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Differential Effect of Diazepam in Unstressed and Stressed Mice

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ABSTRACT

Aim: Investigation of antidepressants like effect of diazepam in unstressed and stressed mice.
Materials and methods: Male Swiss albino mice were used in the present study. Mice were stressed by immobilization for 2h. Mice subjected to immobilization were considered as stressed mice and mice not subjected to immobilization were considered as unstressed mice. Depression like behavioral alterations in unstressed and stressed mice was measured by tail suspension test (TST) followed by forced swim test (FST).
Results: The present study showed that the immobilization stress of 2h significantly enhanced the immobility period of mice in both TST and FST. Fluoxetine (FLX) (20 mg/kg, i.p.) significantly reduced the immobility period of both unstressed and stressed mice significantly as compared to their respective controls. Diazepam (DZP) (2 mg/kg, i.p.) significantly increased the immobility period of the unstressed mice whereas significantly reduced the immobility period of stressed mice in both TST and FST as compared to their respective controls. Administration of DZP (2 mg/kg, i.p.) and FLX (20 mg/kg, i.p.) to the unstressed mice reduced the immobility period of unstressed mice in both TST and FST significantly as compared to the vehicle and DZP (2 mg/kg, i.p.) treated unstressed mice in TST. The co-administration of DZP (2 mg/kg, i.p.) and FLX (20 mg/kg, i.p.) after the immobilization of 2h significantly reduced the immobility period of stressed mice significantly as compared to the vehicle treated stressed mice in both TST and FST.
Conclusion: It is concluded that the administration of DZP (2 mg/kg, i.p.) to the stressed mice reduced the immobility period of stressed mice in both TST and FST. Thus DZP (2 mg/kg, i.p.) exerted the antidepressants like effect in the stressed mice.

INTRODUCTION

GABA is the major inhibitory neurotransmitter present in the brain; synthesized by the enzyme glutamic acid decarboxylase (GAD) (Chen *et al*., 2003). Psychological stress has been shown to modulate the GABAergic neurotransmission in the brain (Hasler *et al*., 2010). The reduced concentrations of GABA had been reported in the plasma, cerebrospinal fluid (CSF), and cortex of the depressed subjects (Sanacora *et al*., 2004; 2006). Thus GABAergic dysfunction in the brain has been found to be responsible for the pathogenesis of depression (Sanacora *et al*., 1999). GABA exerts its effect by the modulation of GABA receptors. $GABA_A$ receptor is the principal inhibitory neurotransmitter receptor in the mammalian brain (Kleingoor *et al*., 1993) and the altered expression or function of these receptors has been implicated in the etiology of anxiety and depressive disorders (Merali *et al*., 2004; Sanacora *et al*., 2004; Bhagwagar *et al*., 2008; Poulter *et al*., 2008; Sequeira *et al*., 2009; Craddock *et al*., 2010; Klempan *et al*., 2009; Levinson *et al*., 2010). Previous studies suggested that the dysregulation of the $GABA_A$ receptor plays key role in the pathogenesis of mood disorders (Hasler *et al*., 2007). Stimulation of $GABA_A$ receptor enhances the release of noradrenaline in ventral NA pathway suggesting that the increasing GABAergic tone might exerts antidepressant effect (Lloyd *et al*., 1989). BZs act as $GABA_A$ receptor agonists (Petty *et al*., 1995) and potentiate the response to submaximal concentrations of GABA (Walters *et al*., 2000). Also the chronic administration of antidepressant drugs induces marked changes in GABAergic function (Sanacora and Saricicek, 2007).

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For e.g. oral administration of fluoxetine (5 mg/kg) for 21 days elevated the CSF levels of GABA (Goren *et al*., 2007); also it has been reported that the chronic treatment with fluoxetine normalizes GABA release in mice (Begenisic *et al*., 2014). Therefore the antidepressants with serotonergic effects known to enhance the function of $GABA_A$ receptor (Matsubara *et al*., 2000) e.g. fluoxetine increase the brain and CSF content of allopregnanolone (Allo), a potent positive allosteric modulator of $GABA_A$ receptors (Pinna *et al*., 2006). Therefore the present study was designed to investigate the effect of the diazepam in unstressed and stressed conditions.

