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Nitriergic Influence in the Compromised Antidepressant Effect of Fluoxetine in Stressed Mice

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ABSTRACT

Objective: Investigation of possible nitriergic mechanism involved in the compromised antidepressant effect of fluoxetine in stressed mice.
Materials and methods: Male swiss albino mice were used in the present study. Mice were stressed by immobilization of 2hrs. 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 and the depression like behavioral alterations in unstressed and stressed mice was measured by TST followed by FST. Nitrite levels were measured in brain homogenates to determine the possible involvement of nitriergic mechanism.
Results: Present study showed that the 2hrs immobilization significantly increased the immobility period of mice in both TST and FST, with the concurrent increase in the levels of nitrite in the brain of stressed mice as compared to the vehicle treated unstressed mice. Fluoxetine (FLX) (20 mg/kg, i.p.); pyrrolidine dithiocarbamate (PDTC) (100 mg/kg, i.p.) and methylene blue (MB) (100 mg/kg, i.p.) significantly reduced the immobility period of stressed mice in both TST and FST as compared to vehicle treated stressed mice. Pre-treatment with PDTC (100 mg/kg, i.p.) followed by the administration of FLX (20 mg/kg, i.p.) did not significantly alter the immobility period and nitrite levels as compared to the FLX (20 mg/kg, i.p.) treated stressed mice. Pre-treatment with MB (100 mg/kg, i.p.) followed by the administration of FLX (20 mg/kg, i.p.) did not significantly alter the immobility period of mice in TST, but significantly reduced the immobility period of mice in FST as compared to the FLX (20 mg/kg, i.p.) treated stressed mice. Also the pre-treatment with MB (100 mg/kg, i.p.) followed by the administration of FLX (20 mg/kg, i.p.) significantly reduced the nitrite levels as compared to the FLX (20 mg/kg, i.p.) treated stressed mice.
Conclusion: It has been concluded that the immobilization stress induced increase production of NO was involved in the compromised antidepressant effect of fluoxetine in stressed mice.

INTRODUCTION

Stress disturbs the physiological homeostasis of body (Bohus *et al.*, 1993) and precipitates mood disorders such as depression (McEwen, 2000). Stress plays a prominent role in the provocation of depression (Caspi *et al.*, 2003) and induces a state analogous to depression (Vollmayr and Henn, 2003). Stress also increase the expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in the brain of animals exposed to stress (Wichers and Maes, 2002) and IL-6 activates NMDA receptors (Maj *et al.*, 1992; Qiu *et al.*, 1995). Also the stress evokes the release of glutamate in the brain that results in the activation of NMDA receptors (Maj *et al.*, 1992); present in linked with the neuronal nitric oxide synthase (nNOS) (Cheah *et al.*, 2006). Therefore the activation of NMDA receptors is responsible for the increased expression of nNOS; responsible for the production of nitric oxide (NO) (Cheah *et al*., 2006). The cytokines such as IL-1 and TNF-α increases the expression of NF-κβ pathway that is responsible for the increase expression of inducible nitric oxide synthase (iNOS) (Cooke *et al.*, 2001; Hua *et al.*, 2008; Alexopoulos and Morimoto, 2011; Madrigal *et al.*, 2002); which once induced continues to produce the larger quantities of NO (Madrigal *et al.*, 2002).

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NF-κβ pathway activation also reduced the production of monoamines and neurotrophic factors in the brain (Wuwongse *et al.,* 2010). NO is a unique neurotransmitter, synthesized from L-arginine by nitric oxide synthases (NOS) (Garthwaite *et al.,* 1988) in two step process; in first step L-arginine is converted to N-hydoxy-L-arginine, and in the subsequent step N-hydoxy-L-arginine is converted into NO and citrulline (Walia *et al.,* 2014). NOS exist in three isoforms: eNOS, iNOS and nNOS with 51-57% homology between isoforms and different localizations, regulation, catalytic properties and inhibitor sensitivity (Walia *et al.,* 2014; Walia, 2016b).

