



# Protective effect of alprazolam in acute immobilization stress-induced certain behavioral and biochemical alterations in mice

Richa Goyal, Kumar Anil

Pharmacology Division, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh-160014, India

**Correspondence:** Anil Kumar, e-mail: kumarujps@yahoo.com

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## Abstract:

Stress can be viewed as a cause of adverse circumstance that induces a wide range of biochemical and behavioral changes. Oxidative stress is a major contributor to the genesis of neurodegenerative and neuropsychiatric problems. In the present study, we investigated the protective effect of alprazolam in acute immobilization-induced various behavioral and biochemical alteration in mice. Mice were immobilized for a period of 6 h. Alprazolam (0.25 and 0.5 mg/kg, *ip*) was administered 30 min before subjecting the animals to acute stress and several behavioral (mirror chamber, actophotometer, tail flick test) and biochemical tests (malondialdehyde level, glutathione, catalase, nitrite and protein) were performed. Acute immobilization stress for a period of 6 h caused severe anxiety, analgesia and decreased locomotor activity in mice. Biochemical analyses revealed an increase in malondialdehyde, nitrite level and depleted glutathione and catalase activity in stressed brain. Pretreatment with alprazolam (0.25 and 0.5 mg/kg, *ip*) significantly reversed immobilization stress-induced anxiety, analgesia and impaired locomotor activity. Biochemically, alprazolam pretreatment decreased malondialdehyde, nitrite activity and restored reduced glutathione level and catalase activity. These results suggest that alprazolam has a neuroprotective effect and can be used in the treatment and management of stress and related disorders.

## Key words:

alprazolam, immobilization stress, lipid peroxidation, anxiety, analgesia, locomotor activity

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**Abbreviation:** DTNB – dithiobisnitrobenzoic acid, GABA –  $\gamma$ -aminobutyric acid,  $H_2O_2$  – hydrogen peroxide, HPA – hypothalamic-pituitary-adrenal axis, MDA – malondialdehyde, NO – nitric oxide, ROS – reactive oxygen species

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## Introduction

Acute stress is experienced in response to emotional and physical threat, which can be real or imagined. Stress has been shown to affect several brain activities

and promote long-term changes in multiple neural systems. Acute stress is a result of a traumatic event that makes a person to feel fear and helplessness. A variety of diverse environmental and stressful stimuli have been reported to alter behavioral pattern, neurotransmitter level and oxidative damage in discrete areas of brain [7, 18]. Acute immobilization stress has been reported to impair motor activity, cause memory dysfunction, modulate anxiety [10], pain perception [27] and depression-like behaviors [11] in the animals. Many of these effects are thought to be mediated by stress-induced neurochemical and hormonal abnormalities that are often associated with oxidative

damage [39]. However, it is unclear that oxidative stress plays a role in the pathogenesis of motor impairment [36], anxiety and nociception. Stress activates hypothalamic-pituitary-adrenal (HPA) axis and influences several neurological functions at both central and peripheral level. Besides, neurotransmitters and neuropeptides also influence HPA axis activity by acting at the hypothalamic or suprahypothalamic level [27]. Adaptive response to psychological stress (HPA axis induction) includes antioxidant defense systems.  $\gamma$ -Aminobutyric acid (GABA) is the major inhibitory neurotransmitter for fast inhibitory synaptic transmission and regulates many physiological and psychological processes [32]. Role of GABAergic system in stress and related conditions have been well documented. Immobilization stress has been reported to alter metabolic and physiological functions of GABA in the CNS [41] and reduce levels of several neurotransmitters, such as noradrenaline, adrenaline [40], dopamine [37] and 5-hydroxytryptamine [21] in the brain. It has also been seen that GABAergic system was significantly altered during stress, particularly benzodiazepine receptor binding sites [3, 5, 12]. High potency benzodiazepines are prescribed for the treatment of acute response to stress [13], episodic anxiety and fluctuations in generalized anxiety and agoraphobia. These major clinical advantages of benzodiazepines are due to high efficacy, rapid onset of action and low toxicity. However, on long-term use, tolerance, dependence and withdrawal effects limits their frequent use [1]. Novel therapeutic uses of benzodiazepines, such as in panic disorders and mania were found with the introduction of two high-potency benzodiazepines; clonazepam and alprazolam which were thought to have serotonergic properties [5]. The degree of changes and the rate of restoration of the initial activity depend on the efficacy of anxiolytics and duration of drug administration. A possible mechanism of this phenomenon can be the GABA-benzodiazepine interaction [31].

