochemical parameters.

Group III: Diazepam and stress control group

Mice were subjected to restraint stress as described in group II. The administration of Diazepam (2 mg/kg, i.p.) was conducted 30 minutes prior to the stress protocol. Animals were then sacrificed immediately after behavioral assessment and the tissue homogenates were prepared for analyzing various biochemical parameters.

Group IV: Trigonelline and stress control group

Mice were exposed to the restraint stress as designated in group II. Trigonelline (25 mg/kg i.p.) was administered 30 minutes before the stress protocol. Animals were then sacrificed immediately after behavioral assessment and the tissue homogenates were prepared for analyzing various biochemical parameters.

Group V: Trigonelline and stress control group

Mice were subjected to the restraint stress as described in group II. Trigonelline (50 mg/kg i.p.) was administered 30 minutes before the stress protocol. Animals were then sacrificed immediately after behavioral assessment and the tissue homogenates were prepared for analyzing various biochemical parameters.

Statistical analysis

To analyze the result, mean ± standard error of mean (SEM) was used. The statistical comparison of results between different groups was carried out by using one-way analysis of variance (ANOVA) and *post-hoc* analysis using Tukey's multiple comparison test.

The p values $< 0.0001, < 0.01, < 0.05$ were well thought out to be statistically substantial.

RESULTS

Effect of several treatments on restraint stress-induced anxiety like behavior in mice

Restraint stress-induced anxiety like behavior as measured in terms of average time spent in the open arm and number of entries in open arm particularly in the restraint stress control group in comparison to that of the control groups treated by vehicle. Administration of trigonelline (25 and 50 mg/kg i.p.) and diazepam (2 mg/kg, i.p.) significantly ($p < 0.05$) and dose-dependently attenuated restraint stress-induced anxiety like behavior as measured in terms of the average time spent in the open arm and number of entries when compared with stress control group (Figs. 1 and 2).

### Effect of several treatments tested on rearing and head dips in hole board test

The frequency of rearing reflects and the index of curiosity or examination of new surroundings was measured with the help of head dips in the hole board test. There was a significant decrease in the frequency of head dips and rearing as compared to the vehicle control group in restrain subjected mice. Pretreatment with trigonelline (25 and 50 mg/kg i.p.) and diazepam (2 mg/kg, i.p.) the frequency of head dips and rearing increased pointedly ($p < 0.05$) when compared to the stress control group (Figs. 3 and 4).

Effect of several treatments on line crossings and rearing in open field test

Motor activity and the frequency of rearing reflect the exploration of novel surroundings indicated by line crossings.
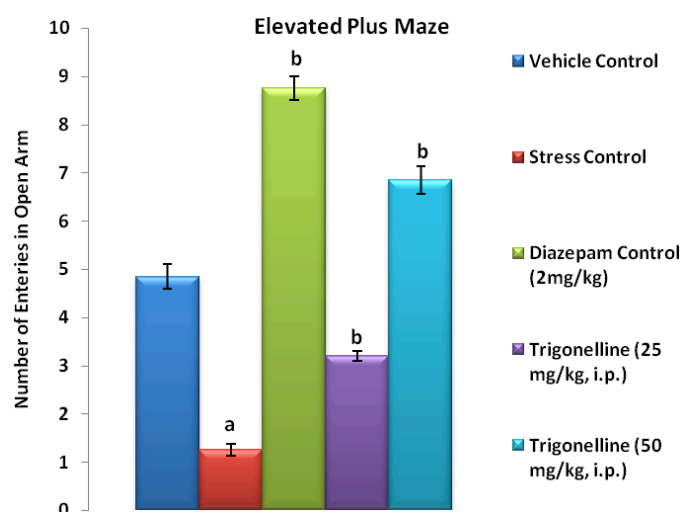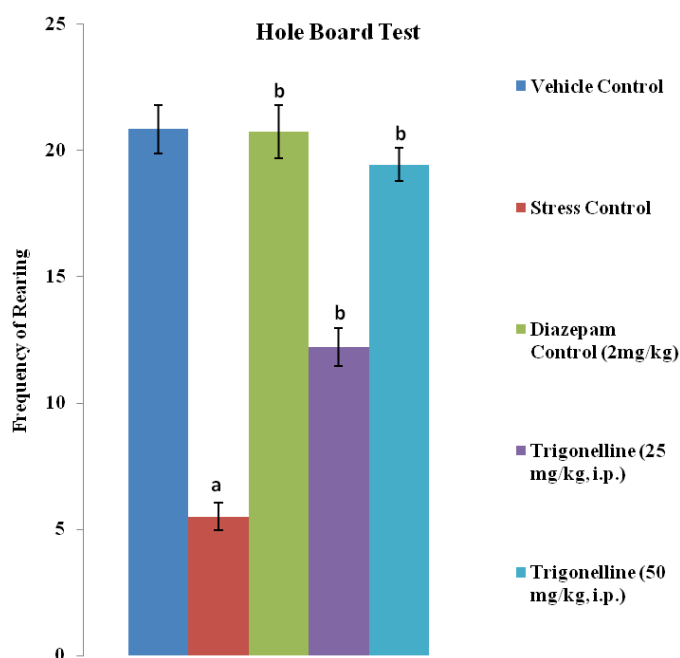