NO modulates the extracellular levels of serotonin (5-HT) (Kaehler *et al.,* 1999). NO inactivate the rate limiting enzyme involved in the synthesis of 5-HT (Kuhn and Arthur, 1996). NO also known to influence release, reuptake and function of 5-HT (Straub *et al.,* 2007). 5-HT is responsible for the regulation of mood and behavior (Rajkumar and Mahesh, 2010) and therefore its deficiency and reduced transmission contribute to depression (Rang *et al.,* 2007).

In laboratory animal depression like behavioral alterations is caused by immobilization stress (Hayase, 2011; Sevgi *et al.,* 2006; Poleszak *et al.,* 2006). Immobilization stress of 2hrs has been shown to increase the immobility period of mice in both TST and FST (Poleszak *et al.,* 2006; Walia, 2016a). Immobilization of 6hrs increased the levels of nitrite in the rodents exposed to immobilization (Madrigal *et al.,* 2002, Gilhotra *et al.,* 2010; Gilhotra and Dhingra, 2011). Also we have previously described that 2hrs immobilization influence the antidepressant effect of fluoxetine in mice (Walia, 2016a). Therefore the present study was designed to determine the possible involvement of the nitriergic mechanism in the compromised antidepressant effect 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).

Drugs and selection of doses

Fluoxetine (FLX) (Cadila Pharmaceuticals, Ahmedabad India); Pyrrolidine dithiocarbamate (PDTC) (Loba chemie India) and methylene blue (MB) (Loba chemie 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 (Conti *et al.,* 2002; Walia, 2016a); MB (100 mg/kg, i.p.) (Klamer *et al.,* 2004) was used as an inhibitor of NOS and soluble guanylyl cyclase (sGC) (Savegnago *et al.,* 2008) and PDTC (100 mg/kg, i.p.) (Gilhotra *et al.,* 2010) was used as an inhibitor of NF-κβ pathway (Madrigal *et al.,* 2002).

Immobilization stress

Stress was produced by immobilizing the mice for 2hrs by taping, all its four limbs and trunk against a wooden board (Mazzon and Cuzzocrea, 2008; Walia, 2016a).

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 total period of immobility 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 duration of immobility was recorded during the next 4 min of the total 6 min testing period (Porsolt *et al.,* 1977).

Biochemical estimations

Nitrite estimation in brain homogenates

Nitrite estimation was carried out in the brain homogenates of mice. In brief, mice were sacrificed by decapitation; brains were removed, rinsed with isotonic saline and weighed. A 10% (w/v) tissue homogenate was prepared with 0.1 M phosphate buffer (pH 7.4). Equal volume of brain homogenate and Greiss reagent (0.1% of N-1-napththyl ethylenediamine dihydrochloride, 1% sulphanilamide and 2.5% o-phosphoric acid) were mixed, the mixture was incubated for 10 min at room temperature and absorbance was measured at 540 nm (Green *et al.,* 1982).

Experimental protocol

Male swiss albino mice were used in the present study. Stress was produced by immobilizing the mice for 2hrs (Mazzon and Cuzzocrea, 2008; Walia, 2016a). 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.) at a 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 (Gilhotra and Dhingra, 2011). 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). Separate

groups of mice were used for the estimation of nitrite levels in brain. Nitrite was estimated in the brain homogenates by the spectrophotometeric assay based on Greiss reaction (Green *et al.*, 1982). Nitrite concentration in supernatant was determined from nitrite standard curve and expressed in µM.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* 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

Effect of different treatments on the immobility period of unstressed and stressed mice in TST and FST was shown in the fig. 1 and fig. 2. In the present study immobilization stress of 2hrs significantly enhanced the immobility period of mice in both TST and FST as compared to the vehicle treated unstressed mice. Administration of FLX (20 mg/kg, i.p.) to the unstressed mice reduced the immobility period in both TST and FST significantly as compared to vehicle treated unstressed mice. Administration of FLX (20 mg/kg, i.p.) to the stressed mice reduced the immobility period in both TST and FST significantly as compared to vehicle treated stressed mice.

