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GABAergic Influence in the Antidepressant Effect of Fluoxetine in Unstressed and Stressed Mice

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ABSTRACT

Objective: To determine the influence of GABA in the antidepressant effect of fluoxetine 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 and 4 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. The combine treatment 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.) before 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; whereas significantly as compared to the FLX (20 mg/kg, i.p.) treated stressed mice in TST only.
Conclusion: It has been concluded that the GABAergic influence is involved in the compromised antidepressant effect of the fluoxetine in stressed mice.

INTRODUCTION

Stress is a stimulus that disturbs the homeostasis of body and increases the vulnerability to mood disorders (Kendler *et al.,* 1999; McEwen, 2003; Lupien *et al.,* 2009). GABAergic transmission is highly sensitive to stressful situations (Orchinik *et al.,* 2001; Caldji *et al.,* 2004; Maggio and Segal, 2009; Surget *et al.,* 2008); the fact is supported by the fact that the psychological stress induces presynaptic down-regulation of prefrontal GABAergic neurotransmission (Hasler *et al.,* 2010).

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GABA is the major inhibitory neurotransmitter present in the brain; synthesized by glutamic acid decarboxylase (GAD) (Chen *et al.,* 2003). GABAergic dysfunction has been found to be responsible for depression because abnormally low cortical concentrations of GABA had been reported in the brains of depressed patients (Sanacora *et al.,* 1999); also the concentrations of GABA had been found to be reduced in the plasma, cerebrospinal fluid (CSF), and cortex of depressed subjects (Sanacora *et al.,* 2006).

The depressed subjects had significantly lower occipital cortex GABA concentrations compared with healthy controls (Sanacora *et al.,* 2004). It has been reported that the chronic administration of antidepressant drugs induces marked changes in GABAergic function (Sanacora and Saricicek, 2007).

For e.g. oral administration of fluoxetine (5 mg/kg) for 21 days elevated the CSF GABA levels by approximately 2-fold ($P < 0.05$) (Gören *et al.,* 2007); also the chronic treatment with fluoxetine administered in drinking water normalizes GABA release in mice (Begenisic *et al.,* 2014). SSRIs treatment known to increase the cortical GABA concentration in the depressed patients and therefore SSRIs act in part to restore the disrupted GABAergic activity (Licata *et al.,* 2014).

$GABA_A$ receptor is the principal inhibitory neurotransmitter receptor in the mammalian brain (Kleingoor *et al.,* 1993) and the altered expression or function of these receptors is increasingly 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).

$GABA_A$ receptor stimulation enhances the noradrenaline release in ventral NA pathway suggesting that the increasing GABAergic tone exerts antidepressant effect (Lloyd *et al.,* Zivkovic, 1989). A possible role of $GABA_A$ receptor dysregulation in mood disorders has been controversial in part due to lack of a consensus about whether benzodiazepines (BZs) are therapeutically effective for the treatment of depression (Hasler *et al.,* 2007). BZs, are the most widely prescribed psychotropic drugs, often used in the patients of depressive disorders, either alone or in combination with the standard antidepressants. BZs act as $GABA_A$ receptor agonists (Petty *et al.,* 1995).

BZs act on $GABA_A$ receptors (Walters *et al.,* 2000), but the binding of BZs agonist does not directly activate the $GABA_A$ receptor but potentiates the response to submaximal concentrations of GABA (Walters *et al.,* 2000). Antidepressants with serotonergic effects known to enhance the function of the $GABA_A$ 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).

We previously reported that the antidepressant effect of fluoxetine gets attenuated in the stressed mice and the immobilization stress of 2h compromised the antidepressant effect of fluoxetine in the stressed mice (Walia, 2016a). Also we reported previously that 2h immobilization significantly increase the levels of the nitric oxide (NO) in the brain of mice (Walia and Gilhotra, 2016). NO is known to influence both the synthesis storage and release of 5-HT (Walia, 2016b; Sezal and Walia, 2015). Besides 5-HT, NO also influence the levels and the function of GABA, and the increase levels of NO has been found to be responsible for the downregulation of $GABA_A$ receptors (Gilhotra and Dhingra, 2011).