Figure 1. Effect of various treatments on the number of mice entries in open arm using elevated plus maze. Results are represented as mean ± SEM with $n = 6$ in each group. Statistical analysis by carried out by one-way ANOVA, followed by Tukey's multiple comparison test. $^a p < 0.05$ when compared to vehicle control group. $^b p < 0.05$ when compared to stress control group.
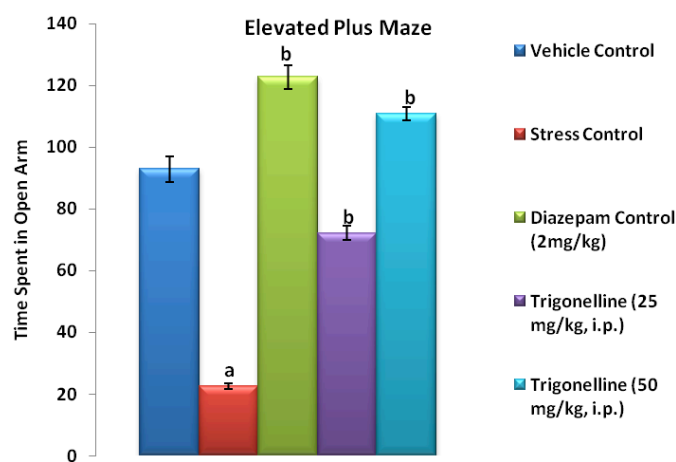
Figure 2. Effect of various treatments on time spent of mice in open arm using elevated plus maze. Results are represented as mean ± SEM with $n = 6$ in each group. Statistical analysis by carried out by one-way ANOVA, followed by Tukey's multiple comparison test. $^a p < 0.05$ when compared to vehicle control group. $^b p < 0.05$ when compared to stress control group.
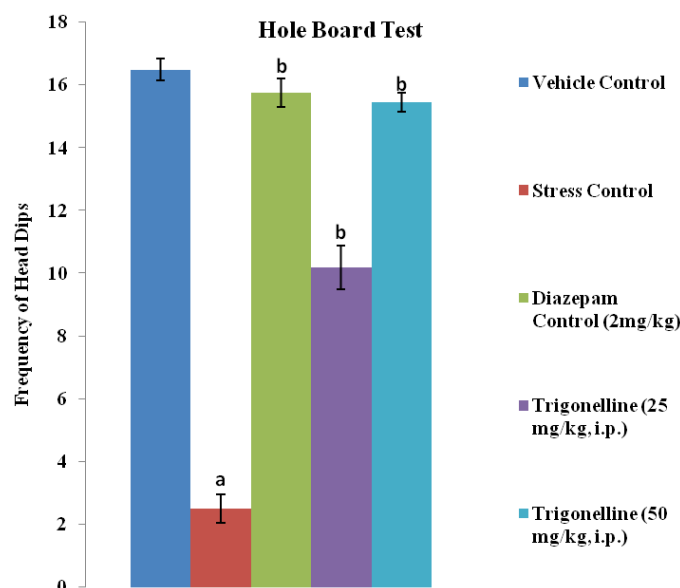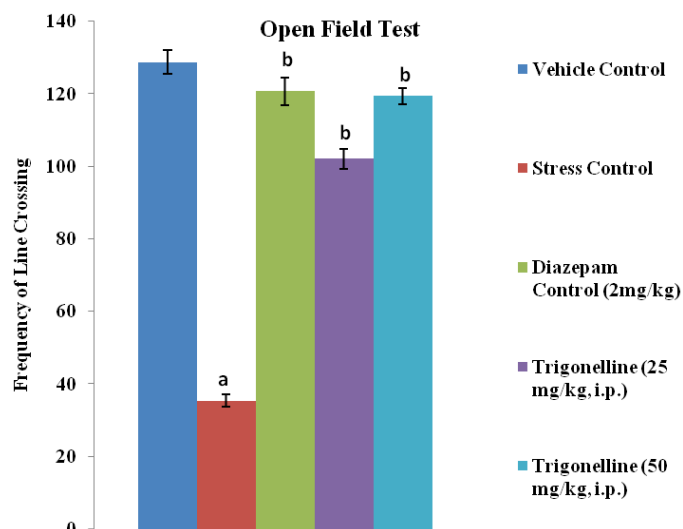
Figure 3. Effect of various treatments on frequency of head dips in the hole board test. Results are represented as mean ± SEM with $n = 6$ in each group. Statistical analysis by carried out by one-way ANOVA, followed by Tukey's multiple comparison test. [a]$p < 0.05$ when compared to vehicle control group. [b]$p < 0.05$ when compared to stress control group.
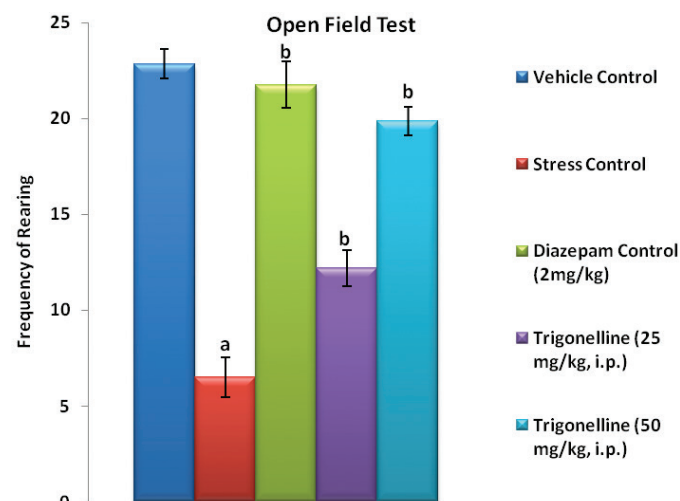

