INTRODUCTION

Stress- potentiated behavior is the physiologically close pathological feature of anxiety in humans [1]. The pathogenesis of anxiety has been linked to alterations in cyclic nucleotide signaling [2]. A significant decrease in intracellular cAMP level has been observed in stressed mice [5]. This reduction in cAMP levels in stress conditions can be explained by increased cAMP metabolism by phosphodiesterase type 4 (PDE4) [6]. Reported enhancers of cAMP; diazepam (PDE4 inhibitor; 2 mg/kg, i. p.) and rolipram (PDE4 inhibitor; 1.25 mg/kg, i. p.) have been reported to produce anxiolytic-like activities in rodents [7]. Forskolin (FK) is activator of adenylate cyclase that has been reproved to increase intracellular cAMP levels [8].

Forskolin has been observed to activate cAMP- activated protein kinase A (PKA) and enhance release of gamma-amino butyric acid (GABA) [9]. Medications that facilitate function of GABA are the best medications to alleviate anxiety [10]. Diazepam (DZP), a classical anti-anxiety drug, has also been reported to inhibit activity of phosphodiesterase 4 and increase levels of cAMP [11-13]. Benzodiazepines are easier to dose, because of their reliable pharmacokinetics, no hepatic autoinduction and metabolism, better dose response curve and predictable half lives. However, Clinical problems associated with the benzodiazepines are the tendency to promote tolerance, dependence and withdrawal [10]. These are relatively addictive substances and DZP, being rapidly absorbed is usually the most abused agent [14].

Here, authors hypothesized that a non-receptor based action may serve to solve the problem of receptor- based adverse effects of GABAergic anxiolytics (benzodiazepines). Therefore, authors went ahead with comparing the anxiolytic of forskolin with that of clinically efficacious anxiolytic; DZP (a benzodiazepine, working through GABAergic agonism) with the purpose of comparing a cAMP mechanism of possible amylolysis with GABAergic mechanism, which stands as mainstay pathway of anxiety therapeutics. Another reason for the choice of DZP is that it has been found to be more effective than other agents in reduction of symptoms of anxiety [10]. Authors have also employed the combination of FK with DZP to explore possible synergy between the two agents.

MATERIALS AND METHODS

Animals

Swiss albino mice (male; 20 - 30g) were employed in the present study. Mice were housed in polypropylene cages at controlled temperature with 12h light/dark cycles. Animals were allowed to habituate to the housing facilities for at least one week before the experiments began and provided with food and water ad libitum. Behavioral studies were conducted in a quiet room. All animal handling procedures were approved by an Institutional Animal Ethics Committee (IAEC) of Maharshi Dayanand University, Rohtak (date of approval: 22.03.2012).

Drugs and treatments

Forskolin (FK; Sigma Chemicals), diazepam (DZP; Ranbaxy Laboratories Ltd.) were used in the present investigation. Normal saline (0.9% sodium chloride) was used as vehicle for DZP, whereas vehicle for forskolin was DMSO. All the drugs or vehicles were provided by the manufacturers.

Elevated plus maze (EPM)

The elevated plus maze (EPM) employed in the present study was a wooden animal maze, comprising two open arms (16 cm × 5 cm), and two closed arms (16 cm × 5 cm × 12 cm), arranged opposite to each other, all connected to a central platform (5 cm × 5 cm). The maze was elevated to a height of 25 cm above the ground. Each mouse was placed separately at the centre of EPM with its head facing towards anyone open arm and observed for 5 min to record the time spent in open arms. Percent time spent by mice on the open arms was determined as follows:

\[
\text{Percent time spent (\%)} = \frac{100 \times \text{Number of seconds spent on open arms}}{300 \text{ total seconds of observation}}
\]
**Light and Dark Box (LDB)**

The light and dark box (LDB) employed in the present study consisted of a rectangular box (45 cm × 27 cm × 27 cm), partitioned into two compartments with the black/dark section comprising one-third and the white/light section comprising the remaining two-thirds of the box connected by a 7.5 cm × 7.5 cm opening in the wall between compartments. Each mouse was placed in the centre of the light compartment and was observed for 5 min for the time spent in the light compartment. Percent time spent by mice in the light compartment was determined as follows:

\[
\text{Percent time spent (\%)} = \frac{\text{Number of seconds spent in light compartment}}{300 \text{ total seconds of observation}} \times 100
\]

**Biochemical estimation of cAMP in plasma of mice**

cAMP levels in plasma of mice were measured using Cayman Chemical commercial cAMP estimation kit. Kit was used as per manufacturer’s protocol and instructions.