MATERIALS AND METHODS

Animals

Male Swiss albino mice were used in the present study. All the mice were kept under controlled conditions of light and environmental and had free access to food and water. The testing was carried out between 9:00 and 16:00 h. The study protocols were approved by Institutional Animal Ethics Committee (IAEC) and care of the animals was carried out in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Ministry of Environment, Forests and Climate change, Government of India.

Drugs and selection of doses

Fluoxetine (FLX) (Cadila Pharmaceuticals, Ahmedabad, India) and Diazepam (DZP) (Neon Laboratories, Thane, India), were used in the present study. The doses were selected on the basis of the relevant previous studies. FLX (20 mg/kg; i.p.) was used as standard antidepressant drug (Walia, 2016a; Walia and Gilhotra, 2016) and DZP (2 mg/kg; i.p.), acts as a $GABA_A$ agonist and DZP (2 mg/kg; i.p.) has been found to increase the levels of the GABA in the brain of mice (Gilhotra and Dhingra, 2011).

Immobilization stress

Stress was produced by immobilizing the mice for 2h by taping, all its four limbs and trunk against a wooden board (Walia, 2016a; Walia and Gilhotra, 2016).

Assessment of depression like behavior in mice

Tail suspension test (TST)

In TST, each mouse was individually suspended at a height of 30 cm from the floor, by adhesive tape placed approximately 1 cm from the tip of the tail. The immobility period was recorded for 6 min. Mouse was considered to be immobile when it did not show any body movement, hung passively and completely immobile (Steru *et al*., 1985).

Forced swim test (FST)

In FST, each mouse was individually forced to swim in the open glass chamber containing fresh water to a height of 15 cm and maintained at 26±1°C. Each mouse shows vigorous movements during the initial 2 min period of the test. The immobility period was recorded during the next 4 min of the total 6 min testing period (Porsolt *et al*., 1977).

Experimental protocol

Male Swiss albino mice were used in the present study (Anki and Walia, 2016). Stress was produced by immobilizing the mice for 2h (Walia, 2016a; Walia and Gilhotra, 2016). Mice subjected to stress were considered as stressed mice and mice not subjected to stress were considered as unstressed mice. All the treatments were administered intraperitoneally (i.p.) in a fixed volume of 10 ml/kg. Unstressed mice were administered 30 min before the testing, whereas the stressed mice were administered, immediately after the mice were freed from the immobilization, and after the 30 min of administration, stressed mice were subjected to the behavioral testing procedure. In case where the combinations of drugs were used, the time elapsed between the two treatments was 10 min. Behavioral testing was performed in stepwise manner i.e. TST followed by FST with 5 min difference between the two testing procedures (Walia, 2016a; Walia and Gilhotra, 2016).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. Values were expressed as Mean ± S.E.M. and $p < 0.05$ was considered as statistically significant.

RESULTS

Effect of different treatment on the immobility period of mice in TST and FST

The effect of different treatments on the immobility period of mice in both TST and FST was shown in the fig. 1 and fig. 2. The present study showed that the immobilization stress of 2h enhanced the immobility period of mice significantly as compared to the vehicle treated unstressed mice in both TST and FST. Thus immobilization stress significantly induced depression or enhances the depression related behavior in the mice subjected to the immobilization of 2h. FLX (20 mg/kg, i.p.) significantly reduced the immobility period in both unstressed and stressed mice significantly as compared to their respective controls. Administration of DZP (2 mg/kg, i.p.) to the unstressed mice increased the immobility period significantly as compared to the vehicle treated unstressed mice in both TST and FST. Administration of DZP (2 mg/kg, i.p.) to the stressed mice significantly reduced the immobility period significantly as compared to vehicle treated stressed mice in both TST and FST. The combine treatment of DZP (2 mg/kg, i.p.) and FLX (20 mg/kg, i.p.) to the unstressed mice significantly reduced the immobility period as compared to the vehicle treated and DZP (2 mg/kg, i.p.) unstressed mice in both TST and FST. Administration of DZP (2 mg/kg, i.p.) and FLX (20 mg/kg, i.p.) to the stressed mice reduced the immobility period of stressed mice significantly as compared to the vehicle treated stressed mice in both TST and FST.