Figure 4. Effect of various treatments on frequency of rearing in the hole board test. Results are represented as mean ± SEM with $n = 6$ in each group. Statistical analysis by carried out by one-way ANOVA, followed by Tukey's multiple comparison test. [a]$p < 0.05$ when compared to vehicle control group. [b]$p < 0.05$ when compared to stress control group.

In restrain subjected mice, the total line crossings as well as frequency of rearing decreased significantly as compared to the vehicle control group. Pretreatment with trigonelline (25 and 50 mg/kg i.p.) and diazepam (2 mg/kg, i.p.) increase the frequency of rearing and the total line crossing in a significant manner ($p < 0.05$) when compared to the stress control group (Figs. 5 and 6).

Figure 5. Effect of various treatments on frequency number of line crossing in open field test. Results are represented as mean ± SEM with $n = 6$ in each group. Statistical analysis by carried out by one-way ANOVA, followed by Tukey's multiple comparison test. [a]$p < 0.05$ when compared to vehicle control group. [b]$p < 0.05$ when compared to stress control group.

Figure 6. Effect of various treatments on frequency number of rearing in open field test. Results are represented as mean ± SEM with $n = 6$ in each group. Statistical analysis by carried out by one-way ANOVA, followed by Tukey's multiple comparison test. [a]$p < 0.05$ when compared to vehicle control group. [b]$p < 0.05$ when compared to stress control group.