Alprazolam is a benzodiazepine derivative that is currently used in the treatment of generalized anxiety, panic attacks with or without agoraphobia and depression. Alprazolam has a quick onset of action and is unlikely to produce dependence or abuse. No tolerance to its therapeutic effect has been reported [42]. Based on the above, the present study was designed to investigate the role of alprazolam against immobilization-induced behavioral alteration and oxidative damage in mice.

## Materials and Methods

### Animals

Albino mice (Laca strain) weighing between 22–30 g bred in Central Animal House facility of the Panjab University, Chandigarh, India were used. The animals were housed under standard laboratory conditions and maintained on natural light and dark cycle and had free access to food and water. Animals were acclimatized to laboratory conditions before the experiment. Each group consists of a minimum of 5 animals. All the experiments were carried out between 09:00 and 15:00 h. The experimental protocols were approved by Institutional Animal Ethics Committee and conducted according to the Indian National Science Academy Guidelines for the use and care of experimental animals.

### Drugs and treatment

Alprazolam (0.25 and 0.5 mg/kg) was dissolved in distilled water and administered intraperitoneally 30 min before the animals were subjected to immobilization stress.

### Immobilization stress in mice

Animals were immobilized for 6 h by taping all the four limbs to board after placing them on their backs using zinc oxide hospital tape. Animals were released by removing the tape after moistening with acetone. In unstressed group, the mice were handled without any stress [7].

### Behavioral assessment

Various behavioral parameters were assessed in mice after 6-h immobilization stress.

#### Measurement of locomotor activity

The locomotor activity was monitored by using actophotometer (IMCORP, India). Before subjecting the animals to locomotor activity test, they were individually placed in activity meter and total activity count was registered for 5 min. The locomotor activity was expressed in terms of total photobeam interruption counts/5 min per animal [36].

### Measurement of anxiety: Mirror Chamber Test

The mirror chamber consisted of a wooden chamber having a mirror chamber enclosed within it. During the 5 min test session, the following parameters were recorded: a) latency to enter the mirror chamber, b) total time spent in mirror chamber, c) number of entries in mirror chamber. Animals were placed individually at the distal corner of the mirror chamber at the beginning of the test. An anxiogenic response was defined as decreased number of entries and time spent in the mirror chamber [20].

### Measurement of antinociception

The nociceptive threshold was determined as the latencies of tail flick reaction in response to radiant heat [6]. Baseline latencies to tail flick withdrawal from the radiant heat source (3–5 s) were established. A cut-off time of 10 s was fixed to prevent any injury to the tail [19].

### Biochemical parameters

All the animals were sacrificed by decapitation on the same day following behavioral assessment. The brains were removed, rinsed in isotonic saline and weighed. A 10% (w/v) tissue homogenate was prepared with 0.1 M phosphate buffer (pH = 7.4). The post-nuclear fraction was obtained by centrifugation of the homogenate at  $12\,000 \times g$  for 20 min at 4°C.

### Lipid peroxidation assay

The quantitative measurement of lipid peroxidation in the whole brain was measured according to the method of Wills [43]. The amount of malondialdehyde formed was measured by the reaction with thiobarbituric acid at 532 nm using Perkin Elmer lambda 20 spectrophotometer. The results were expressed as nanomoles of malondialdehyde per milligram of protein using the molar extinction coefficient of chromophore  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

### Estimation of reduced glutathione

Reduced glutathione in the brain was estimated according to the method of Ellman [9]. Homogenate (1 ml) was precipitated with 1.0 ml of 4% sulfosalicylic acid by keeping the mixture at 4°C for 1 h and

the samples were immediately centrifuged at  $1200 \times g$  for 15 min at 4°C. The assay mixture contained 0.1 ml of supernatant, 2.7 ml of phosphate buffer of pH = 8.0 and 0.2 ml of 0.01 M dithiobisnitrobenzoic acid (DTNB). The yellow color developed was read immediately at 412 nm using Perkin Elmer lambda 20 spectrophotometer. The results were expressed as nanomoles of reduced glutathione per milligram of protein.