Also there was a significant difference in the immobility period of FLX (20 mg/kg, i.p.) treated unstressed mice and FLX (20 mg/kg, i.p.) treated stressed mice in both TST and FST. Administration of PDTC (100 mg/kg, i.p.) to stressed mice significantly reduced the immobility period in both TST and FST as compared to vehicle treated stressed mice. Pre-treatment with PDTC (100 mg/kg, i.p.) followed by the administration of FLX (20 mg/kg, i.p.) to the stressed mice significantly reduced the immobility period in TST as compared to vehicle treated stressed mice. However the pre-treatment with PDTC (100 mg/kg, i.p.) followed by the administration of FLX (20 mg/kg, i.p.) to the stressed mice did not significantly influence the immobility period of stressed mice in both TST and FST as compared to the FLX (20 mg/kg, i.p.) treated stressed mice. Also the combine treatment with PDTC (100 mg/kg, i.p.) and FLX (20 mg/kg, i.p.) to the stressed mice results in the death of all the 5 mice after TST. Administration of MB (100 mg/kg, i.p.) to the stressed mice reduced the immobility period in both TST and FST significantly as compared to vehicle treated stressed mice. Also the administration of MB (100 mg/kg, i.p.) to the stressed mice significantly reduced the immobility period in FST as compared to FLX (20 mg/kg, i.p.) treated stressed mice. Pre-treatment with MB (100 mg/kg, i.p.) followed by the administration of FLX (20 mg/kg, i.p.) reduced the immobility period in both TST and FST significantly as compared to vehicle treated stressed mice. However the pre-treatment with MB (100 mg/kg, i.p.) followed by the administration of FLX (20 mg/kg, i.p.) reduced the immobility period in FST only significantly as compared to FLX (20 mg/kg, i.p.) treated stressed mice.

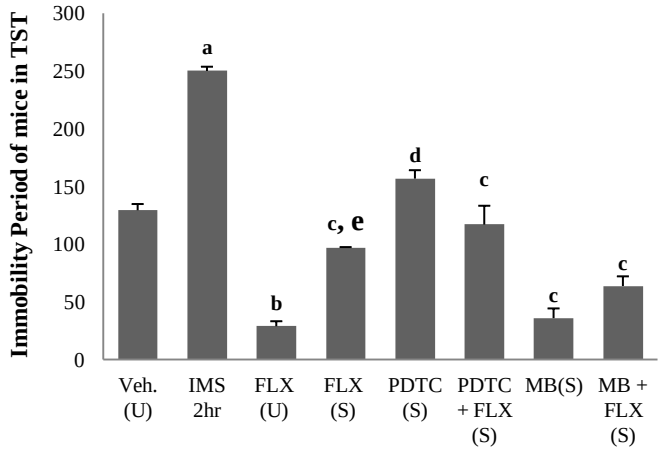

Fig.1. Effect of different treatments on immobility period of mice in TST. n = 5 in each group. Values expressed as the mean ± SEM. Data were analyzed by ANOVA followed by Tukey's *post-hoc* test, F (7, 32) = 54.458. a= $p<0.001$ significant difference from the vehicle treated unstressed mice; b= $p<0.01$ significant difference from the vehicle treated unstressed mice; c= $p<0.001$ significant difference from vehicle treated stressed mice; d= $p<0.05$ significant difference from vehicle treated stressed mice; e= $p<0.001$ significant difference from FLX treated unstressed mice. **Veh (U):** Vehicle treated unstressed mice; **IMS 2hr:** 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; **PDTC (S):** Pyrrolidine dithiocarbamate (100 mg/kg, i.p.) treated stressed mice; **PDTC + FLX (S):** Pyrrolidine dithiocarbamate (100 mg/kg, i.p.) and fluoxetine (20 mg/kg, i.p.) treated stressed mice; **MB (S):** Methylene blue (100 mg/kg, i.p.) treated stressed mice; **MB + FLX (S):** Methylene blue (100 mg/kg, i.p.) and fluoxetine (20 mg/kg, i.p.) treated stressed mice. Doses were mentioned in mg/kg.