Thus it has been suggested that the stress induce NO release modulate the levels of 5-HT and GABA, that might be further responsible for altered antidepressant effect of the agents that modulate the levels and the neurotransmission of these neurotransmitters. Therefore the aim of the present study is to determine the possible GABAergic influence in the antidepressant activity of fluoxetine in stressed mice.

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 (Reg. No. 134/99/CPCSEA).

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 2hrs 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. 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 fixed volume of 10 ml/kg. Unstressed mice were administered 30 min prior to testing whereas the stressed mice were administered immediately before subjecting them to immobilization. In case of the pre-treatment or where the combinations of drugs were used, the time elapsed between the two treatments was 10 min. Behavioral testing was started 10 min after setting the animal free from immobilization. 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

Summary of the 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 significantly enhanced the immobility period of mice as compared to the vehicle treated unstressed mice in both TST and FST. Thus it is suggested that 2h immobilization stress significantly induced depression or enhances the depression in the mice subjected to 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 and 4 mg/kg, i.p.) to the unstressed mice significantly increased the immobility period as compared to vehicle treated unstressed mice. However the administration of DZP (2 and 4 mg/kg, i.p.) before subjecting the mice to the immobilization of 2h significantly reduced the immobility period 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 TST and also significantly reduced the immobility period as compared to the FLX (20 mg/kg, i.p.) treated stressed mice in FST only. The co-administration of DZP (2 mg/kg, i.p.) and FLX (20 mg/kg, i.p.) before the immobilization of 2h significantly reduced the immobility period as compared to the vehicle treated stressed mice in both TST and FST; whereas compare to the FLX (20 mg/kg, i.p.) treated stressed mice in FST only.