### Nitrite estimation

Nitrite is the stable end product of nitric oxide (NO) in living systems. Accumulation of nitrite was measured in cell-free supernatants from brain homogenates by spectrophotometric assay based on Greiss reagent 15 (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% phosphoric acid) and incubated at room temperature for 10 min to yield a chromophore. Absorbance was read at 543 nm spectrophotometrically. The nitrite concentration was calculated from a standard curve using sodium nitrite as standard and expressed as micromoles nitrite per milliliter of homogenate [15].

### Protein estimation

The protein content was measured according to the method of Lowry using bovine serum albumin as standard [25].

### Catalase estimation

Catalase activity was determined by the method of Luck [26], wherein the breakdown of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of  $\text{H}_2\text{O}_2$ , phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%), and the change in absorbance was recorded at 240 nm. The results were expressed as micromoles of  $\text{H}_2\text{O}_2$  decomposed per milligram of protein/min.

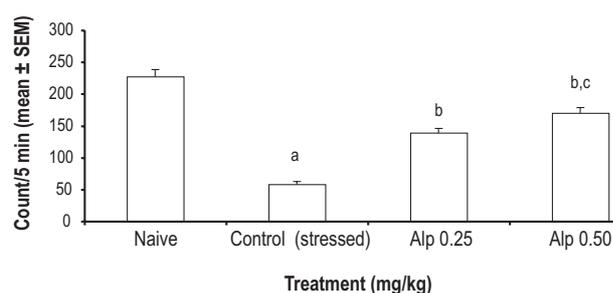
### Statistical analysis

All the values are expressed as the mean  $\pm$  SEM. The data were analyzed by using one-way analysis of variance followed by Tukey's test;  $p < 0.05$  was considered statistically significant.

## Results

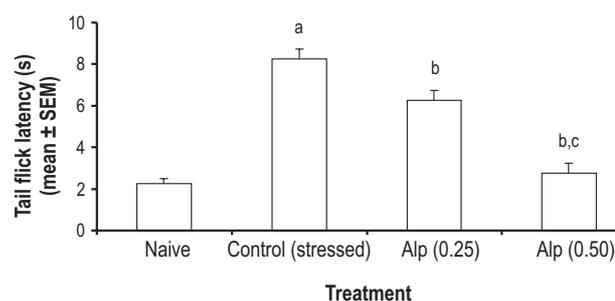
### Behavioral measurements (locomotor, anxiety and analgesic activity)

Six-hour acute immobilization stress elicited significant locomotor activity impairment (as indicated by



**Fig. 1.** Effect of alprazolam (Alp) on locomotor activity in acute immobilization stress. <sup>a</sup>  $p < 0.05$  as compared to naive, <sup>b</sup>  $p < 0.05$  as compared to control, <sup>c</sup>  $p < 0.05$  as compared to Alp (0.25 mg/kg). (One-way ANOVA followed by Tukey's test)

decreased ambulatory movements), anxiety-like-behavior (increased latency to enter mirror chamber, decreased number of entries and time spent in the mirror chamber) and antinociceptive effect (increased tail flick latency). Pretreatment with alprazolam (0.25 and 0.5 mg/kg, *ip*) significantly improved ambulatory movements (Fig. 1), had antianxiety effect (decreased latency to enter mirror chamber, increased number of



**Fig. 2.** Effect of alprazolam (Alp) on pain perception in acute immobilization stress. <sup>a</sup>  $p < 0.05$  as compared to naive, <sup>b</sup>  $p < 0.05$  as compared to control, <sup>c</sup>  $p < 0.05$  as compared to Alp (0.25 mg/kg). (One-way ANOVA followed by Tukey's test)

**Tab. 1.** Antianxiety effect of alprazolam in the mirror chamber test

Drug treatment (mg/kg)	Latency to enter (sec) mirror chamber (mean ± SEM)	No. of entries in mirror chamber (mean ± SEM)	Time spent in mirror chamber (sec) (mean ± SEM)
Naive (without stress)	39.0 ± 8.81	3.0 ± 0.71	120.0 ± 10.21
Control (stressed)	82.0 ± 5.09 <sup>a</sup>	0.5 ± 0.29 <sup>a</sup>	1.25 ± 0.75 <sup>a</sup>
Alp (0.25)	42.25 ± 1.37 <sup>b</sup>	2.25 ± 0.25 <sup>b</sup>	48.0 ± 3.39 <sup>b</sup>
Alp (0.50)	32.50 ± 4.35 <sup>b,c</sup>	3.5 ± 0.64 <sup>b</sup>	81.25 ± 3.95 <sup>b,c</sup>