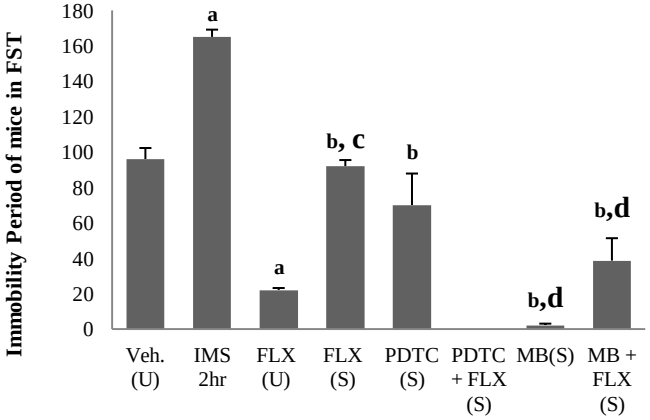

Fig. 2. Effect of different treatments on immobility period of mice in FST. n = 5 in each group. Values expressed as the mean ± SEM. Data were analyzed by ANOVA followed by Tukey's *post-hoc* test, F (6, 28) = 61.102. a= $p<0.001$ significant difference from the vehicle treated unstressed mice; b= $p<0.001$ significant difference from vehicle treated stressed mice; c= $p<0.001$ significant difference from FLX treated unstressed mice; d= $p<0.001$ significant difference from FLX treated stressed mice. **Veh (U):** Vehicle treated unstressed mice; **IMS 2hr:** 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; **PDTC (S):** Pyrrolidine dithiocarbamate (100 mg/kg, i.p.) treated stressed mice; **PDTC + FLX (S):** Pyrrolidine dithiocarbamate (100 mg/kg, i.p.) and fluoxetine (20 mg/kg, i.p.) treated stressed mice; **MB (S):** Methylene blue (100 mg/kg, i.p.) treated stressed mice; **MB + FLX (S):** Methylene blue (100 mg/kg, i.p.) and fluoxetine (20 mg/kg, i.p.) treated stressed mice. Doses were mentioned in mg/kg.

Effect of different treatment on Nitrite levels

Effect of different treatments on the nitrite levels in the brain of mice was shown in fig. 3. Immobilization stress of 2hrs enhanced the levels of nitrite in the brain of mice significantly as compared to the vehicle treated unstressed mice. Administration of FLX (20 mg/kg, i.p.) to unstressed mice reduced the nitrite levels significantly as compared to the vehicle treated unstressed mice. Administration of FLX (20 mg/kg, i.p.) to the stressed mice reduced the nitrite levels significantly as compared to the vehicle treated stressed mice. Pre-treatment with PDTC (100 mg/kg, i.p.) followed by the administration of FLX (20 mg/kg, i.p.) to stressed mice significantly reduced the nitrite levels as compare to the vehicle treated stressed mice. Pre-treatment with MB (100 mg/kg, i.p.) followed by the administration of FLX (20 mg/kg, i.p.) to the stressed mice significantly reduced the nitrite levels as compared to the vehicle and FLX (20 mg/kg, i.p.) treated stressed mice.