**Plasma corticosterone estimation**

Estimation of corticosterone level in plasma of mice was performed by the method of Bartos and Pesez [15].

**Measurement of spontaneous locomotor activity**

The effects of various treatments on spontaneous locomotor activity of animals were measured using a rodent activity cage (actophotometer, Inco, Ambala). The locomotor activity scores for each animal were recorded for a period of 10 min before and after drug treatments.

**Experimental protocol**

Experimental animal groups employed in the present study consisted of six mice each (n = 6). Unstressed mice were exposed to EPM and LDB for normal duration (5 min), sufficient to assess the anxiety levels in rodents [16]. Stress was produced in mice by immobilizing them for 6 h by taping all their four limbs and trunk on a wooden board. The stress protocol adopted in the present study has earlier been utilized by several relevant studies and is reported to induce relevant pathological changes in rodents, thus stands scientifically relevant to our hypothesis [17, 18].

Corticosterone levels were estimated in the plasma of mice, subjected to such immobilization, so as to ensure the relevant biochemical change in stressed mice. Mice with a significantly increased plasma corticosterone levels were included in the study and were called as stressed mice. Mice, the subjected to immobilization stress were called as unstressed mice and mentioned accordingly hereafter in the manuscript. However, for clarity of results and discussion, mice were clearly indicated as “vehicle-treated” or “drug-treated” unstressed or stressed mice throughout the manuscript. Behavioral tests were performed in independent groups of mice carefully in a stepwise manner i.e., mice in each group were subjected to EPM followed by light/dark box. In unstressed mice, behavioral testing was done 30 min after administration of the drug(s).

All the drugs were administered intraperitoneally (i.p.) 30 min before behavioral testing in unstressed mice and immediately before immobilization in stressed mice. In case of stressed mice, behavioral testing was started 10 min after setting the animals free from immobilization. Whenever, combinations of drugs were employed, pre-treatments were administered 15 min earlier than the subsequent administration. The dosage schedule in the stressed group assures that the treatment(s) employed inhibited any change(s) occurring immediately after and during immobilization, thereby producing the net change in behavior or biochemical parameter of mice under investigation [19-22]. The experimental protocol was subjected to animal reduction ethics; hence, biochemical estimation of cAMP was conducted only in the selected groups that served to substantiate its role in testing the hypothesis of the present study. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of Maharshi Dayanand University, Rohtak (date of approval: 22.03.2012).

**Statistical analysis**

The Statistical analysis was performed by using one way ANOVA followed by Tukey’s post hoc test by using the Software Graph Pad In stat; version 3.05 (Graph Pad Software, San Diego, CA, USA).

**RESULTS**

Effect of different treatments on behavior of unstressed and stressed mice on EPM and LDB

Mice, subjected to behavioral paradigm (EPM and LDB) for 5 min duration were found to show anxiety-like behavior as characterized by their longer stay in closed arm/dark compartment of EPM and LDB respectively in total 5 min observation time. 6h immobilization served to significantly enhance an anxiety-like behavior of mice on EPM and LDB as compared to that of unstressed mice. Administration of FK (5 mg/kg, i.p.) produced a significant antianxiety-like behavior in unstressed and stressed mice. Administration of DZP (2 mg/kg, i.p.) produced a significant anti-anxiety-like behavior of unstressed control mice on EPM and LDB, but not in stressed mice. Pre-treatment of DZP produced a significant antianxiety-like behavior in forskolin-treated unstressed and stressed mice [fig. 1 and 2].