Effect of several treatments on social and non-social behavior in social interaction test

In restraint stress subjected mice, time of avoidance (non-social behavior) increased significantly ($p < 0.05$) in comparison to the time of following (social behavior) in the vehicle control group. However, in pretreatment with trigonelline (25 and 50 mg/kg i.p.) and diazepam (2 mg/kg, i.p.) protocols, time following the partner (social behavior) was principally shown as related to the vehicle control group (Fig. 7).
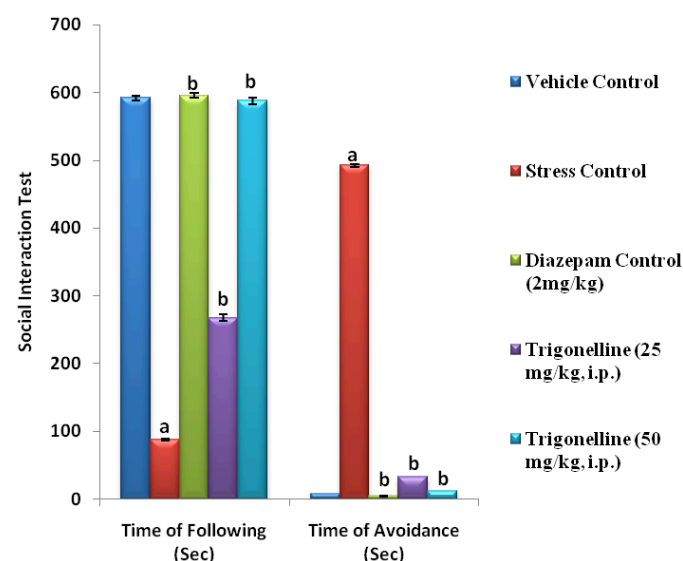
Effect of several treatments on changes in GSH levels

Restraint stress resulted in a decrease in brain GSH levels in comparison to the vehicle control group. Pretreatment with
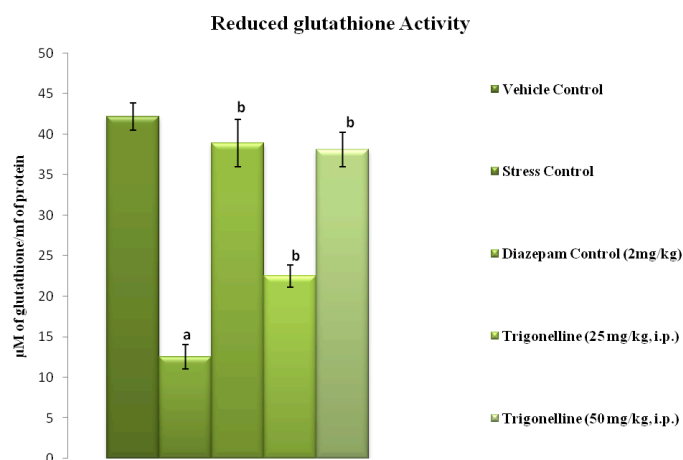
trigonelline (25 and 50 mg/kg, i.p.) significantly ($p < 0.05$) and dose-dependently increased the brain GSH levels. Pretreatment with the standard drug diazepam (2 mg/kg, i.p.) increased the glutathione levels in a dose-dependent manner (Fig. 8).

Effect of several treatments on changes in lipid peroxidation (LPO) levels

Restraint stress resulted in an increase in brain TBARS levels as compared to the vehicle control group. Pretreatment with trigonelline (25 and 50 mg/kg i.p.) and diazepam (2 mg/kg, i.p.) decreased the brain TBARS levels significantly ($p < 0.05$) when compared to the stress control group (Fig. 9).

Effect of several treatments on changes in the CAT level

Restraint stress resulted in a decrease in brain CAT levels when compared to the vehicle control group. However, pretreatment with trigonelline (25 and 50 mg/kg i.p.) and diazepam (2 mg/kg, i.p.) increased the brain CAT levels in a significant manner ($p < 0.05$) when compared to the stress control group (Fig. 10).