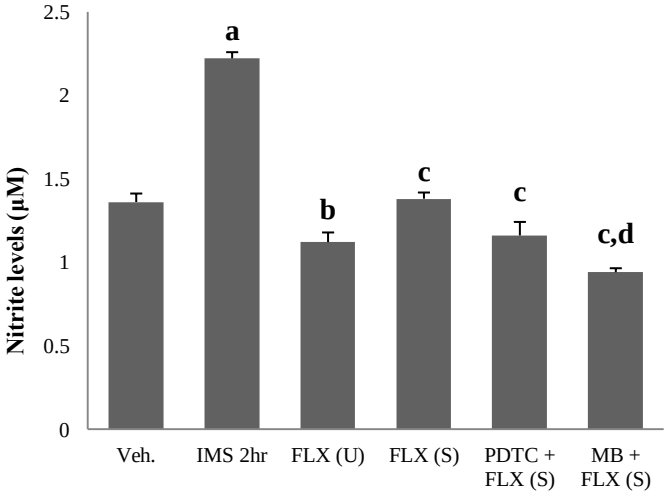

Fig.3. Effect of different treatments on nitrite levels in the brain homogenates of mice. n = 5 in each group. Values expressed as the mean ± SEM. Data were analyzed by ANOVA followed by Tukey's *post-hoc* test, F (5, 24) = 76.045. a= p<0.001 significant difference from the vehicle treated unstressed mice; b= p<0.05 significant difference from the vehicle treated unstressed mice; c= p<0.001 significant difference from the vehicle treated stressed mice; d= p<0.001 significant difference from the FLX treated stressed mice. **Veh (U):** Vehicle treated unstressed mice; **IMS 2hr:** 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; **PDTC (S):** Pyrrolidine dithiocarbamate (100 mg/kg, i.p.) treated stressed mice; **PDTC + FLX (S):** Pyrrolidine dithiocarbamate (100 mg/kg, i.p.) and fluoxetine (20 mg/kg, i.p.) treated stressed mice; **MB (S):** Methylene blue (100 mg/kg, i.p.) treated stressed mice; **MB + FLX (S):** Methylene blue (100 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 depressed mood, anhedonia, loss of energy, and low self esteem (Wong and Licinio, 2004). Stress produces alterations in neurotransmitters and neuroendocrine systems (McIntyre *et al.*, 1999; Konstandi *et al.*, 2000) and plays a key role in the pathogenesis of depression (Caspi *et al.*, 2003; Paykel, 2001). Exposure of rodents to uncontrollable stressors in unpredictable manner induces depressive behavior (Willner *et al.*, 1987). Stress-induced depression like behavioral alterations can be assessed by using TST and FST in rodents. Immobility period in TST and FST reflects the behavioral despair, a condition similar to depression in human (Cryan *et al.*, 2005a;b; Steru *et al.*, 1985; Willner, 1984). The present study showed that the exposure of mice to the immobilization stress of 2hrs significantly increased the immobility period of mice in both TST and FST as shown in fig. 1 and 2. Thus it is established that the immobilization of 2hrs enhanced the depression or produces acute depression in the mice exposed to immobilization stress of 2hrs. The similar findings had been reported previously also (Poleszak *et al.*, 2006; Walia 2016a). Stressors such as immobilization stress induced depression like behavioral alterations in the rodents (Hayase, 2011; Sevgi *et al.*, 2006; Poleszak *et al.*, 2006). Immobilization stress of 6hrs increased the levels of nitrite in the rodents exposed to immobilization (Madrigal *et al.*, 2002, Gilhotra *et al.*, 2010; Gilhotra and Dhingra, 2011). The present study showed that the immobilization of 2hrs significantly increased the nitrite levels in the brain of the mice exposed to the stressed as compare to the unstressed mice. It is therefore suggested that the 2hrs immobilization stress induced the production of NO in the brain of the mice exposed to the immobilization and the increased production of the NO may be responsible enhancement of depression like behavioral alterations in mice. Fluoxetine, a standard antidepressant drug; belongs to the category of SSRIs and reduces the immobility period of mice in both TST and FST (Walia, 2016a; Cryan *et al.*, 2005a; 2005b). Also in the present study, FLX (20 mg/kg, i.p.) reduced the immobility period of both unstressed and stressed mice significantly as compared to their respective controls as shown in fig.1 and 2. FLX (20 mg/kg, i.p.) reduced the nitrite levels significantly both in the unstressed and stressed mice with respect to their respective control as shown in fig. 3. However the immobility period of FLX (20 mg/kg, i.p.) stressed mice is significantly higher as compared to FLX (20 mg/kg, i.p.) treated unstressed mice as shown in fig.1, 2 and 3. Therefore the increased levels of the nitrite in the brain of FLX (20 mg/kg, i.p.) treated stressed mice compromised the antidepressant effect of FLX (20 mg/kg, i.p.) in stressed mice and therefore responsible for the higher immobility period as compared to the FLX (20 mg/kg, i.p.) treated unstressed mice.

Several mechanisms had been proposed by which stress increased the levels of NO in the brain. It has been suggested that the stress results in the release of glutamate in the brain of the rodents exposed to the stress (Moghaddam, 1993). Glutamate so released results in the activation of NMDA receptors (Nowak *et al.*, 1995; Okano *et al.*, 1995); linked with the nNOS via PSD95 (Brenman and Bredt, 1997). NMDA receptors activation exposes nNOS directly to the flux of Ca++ entering the ion channel for the production of NO (Garthwaite *et al.*, 1988; Liu *et al.*, 2004). This is further supported by the fact that the large amount of the glutamate has been found in the CNS of the depression patients (Altamura *et al.*, 1993; Levine *et al.*, 2000; Mathis *et al.*, 1988). Another mechanism suggested that the exposure to the