![Fig. 1: Effect of different treatments on percentage of time spent by mice on open arms of elevated plus maze. n=6 in each group.](image)

Values are expressed as mean ± S. E. M. Data was analyzed by one-way ANOVA followed by Tukey’s post hoc Test. F (9, 50) = 73.53; p<0.0001, a = p<0.05 significant difference from unstressed control mice. b = p<0.01 significant difference from vehicle-treated unstressed mice, c = p<0.001 significant difference from unstressed mice, d = p<0.05 significant difference from unstressed mice; e = p<0.05 significant difference from diazepam-treated unstressed mice; f = p<0.05 significant difference from vehicle-treated unstressed mice; g = p<0.05 significant difference from forskolin-treated stressed mice and diazepam-treated stressed mice. SALU: normal saline-treated unstressed mice; DMSOU: dimethyl sulfoxide-treated unstressed mice; SALS: normal saline-treated stressed mice; DMSOS: dimethyl sulfoxide-treated stressed mice; FKU: forskolin-treated unstressed mice; FKS: forskolin-treated stressed mice; DZPU: Diazepam-treated stressed mice; DZPS: Diazepam-treated stressed mice. Values mentioned are doses in mg/kg, i.p.

![Fig. 2: Effect of different treatments on percentage of time spent by mice in light compartment of light/dark box. n=6 in each group.](image)

Values are expressed as mean ± S. E. M. Data was analyzed by one-way ANOVA followed by Tukey’s post hoc Test. F (9, 50) = 43.20; p<0.0001, a = p<0.05 significant difference from unstressed control
FK (5 mg/kg, i. p.) and DZP (2 mg/kg, i. p.) alone [fig. 3].

Effect of selected treatments on cAMP levels in plasma of mice

6h immobilization stress significantly decreased cAMP levels in the plasma of mice, as compared to that in unstressed control mice. FK (5 mg/kg, i. p.) significantly increased the cAMP levels in the plasma of unstressed mice but not in stressed mice. Similarly, DZP (2 mg/kg, i. p.) significantly increased the cAMP levels in the plasma of unstressed mice. On the other hand, administration of DZP (2 mg/kg, i. p.) in combination with FK (5 mg/kg, i. p.) significantly increased cAMP levels in the plasma of unstressed as well as stressed mice as compared to that in stressed mice, treated with FK (5 mg/kg, i. p.) and DZP (2 mg/kg, i. p.) alone [fig. 3].

Values are expressed as mean ± S. E. Data was analyzed by one-way ANOVA followed by Tukey’s Post hoc Test, F (2, 15) = 6.76; p<0.0001, a = p<0.05 significant difference from normal saline-treated unstressed mice and DMSO- treated unstressed mice. ALU: normal saline- treated unstressed mice; DMSOU: dimethyl sulfoxide-treated unstressed mice; SALS: normal saline- treated stressed mice; DMSOS: dimethyl sulfoxide- treated stressed mice; FKU: forskolin- treated unstressed mice; FKS: forskolin- treated stressed mice; DZPU: diazepam- treated unstressed mice; DZPS: diazepam- treated stressed mice FK+DZP (U): forskolin and diazepam - treated unstressed mice; FK+DZP(S): forskolin and diazepam- treated stressed mice. Values mentioned are doses in mg/kg, i. p.

Effect of selected treatments on corticosterone levels in the plasma of mice

6h immobilization stress produced a significant increase in plasma corticosterone levels in mice, as compared to that of normal saline and DMSO- treated unstressed mice [fig. 4].

Effect of different treatments on spontaneous locomotor activity of mice

Drug treatments used in the present study did not produce any significant change in the spontaneous locomotor activity of unstressed or stressed mice as compared to respective unstimmed or stressed controls (results not shown).