Effect of several treatments on changes in SOD and GSH levels

Restraint stress resulted in a decrease in brain SOD and GSH levels when compared to the vehicle control group. Pretreatment with trigonelline (25 and 50 mg/kg i.p.) and diazepam (2 mg/kg, i.p.) increased the superoxide dismutase and GSH levels in a significant manner ($p < 0.05$) when compared to the stress control group (Figs. 11 and 12).

Figure 7. Effect of various treatments on the time of following and avoidance(s) in the social interaction test. Results are represented as mean ± SEM with $n = 6$ in each group. Statistical analysis by carried out by one-way ANOVA, followed by Tukey's multiple comparison test. [a]$p < 0.05$ when compared to vehicle control group. [b]$p < 0.05$ when compared to stress control group.
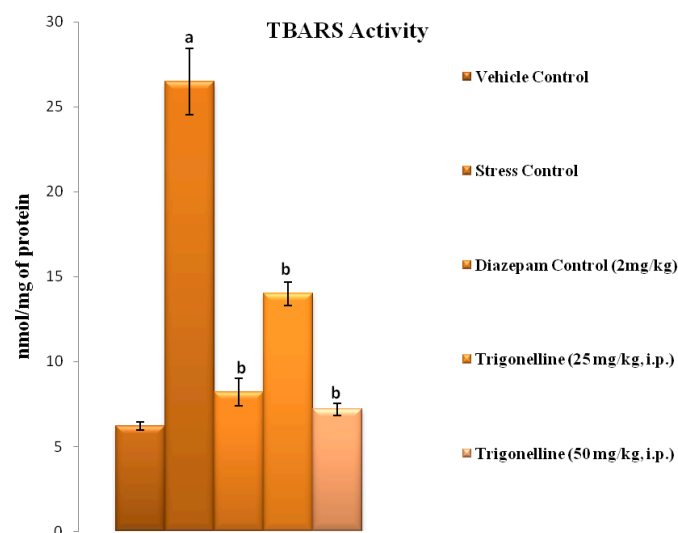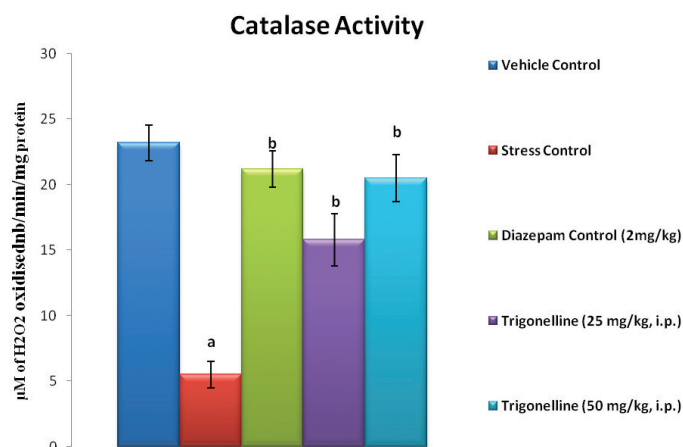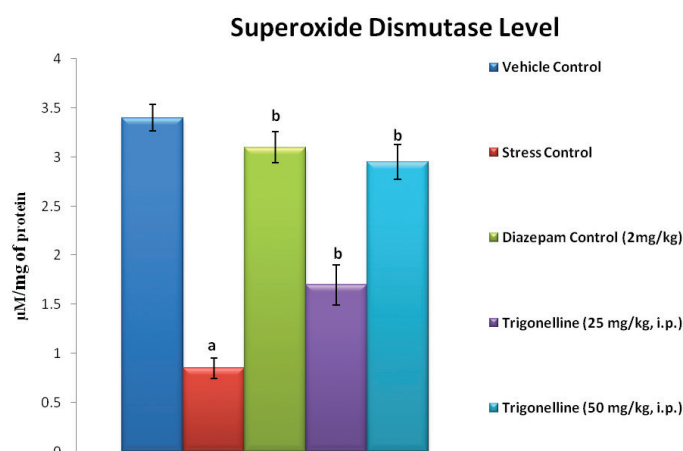

Figure 9. Effect of various treatments on changes in LPO (TBARS) levels. Results are represented as mean ± SEM with $n = 6$ in each group. Statistical analysis by carried out by one-way ANOVA, followed by Tukey's multiple comparison test. [a]$p < 0.05$ when compared to vehicle control group. [b]$p < 0.05$ when compared to stress control group.