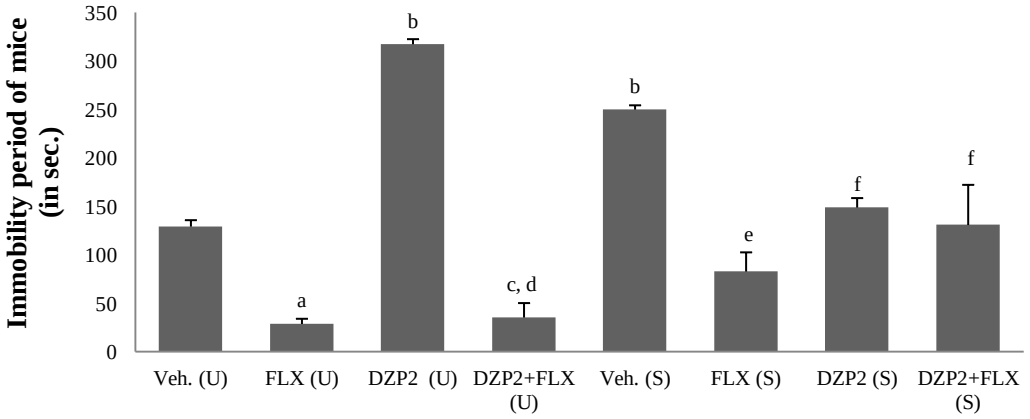

Fig. 1: Effect of different treatments on immobility period of mice (in sec.) in TST (n = 5 in each group). Values expressed as the mean ± SEM. Data were analyzed by ANOVA followed by Tukey's *post-hoc* test, F (7, 32) = 31.654. a=p<0.01 significant difference from the vehicle treated unstressed mice; b=p<0.001 significant difference from the vehicle treated unstressed mice; c=p<0.05 significant difference from the vehicle treated unstressed mice; d=p<0.001 significant difference from the diazepam treated unstressed mice; e=p<0.001 significant difference from the vehicle treated stressed mice; f=p<0.01 significant difference from the vehicle treated stressed mice. **Veh. (U):** Vehicle treated unstressed mice; **Veh. (S):** Vehicle treated stressed mice; **FLX (U):** Fluoxetine (20 mg/kg, i.p.) treated unstressed mice; **FLX (S):** Fluoxetine (20 mg/kg, i.p.) treated stressed mice; **DZP2 (U):** Diazepam (2 mg/kg, i.p.) treated unstressed mice; **DZP2 (S):** Diazepam (2 mg/kg, i.p.) treated unstressed mice; **DZP2 + FLX (U):** Diazepam (2 mg/kg, i.p.) and fluoxetine (20 mg/kg, i.p.) treated unstressed mice; **DZP2 + FLX (S):** Diazepam (2 mg/kg, i.p.) and fluoxetine (20 mg/kg, i.p.) treated stressed mice. Doses were mentioned in mg/kg.