Values are expressed as mean ± S. E. Data was analyzed by one-way ANOVA followed by Tukey’s Post hoc Test, F (9, 50) = 3.4; p<0.0001, a = p<0.05 significant difference from FK treated mice, b = p<0.01 significant difference from FK and DZP treated mice, c = p<0.001 significant difference from unstressed control mice, d = p<0.05 significant difference from vehicle- treated unstressed mice; e = p<0.05 significant difference from dexamethasone (DZP)- treated stressed mice; f = p<0.05 significant difference from vehicle- treated stressed mice; g = p<0.05 significant difference from forskolin- treated stressed mice and diazepam- treated stressed mice. ALU: normal saline- treated unstressed mice; DMSOU: dimethyl sulfoxide- treated unstressed mice; SALS: normal saline- treated stressed mice; DMSOS: dimethyl sulfoxide- treated stressed mice; FKU: forskolin- treated unstressed mice; FKS: forskolin- treated stressed mice; DZPU: diazepam- treated unstressed mice; DZPS: diazepam- treated stressed mice FK+DZP (U): forskolin and diazepam - treated unstressed mice; FK+DZP(S): forskolin and diazepam- treated stressed mice. Values mentioned are doses in mg/kg, i. p.

Discussion

Forced immobilization combines emotional stress (escape reaction) and physiological stress (muscle work) and does not involve painful stimuli; therefore, this form of stress is probably more akin to physiological stress [23]. Stress initiates a series of underlying mechanisms and cascades and one of the several implicated chemicals that is reduced in stress, is cAMP [5]. EPM and LDB offer a similar environment of light and dark transition, so, this combination of tests in the series may serve to enhance the robustness of the procedure of anxiety assessment. We have found that stress- exposed mice were more anxious in their behavior as compared to unstressed mice. 2h immobilization stress is known to potentiate anxiety in rodents [18]. 6h immobilization stress, used in the present investigation, significantly potentiated anxiety in mice and significantly reduced cAMP levels in the plasma of mice. Pharmacological evidence indicates that cAMP elevation elicits anxiolytic activity in stressed conditions [13]. Beer et al. [2] have discussed that the anxiolytic activity produced by such pharmacological interventions may be based on the physiological implications such as (a) potent anxiety- reducing drugs are potent inhibitors of brain phosphodiesterase (b) dibutyl cyclic adenosine monophosphate has the ability to reduce anxiety, (c) the methyl xanthines show significant anxiety-reducing effects. The reduction in cAMP levels in stress conditions has been attributed to increased cAMP metabolism by PDE4 [6]. In the present study, forskolin has served to enhance the levels of plasma cAMP and reduce the anxiety in unstressed mice. Though, FK per se has not been able to produce a significant anxiolysis in stressed mice, its anti-anxiety- like activity has been enhanced by diazepam co-administration in stressed mice, similar to such activity in unstressed mice. These findings may further be supported by the results of pharmacological interventions, which report that FK, a cAMP elevating drug, may also elevate GABA release by PKA activation [9]. Further, FK has also been shown to increase synaptic GABA release frequency [24]. These effects on GABA may explain its noted synergy with DZP in stressed mice.

DZP has also been observed to inhibit a specific subtype of PDE4 i.e. PDE4B, known to be a part of pathogenetic events leading to anxiety [11]. The inability of DZP to produce antianxiety activity in stressed mice has been explained elsewhere [22,25]. Of course, the advent of more non-addictive and safer anxiety medications led to under use of GABA-based substances. Agents with better side effect profile and equal effectiveness are always under investigation by workers engaged in academic as well as commercial research. Studies in laboratories have suggested that the development of dependence on benzodiazepines may be associated with a reduction in the sensitivity of GABA-A receptor [26]. Similar reports of GABAergic receptor system disturbances by immobilization stress indicate the possibility of the similar compromise in effect of DZP in stressed conditions and FK (an agent found to reverse stress- induced changes) may be a future promise to overcome such situations.
CONCLUSION

The present findings contribute to suggest a non–receptor mediated anti-anxiety action of a novel agent, acting through cAMP elevation, thus avoiding receptor-mediated adverse effect profile of the conventional anxiolytics. These findings are even important in light of reports that under stressed conditions, psycho-pathological symptoms appear to precipitate and manifest to even more extent. Therefore, the present findings help to provide a clue to possible utility of FK in stress- potentiated anxiety alone or in co-administration with DZP. The findings of the present study may further be strengthened by future studies on FK in combination with other drugs in the benzodiazepine and non-benzodiazepine category.

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CONFLICT OF INTEREST

The authors have none conflict of interest

REFERENCES