Figure 8. Effect of various treatments on changes in GSH levels. Results are represented as mean ± SEM with n = 6 in each group. Statistical analysis by carried out by one-way ANOVA, followed by Tukey's multiple comparison test. [a]$p < 0.05$ when compared to vehicle control group. [b]$p < 0.05$ when compared to stress control group.

Figure 10. Effect of various treatments on changes in CAT levels. Results are represented as mean ± SEM with $n = 6$ in each group. Statistical analysis by carried out by one-way ANOVA, followed by Tukey's multiple comparison test. [a]$p < 0.05$ when compared to vehicle control group. [b]$p < 0.05$ when compared to stress control group.
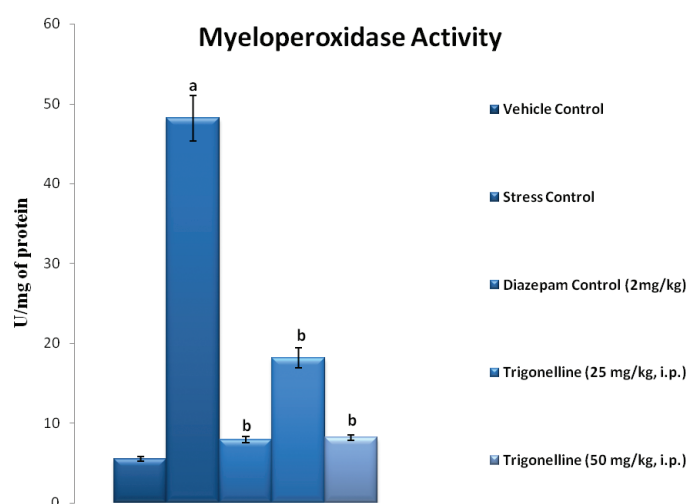
Superoxide Dismutase Level

Figure 11. Effect of various treatments on changes in SOD levels. Results are represented as mean ± SEM with $n = 6$ in each group. Statistical analysis by carried out by one-way ANOVA, followed by Tukey's multiple comparison test. [a]$p < 0.05$ when compared to vehicle control group. [b]$p < 0.05$ when compared to stress control group.

Myeloperoxidase Activity

Figure 12. Effect of various treatments on changes in myeloperoxidase levels. Results are represented as mean ± SEM with $n = 6$ in each group. Statistical analysis by carried out by one-way ANOVA, followed by Tukey's multiple comparison test. [a]$p < 0.05$ when compared to vehicle control group. [b]$p < 0.05$ when compared to stress control group.

DISCUSSION

Exposure to stress stimuli brings with it a multitude of variations in the body, including behavioral changes, activation of autonomic control, and the secretion of many hormones such as corticosterone and ACTH (Gonzalo *et al.*, 2003; Kar *et al.*, 1999). Many behavioral alterations were reported in acute stress, such as decrease in exploratory, spontaneous activity behavior, and change in social behavior. It is also evident that exposure to chronic stress induces a state of depression in animal models (Swiergiel *et al.*, 2008; Willner *et al.*, 1998) and in humans (Van Praag, 2004). In agreement with the above findings, restraining of Swiss albino mice in our study restrained stress for 3.5 hour resulted in significant enhancement in oxidative stress and decline

in spontaneous activity, alteration in orientational investigating activity and social behavior. In the hole board test, there was a decline in the occurrence of head dips and rearing. In social interaction studies, there was decline in follow-up time and intensification in the time of avoidance behavior.

In order to achieve acute stress in mice, the most successful animal model is the stress-induced restraint (Kumari *et al.*, 2007; Stamp *et al.*, 1999). Also, such type of stress is a type of physical stress that is helpful in studying the stress-induced neurodegeneration and post-traumatic stress disorders (Adamec and Shallow, 1993; Southwick *et al.*, 1994).