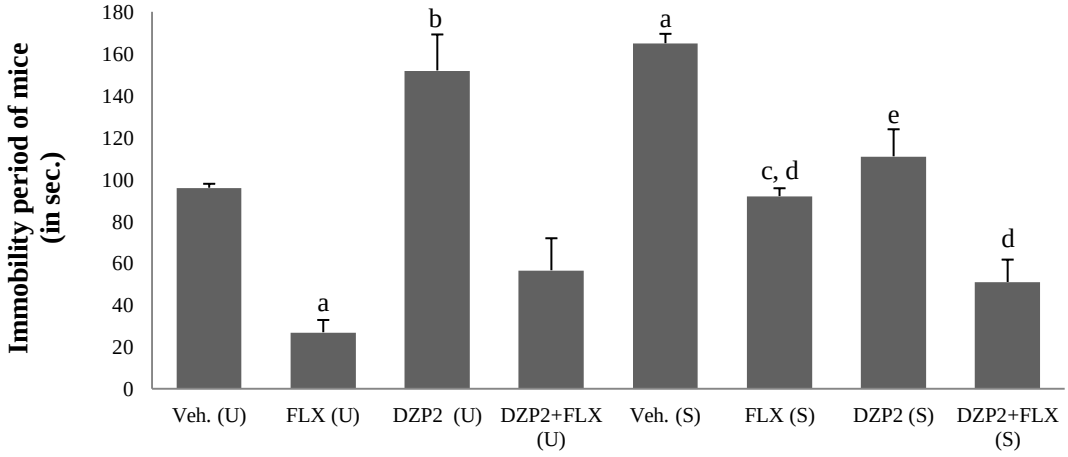

Fig.2: Effect of different treatments on immobility period of mice (in sec.) in FST (n = 5 in each group). Values expressed as the mean ± SEM. Data were analyzed by ANOVA followed by Tukey's *post-hoc* test, F (7, 32) = 21.420. a=p<0.01 significant difference from the vehicle treated unstressed mice; b=p<0.05 significant difference from the vehicle treated unstressed mice; c=p<0.01 significant difference from the fluoxetine treated unstressed mice; d=p<0.001 significant difference from the vehicle treated stressed mice; e=p<0.05 significant difference from the vehicle treated stressed mice. **Veh. (U):** Vehicle treated unstressed mice; **Veh. (S):** Vehicle treated stressed mice; **FLX (U):** Fluoxetine (20 mg/kg, i.p.) treated unstressed mice; **FLX (S):** Fluoxetine (20 mg/kg, i.p.) treated stressed mice; **DZP2 (U):** Diazepam (2 mg/kg, i.p.) treated unstressed mice; **DZP2 (S):** Diazepam (2 mg/kg, i.p.) treated unstressed mice; **DZP2 + FLX (U):** Diazepam (2 mg/kg, i.p.) and fluoxetine (20 mg/kg, i.p.) treated unstressed mice; **DZP2 + FLX (S):** Diazepam (2 mg/kg, i.p.) and fluoxetine (20 mg/kg, i.p.) treated stressed mice. Doses were mentioned in mg/kg.

DISCUSSION

Depression is a psychiatric disorder characterized by the feeling of hopelessness, helplessness, altered thoughts, suicidality, sleep disturbances etc. It has been suggested that depression may occur due to the alteration in the various pathways linked with the synthesis and the metabolism of the neurotransmitters implicated in the pathogenesis of depression (Walia, 2016b). Depression related behavior can be measured by the most commonly used behavioral test TST and FST (Steru *et al*., 1985; Porsolt *et al*., 1977). Stress induces depression like behavioral alteration in the laboratory animals subjected to the stress (Hyase, 2011). Present

study showed that the immobilization stress of 2h enhanced the immobility period of the mice in both TST and FST significantly as compared to the vehicle treated unstressed mice. Also we previously proved that the immobilization stress of 2h enhanced the depression related behavior of the mice (Walia, 2016; Walia and Gilhotra, 2016). Previous studies suggested that the GABAergic transmission is highly sensitive to stressful situations (Orchinik *et al*., 2001; Caldji *et al*., 2004; Maggio and Segal, 2009; Surget *et al*., 2008) and the reduction in the levels of GABA had been found to be associated with depression (Gold *et al*, 1980; Price *et al*., 2009; Lener *et al*., 2016). Therefore the dysfunctioning of the GABAergic system contributes to depression (Sanacora *et al*., 1999). Therefore the immobilization stress of 2h might results in the alterations in the neurotransmission of GABA that might be responsible for the enhancement of depression related behavior in the stressed mice.

Fluoxetine is widely used in the treatment of the mood disorders (Caiati and Cherubini, 2013). It has been reported that the administration of fluoxetine (5 mg/kg) for 21 days elevated the CSF GABA levels by approximately 2-fold (Goren *et al*., 2007). Fluoxetine also acts as a positive allosteric modulator of $GABA_A$-receptor (Robinson *et al*., 2003). Administration of fluoxetine (20 mg/kg; i.p.) reduced the immobility period of unstressed and stressed mice in both TST and FST significantly as compared to their respective controls. Also the immobility period of fluoxetine (20 mg/kg; i.p.) treated stressed mice was greater than the fluoxetine (20 mg/kg; i.p.) treated unstressed mice in both TST and FST. Thus the immobilization stress of 2h compromised the antidepressant effect of the fluoxetine (20 mg/kg; i.p.) in the stressed mice. Thus the results of the present study were in the agreement with our previous findings (Walia, 2016; Walia and Gilhotra, 2016).