immobilization stress results in the release of the pro-inflammatory cytokines such as IL-1 and TNF-α in the brain of rodents exposed to immobilization stress (Wichers and Maes, 2002). IL-1 and TNF-α increases the expression of NF-κβ pathway which is further responsible for the increase expression of iNOS (Cooke *et al.,* 2001; Hua *et al.,* 2008; Alexopoulos and Morimoto, 2011; Madrigal *et al.,* 2002) which produces larger quantities of NO (Madrigal *et al.,* 2002). Also it has been reported that the exposure to stressful conditions increased the expression of iNOS (Madrigal *et al.,* 2001; Olivenza *et al.,* 2000). Thus stress induced production of NO may either be due to the increased expression of nNOS or iNOS in the brain of the rodents exposed to immobilization stress.

Since the increased expression of the iNOS is followed by the activation of NF-κβ pathway (Madrigal *et al.,* 2002); therefore we checked the possible involvement of the NF-κβ pathway in the compromised antidepressant effect of fluoxetie in the stressed mice. We administered the stressed mice with NF-κβ pathway inhibitor, PDTC (100 mg/kg, i.p.) alone and in combination with FLX (20 mg/kg, i.p.). It was found that PDTC (100 mg/kg, i.p.) reduced the immobility period of stressed mice significantly as compared to the vehicle treated stressed mice in both TST and FST as shown in fig.1 and fig.2. But the pre-treatment with PDTC (100 mg/kg, i.p.) followed by the administration of the FLX (20 mg/kg, i.p.) to the stressed mice did not modulate the immobility period and the nitrite levels significantly as compared to the FLX (20 mg/kg, i.p.) treated stressed mice in the present study. Also the administration of PDTC (100 mg/kg, i.p.) in combination with FLX (20 mg/kg, i.p.) led to the death of all the 5 mice when they were tested in the FST after TST.

Therefore the present study suggested that pre-treatment with the PDTC did not significantly modulate the antidepressant effect of fluoxetine in stressed mice. However the increased levels of NO was responsible for the compromised antidepressant effect of the fluoxetine in the stressed mice. Therefore to determine the possible role of NOS and sGC, we administered the stressed mice with the inhibitor of NOS and sGC, i.e. MB (100 mg/kg, i.p.) alone and in combination with in combination with FLX (20 mg/kg, i.p.).

Administration of MB (100 mg/kg, i.p.) to the stressed mice significantly reduced the immobility period of stressed mice significantly as compared to the vehicle treated stressed mice in both TST and FST as shown in fig.1 and 2. Also the pre-treatment of stressed mice with MB (100 mg/kg, i.p.) followed by the administration of FLX (20 mg/kg, i.p.) reduced the immobility period in FST and nitrite levels of the stressed mice significantly as compared to the vehicle and FLX (20 mg/kg, i.p.) treated stressed mice as shown in fig. 2 and 3. Also it has been reported that the MB exerted antidepressant effect (Savegnago *et al.,* 2008) in the stressed mice. The results of the present study showed that MB (100 mg/kg, i.p.) significantly modulate the antidepressant effect of FLX (20 mg/kg, i.p.) in stressed mice. Therefore it has been suggested that the immobilization stress of 2hrs increased the production of NO in the brain of the mice exposed to the immobilization and NO further stimulate the enzyme sGC responsible for the production of cGMP (Paul and Ekambaram, 2011; Moncada and Higgs, 2006). cGMP is known to produce the depressive effect by influencing the 5-HT transporters (Miller and Hoffman, 1994). cGMP mediated enhancement of 5-HT transporters results in the reduction of the 5-HT levels (Sezal and Walia, 2015). Therefore the combine administration of the MB (100 mg/kg, i.p.) and FLX (20 mg/kg, i.p.) to the stressed mice potentiated the antidepressant effect of FLX (20 mg/kg, i.p.) in stressed mice.

CONCLUSION

It has been concluded that the stress mediated increased production of NO plays a key role in the compromised antidepressant effect of the fluoxetine in stressed mice. Immobilization stress results in the increased production of NO, which stimulate the enzyme sGC, which further increase the production of cGMP and cGMP is known to alter the level of 5-HT by influencing 5-HT transporters. Thus NO-cGMP pathway plays a key role in the compromise antidepressant effect of fluoxetine in stressed mice.

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