Values are expressed as the mean ± SEM. <sup>a</sup>  $p < 0.05$  as compared to naive, <sup>b</sup>  $p < 0.05$  as compared to control, <sup>c</sup>  $p < 0.05$  as compared to Alp (0.25 mg/kg). (One-way ANOVA followed by Tukey's test)

**Tab. 2.** Effect of alprazolam on immobilization-induced biochemical alteration in the whole brain of mice

Treatment (mg/kg)	LPO (moles of MDA/mg protein)	Red GSH (micromoles of GSH/mg protein)	Nitrite (µg/ml)	Catalase (µ Mole of H <sub>2</sub> O <sub>2</sub> /min/mg protein)
Naive	0.168 ± 0.03	0.065 ± 0.0018	318 ± 2.94	0.7050 ± 0.033
Control	0.611 ± 0.03 <sup>a</sup>	0.0147 ± 0.002 <sup>a</sup>	649.75 ± 4 <sup>a</sup>	0.128 ± 0.0023 <sup>a</sup>
Alp (0.25)	0.244 ± 0.02 <sup>b</sup>	0.0405 ± 0.005 <sup>b</sup>	422 ± 5.49 <sup>b</sup>	0.386 ± 0.021 <sup>b</sup>
Alp (0.50)	0.181 ± 0.02 <sup>b,c</sup>	0.0245 ± 0.003 <sup>b</sup>	509 ± 4.65 <sup>b</sup>	0.519 ± 0.018 <sup>b,c</sup>

Values are expressed as the mean ± SEM. <sup>a</sup>  $p < 0.05$  as compared to naive, <sup>b</sup>  $p < 0.05$  as compared to control, <sup>c</sup>  $p < 0.05$  as compared to Alp (0.25 mg/kg). (One-way ANOVA followed by Tukey's test)

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entries and duration in mirror chamber) (Tab. 1) and reduced tail flick latency (Fig. 2). The effects were significant as compared to control (stressed) ( $p < 0.05$ ).

### Biochemical estimations

Six-hour acute immobilization stress significantly increased malondialdehyde, nitric oxide levels (as indicated by a rise in whole brain nitrite level), depleted reduced glutathione levels and catalase activity as compared to naive animals ( $p < 0.05$ ). Pretreatment with alprazolam (0.25 and 0.50 mg/kg *ip*) significantly attenuated malondialdehyde level, nitrite activity and restored depleted reduced glutathione and catalytic activity as compared to control ( $p < 0.05$ ) (Tab. 2).

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## Discussion

Stressful life events contribute to the development of many neurodegenerative and neuropsychiatric disorders including depression and anxiety. However, their relationship with stress is still controversial. Moreover, neither neurobiological basis of anxiety nor the mechanisms responsible for neurochemical regulation by stressful stimuli are well understood. Many of these effects are thought to be mediated by stress-induced neurochemical and hormonal abnormalities that are often associated with oxidative stress. The stress response in both humans and animals has been shown to involve a cascade of biological events initiated by corticotropin releasing factor (CRF). CRF is the primary physiologic regulator of the hypothalamic-pituitary-adrenal (HPA) axis and serves to globally coordinate the mammalian stress response. Stressful stimuli increase the release of corticotropin releasing factor from hypothalamic paraventricular nucleus, causing the secretion of ACTH from the anterior pituitary, which in turn stimulates the secretion of corticosterone from the adrenal cortex [35]. Besides, the body's principal physiological responses to stress stimuli are mediated by the sympathoadrenal system, functionally separable into the sympathetic nervous system and the adrenal medulla and the HPA axis. Acute activation of sympathoadrenal system gives rise to increased production of epinephrine and nor-