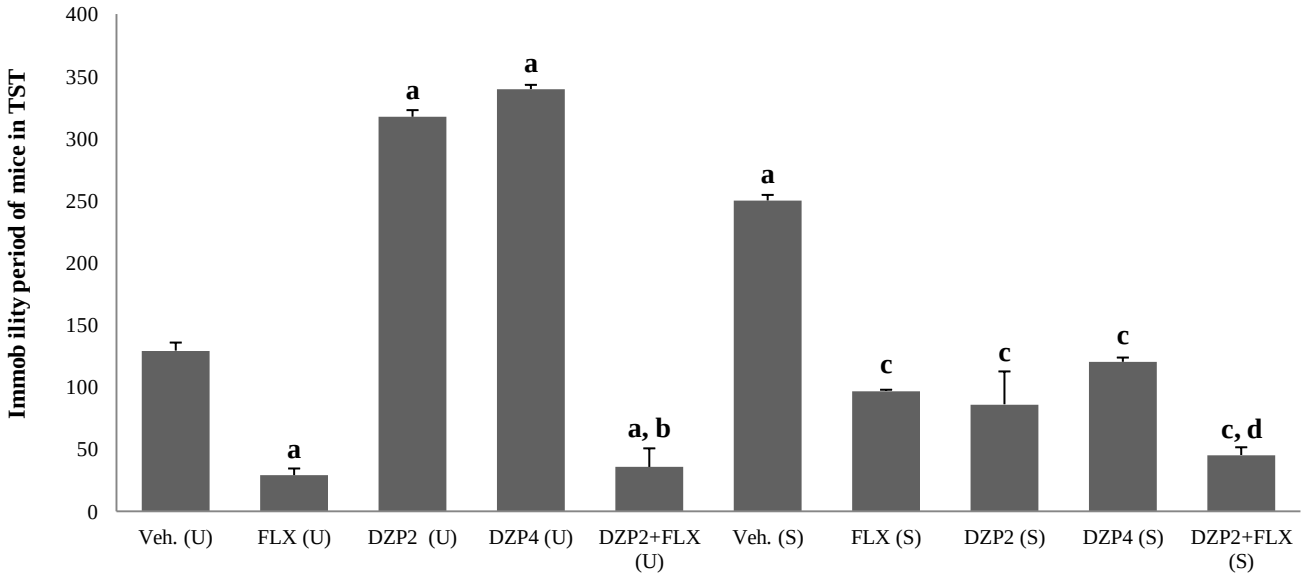

Fig.1. Effect of different treatments on immobility period of mice in TST. Values are expressed as mean±SEM, n=5 in each group. Data was analyzed by one way ANOVA followed by Tukey's Post hoc test, $F(9, 40) = 121.98$. a=p<0.001 significant difference from the vehicle treated unstressed mice; b=p<0.001 significant difference from the DZP (2 mg/kg, i.p.) treated unstressed mice; c=p<0.001 significant difference from the vehicle treated stressed mice; d=p<0.05 significant difference from the FLX (20 mg/kg, i.p.) 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; DZP4 (U): Diazepam (4 mg/kg, i.p.) treated unstressed mice; DZP2 (S): Diazepam (2 mg/kg, i.p.) treated stressed mice; DZP4 (S): Diazepam (4 mg/kg, i.p.) treated stressed 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.

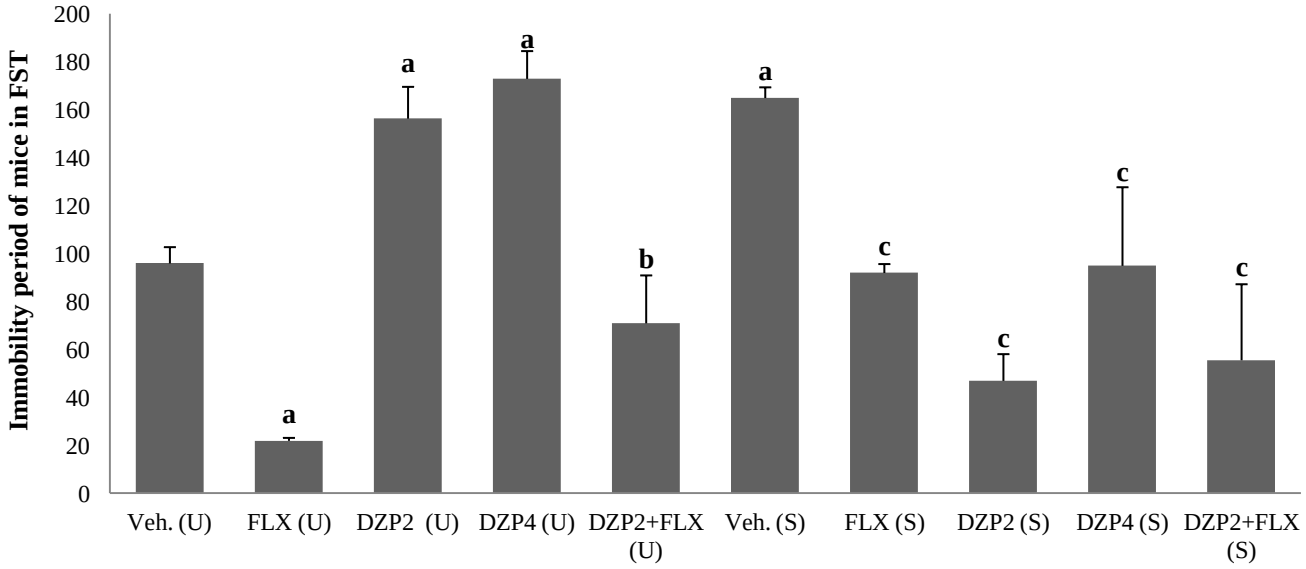

Fig.2. Effect of different treatments on immobility period of mice in FST. Values are expressed as mean±SEM, n=5 in each group. Data was analyzed by one way ANOVA followed by Tukey's Post hoc test, F (9, 40) = 34.355. a=p<0.001 significant difference from the vehicle treated unstressed mice; b=p<0.05 significant difference from the FLX (20 mg/kg, i.p.) treated unstressed mice; c=p<0.001 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; **DZP4 (U):** Diazepam (4 mg/kg, i.p.) treated unstressed mice; **DZP2 (S):** Diazepam (2 mg/kg, i.p.) treated stressed mice; **DZP4 (S):** Diazepam (4 mg/kg, i.p.) treated stressed 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