GABAergic dysfunction contributes to depression therefore the agents that modulates the GABAergic pathways positively might exerts antidepressants like effect. Therefore we administered the unstressed and stressed mice with the DZP (2 mg/kg; i.p.). Administration of DZP (2 mg/kg; i.p.) to the unstressed mice increased the immobility of the mice significantly as compared to the vehicle treated unstressed mice in both TST and FST. However administration of DZP (2 mg/kg; i.p.) in stressed mice reduced the immobility period of stressed mice in both TST and FST significantly as compared to the vehicle treated stressed mice as shown in fig.1 and 2. Thus the results of the present study suggested that the DZP (2 mg/kg; i.p.) exerted antidepressants like effect in the stressed mice in both TST and FST.

Diazepam belongs to the category of BZs (Eghbali *et al*., 1997) and acts as a positive allosteric modulator of $GABA_A$ receptor; upon binding $GABA_A$ complex undergoes a conformational change, resulting in increased affinity of the receptor for the endogenous GABA ligand and this results in the increase in the neuronal chloride-ion influx resulting in the hyperpolarization of the postsynaptic membranes (Nutt and Malizia, 2001). Diazepam enhances the binding of GABA to its receptors (Skerritt and Macdonald, 1984) and increased the frequency of GABA receptor currents with minimal effect on the duration of bursts (Twyman *et al*., 1989). Stress evokes the release of glutamate in the brain that results in the activation of NMDA receptors responsible for excitotoxicity (Maj *et al*., 1992) and the diazepam through its GABA facilitatory action may exerts the antidepressant effect. This might be the possible reason why diazepam reduced the immobility period of stressed mice.

It has been reported that the co-administration of BZs with SSRIs led to greater treatment response, as well as faster onset of efficacy (Fava *et al*., 2006; Fava *et al*., 2011). In the present study, administration of DZP (2 mg/kg; i.p.) followed by the administration of fluoxetine (20 mg/kg; i.p.) to the unstressed mice reduced the immobility period of unstressed mice significantly as compared to the vehicle treated unstressed mice in both TST and FST.

However the administration of DZP (2 mg/kg; i.p.) followed by the administration of fluoxetine (20 mg/kg; i.p.) in stressed mice reduced the immobility period of mice significantly as compared to vehicle treated stressed mice in both TST and FST. However the combine administration of DZP (2 mg/kg; i.p.) and fluoxetine (20 mg/kg; i.p.) in the unstressed and stressed mice did not significantly modulates the antidepressant effect of fluoxetine (20 mg/kg; i.p.). However the clinical study reported that the adult outpatients treated with fluoxetine 20 mg + clonazepam 0.5-1.0 mg showed the accelerated the response of treatment, decreasing anxiety, sleep disturbances and suppressed the SSRIs side-effects (Londborg *et al*., 2000).

SSRIs are reported to increase the risk of the suicidal ideations and attempts in the patients prescribed with SSRIs (Walia, 2016c) and the main reason behind this is the development of the akathisia; that occurs frequently with the SSRIs treatment (Healy *et al*., 2006). Therefore to minimize the incidence of akathisisa, agitation and anxiety, benzodiazepines had been co-prescribed with SSRIs (Healy, 1999).

CONCLUSION

Stress induces depression related behavior in the organisms exposed to the stress. It has been reported that the immobilization stress of 2h compromised the antidepressant effect of the fluoxetine in the stressed mice. Further the SSRIs have been found to increase the emergence of suicidal behaviors in the prescribed subjects. BZs are effective in the treatment of the stress induced psychiatric disorders. Both SSRIs and BZs increase the levels of GABA in the brain and are generally used in the combinations. However in the present study the co-administration of the diazepam and fluoxetine did not significantly modulate the antidepressant effect of the fluoxetine in the unstressed and the stressed mice.

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