epinephrine by the adrenal medulla. It is generally well-accepted that stress induces a wide range of biochemical and behavior changes [33]. Accumulating data also demonstrated that stress causes several neurobehavioral and neuropsychiatric deficits such as anxiety and motor activity [7]. Individuals exposed to stressful conditions have an increase in pain threshold, known as stress-induced analgesia, which may present a different neurochemical basis related to stress severity. A large body of evidence also favor a nonopioid mediation of stress-induced analgesia [1, 2]. A single prolonged acute stress enhances hypothalamic-pituitary-adrenal negative feedback. In the present study, 6-h immobilization caused impairment of locomotor activity, anxiety and antinociception in animals. Acute stress has been reported to influence significantly motor activity, anxiety and antinociceptive effect [28, 38, 41]. Hyperactivity of central nervous system has also been strongly implicated in the pathophysiology of anxiety. Immobilization stress has also been reported to induce 2–3-fold rise of plasma cortisol level [8]. Increased cortisol level has been linked with anxiety-like behavior and painful response in humans [4, 17]. Oxidative stress has been implicated in the pathophysiology of many neurological disorders. Experiments indicate the existence of an association between stress and disease in which reactive oxygen species are involved [23]. Stress may also cause the formation of oxidants and induce oxidative change to lipids, resulting in alterations in membrane functions, protein damage, reduction in intracellular antioxidant defense in different areas of the brains. Stress stimulates numerous pathways leading to increased production of oxidants. Oxidative stress can cause cellular damage and neurodegeneration by inducing the reactive oxygen species (ROS) that oxidize vital cellular components, such as lipids, proteins and DNA. In the present study, 6-h immobilization caused significant oxidative damage in animals brain as indicated by raised lipid peroxidation, nitrite. Nitric oxide (NO) is a multifunctional messenger in many vertebrates. NO plays an important but controversial role in injury produced by immobilization stress. Many studies have measured stress-induced lipid peroxidation in different tissues [24]. Liu et al. [24] also reported a significant increase in lipid peroxidation in the hippocampus, midbrain, cerebral cortex and cerebellum. Gupta and Hasan [16] suggested that restraint stress of 24-h increased lipid peroxidation in aged brains. If it is true that oxygen radicals form abun-

dantly during stress, then changes in other antioxidative systems could be expected. The glutathione system is the most important antioxidant system. Liu and colleagues [23, 24] reported that long-term immobilization of rats activated GSH peroxidase and glutathione transferase, two key antioxidant enzymes in the glutathione cycle; and that depletion of GSH exacerbated oxidative damage. In the present study, 6-h immobilization stress induced oxidative damage as indicated by increased lipid peroxidation, nitrite activity and depleted reduced glutathione and catalase activity in stressed brains. Antioxidant defense mechanisms include removal of oxygen, scavenging of reactive oxygen/nitrogen species or their precursors, inhibition of ROS formation, binding of metal ions needed for the catalysis of ROS generation and up-regulation of endogenous antioxidant defenses [39]. Stress has been known to increase the MDA levels and decrease the reduced glutathione activity [31, 32, 34, 35]. GABA a major inhibitory neurotransmitter has been implicated in stress and related disorder [34, 45]. The anxiogenic behavior found at the elevated plus maze (EPM) has been related to the reduced levels of BDZ receptor levels in specific brain areas [13]. It has also been seen that GABAergic system influences significantly in stress; particularly the benzodiazepine receptor binding [3, 22, 29, 30, 44]. In the present study, pretreatment with alprazolam, a positive GABAergic modulator improved impaired locomotor activity, had antianxiety-like effect and reduced pain threshold, suggesting the possible involvement of GABAergic mechanism in stressed conditions. Besides, alprazolam has also been reported to mark inhibitory effect on both spontaneous and stimulated HPA axis activity [14]. Stress activates HPA axis and influences several biological effects at both central and peripheral level. Further pretreatment with alprazolam, a positive GABAergic modulator significantly attenuated immobilization-induced oxidative damage, suggesting role of alprazolam in stress conditions. However, it remains to be elucidated whether GABAergic mechanism is involved in its protective effect against immobilization stress.

In summary, the present study has shown that alprazolam is effective in ameliorating immobilization stress-induced behavioral alterations and oxidative stress. The present findings further support the therapeutic potential of alprazolam in the treatment of stress-related disorders.

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