Stress induces depression like behavioral alteration in the laboratory animals subjected to the stress (Hyase, 2011). Depression like behavioral alteration in the laboratory animals can be measure by using TST and FST (Porsolt *et al.,* 1977; Steru *et al.,* 1985). In the present study we used TST followed by FST; the main reason behind is to reduce the use of number of animals. However we used TST followed by FST because after FST the mice wets completely and therefore cannot be used immediately in the TST. Also FST is a more stressful procedure than TST. FST is known to produce the hypothermia in the mice or the animals subjected to the FST. Therefore to avoid such problems we used TST followed by FST in the present study.

Depression like behavioral alteration can be produced by the stressors such as immobilization stress (Hyase, 2011). The results of the present study showed that the immobilization stress enhanced the immobility period of the mice in both TST and FST significantly as compared to the vehicle treated unstressed mice. Also the immobilization stress of 2h has been shown to increase the immobility period of the mice in the various previous studies (Walia, 2016; Walia and Gilhotra, 2016). GABAergic transmission is highly sensitive to stressful situations (Orchinik *et al.,* 2001; Caldji *et al.,* 2004; Maggio and Segal, 2009; Surget *et al.,* 2008). Also the altered levels of GABA had been reported in the patients of depression (Gold et al, 1980; Price *et al.,* 2009). Further the network dysfunction in association with altered brain levels of glutamate and GABA have been identified in both animal and human studies of depression (Lener *et al.,* 2016). Thus the dysfunction of the GABAergic system is associated with major depression (Sanacora *et al.,* 1999) and therefore the treatment with the GABAergic agents or GABA modulating drugs may exerts the beneficial effects (Birkenhäger *et al.,* 1995). For example in one study alprazolam has been shown to reduce the anhedonia (Petty *et al.,* 1995). In the present study the 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 antidepressant effect of the fluoxetine (20 mg/kg; i.p.) was compromised by the immobilization stress of 2h. Fluoxetine (Prozac), is an antidepressant drug that selectively inhibit the reuptake of 5-HT and is widely used in the treatment of the mood disorders (Caiati and Cherubini, 2013). Fluoxetine was ineffective in changing the CSF GABA levels at the dose of 2.5 mg/kg but produced a significant increase in the perfusates following injection of 5 mg/kg of fluoxetine. Also the oral fluoxetine administration (5 mg/kg) for 21 days also elevated the CSF GABA levels by approximately 2-fold (Goren *et al.,* 2007). Robinson et al show that fluoxetine acts as a positive allosteric modulator on $GABA_A$-receptor (Robinson *et al.,* 2003). Low fluoxetine concentrations (1 nM) enhanced GABA-stimulated Cl- uptake by a rat cerebral cortical vesicular preparation whereas the higher concentrations (100 microM and 1 mM), inhibited GABA-stimulated Cl^- uptake (Tunnicliff *et al.,* 1999). Chronic fluoxetine

can increase the intrinsic excitability in some interneurons (Zhong and Yan, 2011) and increase CSF GABA concentrations (Gören *et al.,* 2007) while at the same time reducing 5-HT effect on phasic GABA activation (Zhong and Yan, 2004; Zhong and Yan, 2011) it is unclear whether fluoxetine's GABA-enhancing effects are beneficial for mood. Antidepressants with serotonergic effects enhanced the function of $GABA_A$ receptor complex (Matsubara *et al.,* 2000). SSRIs cause an acute increase in brain GABA levels (Bhagwagar *et al.,* 2004; Sanacora *et al.,* 2002). SSRI treatment has a slight influence on GABAergic transmission in the hippocampus (Choi *et al.,* 2010). SSRIs treatment increases the cortical GABA levels in the depressed patients and suggest that this results from an action of SSRIs on GABA neurons rather than as a secondary consequence of mood improvement (Bhagwagar *et al.,* 2004). SSRIs thus act in part to restore the disrupted GABAergic activity (Licata *et al.,* 2014). Preclinical studies demonstrate that GABA modulating agents are active in commonly used rodent behavioral models of antidepressant activity, and that chronic administration of antidepressant drugs induces marked changes in GABAergic function (Sanacora and Saricicek, 2007).