The various study groups used specific time intervals for restraining or immobilization, such as 1 hour (Stamp *et al.*, 1999), 2 hours (Zafar *et al.*, 1997), 2.5 hours (Dronjak and Gavrilovic, 2006), 3 hours (Zafir and Banu, 2009), 4 hours (Komori *et al.*, 2003), and 6 hours (Dhir *et al.*, 2006; Kumari *et al.*, 2007) for variable degree induction of acute stress. In the present study, mice were stressed for 3.5 hours to induce acute stress because during the pilot studies this time was found to generate reproducible and optimal stress in mice.

In the current study, treatment with trigonelline (25 and 50 mg/kg, i.p.), a competitive Nrf2 factor inhibitor (Arlt *et al.*, 2013), pointedly attenuated the restraint stress-induced behavioral alterations suggesting that the pharmacological modulation of Nrf-2 may be employed for counteracting the stress-associated behavioral alterations.

The pharmacological effect of trigonelline modulates the activity of Nrf2-dependent proteasome system and its obstacle to tumor necrosis factor-related apoptosis-inducing ligand and anticancer drug-induced apoptosis (Arlt *et al.*, 2013). Nrf2-linked pathways under normal conditions are involved in defensive mechanisms against oxidative stress by regulating levels of antioxidant enzymes. The deregulation of Nrf2 was thus related to the aging, pathogenesis of neurodegenerative diseases, and stressful conditions (Djordjevic *et al.*, 2015). Activity Nrf2 is strictly regulated by a fine balance between positive and negative modulators. It was inspected that the persistently augmented initiation of the Nrf2/Keap1 pathway in the hippocampus enhanced the neuroinflammation and oxidative stress at the cellular level (Shetty *et al.*, 2017). Nrf2 is a transcription factor well known for its role in regulating antioxidant proteins that defend against oxidative stress. Nrf2 has its role in modulating antioxidant proteins that picket contrary to oxidative damage caused by neuronal injury and inflammation (Flower *et al.*, 2015). It is evident that the overactivation of Nrf2 in neuronal oxidative stress was confirmed by investigating the nuclear translocation in hippocampal neurons and level of triggered Nrf2 in hippocampal lysates (Sandberg *et al.*, 2014). Therefore, it is vivid that the Nrf2 system dysregulation in the brain contributes to oxidative stress and injury.

Trigonelline has demonstrated its therapeutic potential for antianxiety, memory enhancement, and neuroprotection due to its axonal extension and neuronal excitability potential in neuropathy due to modulation of cell regeneration, hypolipidemic, and antidiabetic action due to insulin secretion, in antimicrobial and anti-cancer therapy due to its reactive oxygen species scavenging properties (Bothwell and Gillete, 2018; Mohamadi *et al.*, 2018; Ribeiro *et al.*, 2017).
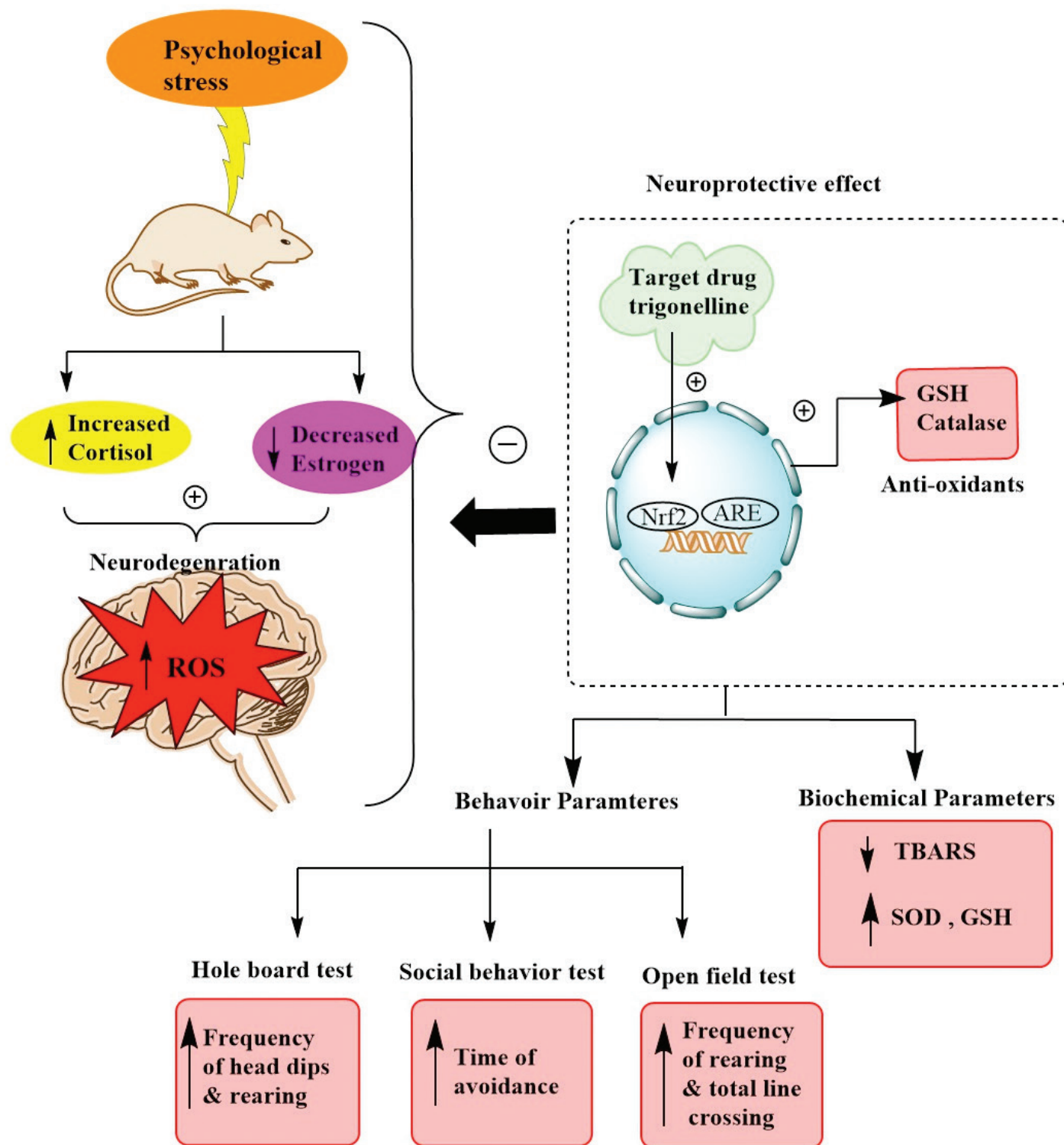
Figure 13. Graphical presentation of the restraint stress-induced behavioral and biochemical alterations in mice and the neuroprotective effect of Trigonelline.

Oxidative stress levels of TBARS, GSH, CAT, SOD, and MPO were distinctly modulated by trigonelline treatment in albino mice, underscoring trigonelline's contribution to the antioxidant and anti-inflammatory effects in restraint stress. In our study, mice treated with trigonelline (25 and 50 mg/kg, i.p.) for 1 day showed decreased TBARS and MPO contents, and increased superoxide dismutase, CAT, and glutathione levels in the brain.

These findings indicate that trigonelline has beneficial effects in the restrained stress by modulating the levels of regulating antioxidant enzyme and decreasing LPO by scavenging free radicals.

CONCLUSION

Restraint stress in animals has been characterized from the behavioral level to the neurochemical concomitants of stress. The use of restraint directly modulates the pathophysiology and neurobiology of animals. It was found that the dysfunction of Nrf2 occurred under stressful environments, leading to an increase in neuroinflammatory mediators, and is responsible for behavioral changes, whereas inducing oxidative stress is responsible for the biochemical changes. In the current study, trigonelline (25 and 50 mg/kg, i.p.) dose-dependently and significantly inhibited the increased levels of MPO and TBARS and reduced the level of protective enzymes, such as GSH, SOD, and CAT by modulating the dysfunctioning of Nrf2 in the brain and also shows neuroprotective effects in restraint stress-induced behavioral alterations (Fig. 13).

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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