If the GABAergic dysfunction contributes to depression therefore the agents that increase the levels of the GABA might exerts the antidepressants like effect. To determine the possible GABAergic influence in the compromised antidepressant effect of fluoxetine (20 mg/kg; i.p.) in stressed mice; we administered diazepam in unstressed and stressed mice alone and in combination with the fluoxetine to determine the possible beneficial outcomes. Administration of DZP (2 and 4 mg/kg; i.p.) in unstressed mice enhanced the immobility period of unstressed mice in a dose dependent manner in both TST and FST significantly as compared to the vehicle treated unstressed mice as shown in fig.1 and 2. However the administration of DZP (2 and 4 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.. However the immobility period of the stressed mice treated with DZP (4 mg/kg; i.p.) was greater than the immobility period of the stressed mice treated with DZP (2 mg/kg; i.p.). Diazepam belongs to the category of BZs, widely used clinically for relief of anxiety and for sedation (Eghbali *et al.,* 1997). Diazepam acts as the 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). The hyperpolarization further result in the subsequent inhibition of the firing threshold and result is CNS depression (Nutt and Malizia, 2001; Battistin *et al.,* 1984; Sieghart, 1995). Diazepam enhances the binding of GABA to its receptors (Skerritt and Macdonald, 1984). Also the diazepam 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. Co-administration of BZs with SSRIs can lead to significantly 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.) in unstressed mice increased the immobility period of the mice in both TST and FST significantly as compared to fluoxetine (20 mg/kg; i.p.) treated unstressed mice. 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 in both TST and FST significantly as compared to vehicle treated stressed mice. 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 in TST significantly as compared to the fluoxetine (20 mg/kg; i.p.) treated stressed mice. It has been reported that the adult outpatients treated with fluoxetine 20 mg + clonazepam 0.5-1.0 mg accelerated the response of treatment, decreasing anxiety, sleep disturbances and suppressed the SSRIs side-effects (Londborg *et al.,* 2000). Extended clonazepam cotherapy of fluoxetine appeared safe and effective for depressed outpatients: it was superior to fluoxetine alone early in treatment and again following fluoxetine dose increase. Cotherapy might be considered at the start of fluoxetine treatment, especially for those with insomnia, and when a dose increase of fluoxetine is anticipated (Smith *et al.,* 2002). SSRIs are reported to increase the risk of the suicidality (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) and it may be one of the main causes of suicidality associate with the SSRIs therapy (Rothschild and Locke, 1991). Therefore to minimize the incidence of akathisisa, agitation and anxiety, benzodiazepines had been co-prescribed with SSRIs (Healy, 1999).

CONCLUSION

It is concluded that stress is mainly responsible for the induction of psychiatric disorders such as depression. BZs are effective in the treatment of stress induced psychiatric disorders such as anxiety and depression. Both SSRIs and BZs have been found to increase the levels of GABA in the brain. However the use of BZs led to decline in attention/concentration, problem solving skills, general intelligence, psychomotor speed, memory etc. SSRIs are effective in the treatment of stress induced psychiatric disorders but the use of the SSRIs increased the risk of suicidality in the prescribed patients. SSRIs induced akathisia is mainly responsible for the emergence of suicidality. Therefore SSRIs are often co-prescribed with BZs. Also the present study showed the superiority of combine treatment of BZ and SSRI as compared to SSRI alone.

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