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Original Article

A NOVEL NON–RECEPTOR AND NON- GABAERGIC ANTIANXIETY-LIKE ACTIVITY OF FORSKOLIN: SYNERGY WITH DIAZEPAM

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ABSTRACT

Objective: Clinical problems associated with the benzodiazepines like dependence, withdrawal or tolerance may lead to under use of substances based on gamma-amino butyric acid (GABA). Non-GABAergic and substances that elevate cyclic adenosine monophosphate (cAMP) have shown anti-anxiety activity. Therefore, present investigation aimed to explore a non-GABAergic mechanism and non-receptor mediated anti-anxiety activity of a cAMP elevating agent, forskolin.

Methods: Elevated plus maze and light/dark box were employed to measure effect of forskolin on anxiety and the noted activity was compared with that of diazepam. cAMP levels were also measured in plasma of mice.

Results: Forskolin produced a significant antianxiety- like activity in unstressed mice and stressed mice. Diazepam produced a significant antianxiety- like activity in unstressed mice but not in stressed mice. The noted antianxiety activity of forskolin was accompanied by a significant elevation of cAMP levels.

Conclusions: The present findings contribute to suggest a non– receptor mediated anti-anxiety action of a forskolin, acting through cAMP elevation, thus avoiding receptor-mediated adverse effect profile of the conventional anxiolytics.

Keywords: cAMP, Anxiety, Forskolin, Stress.

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]. 3, 5- cyclic adenosine monophosphate (cAMP) is reported to be a predominant cyclic nucleotide that plays a role in anxiety [3,4]. 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].

cAMP 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 anxiolysis 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 anxiolysis 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 administered intraperitoneally (i. p.) in a volume of 10 ml/kg body weight of mice.

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:

$$\text{Percent time spent (\%)} = 100 \times \frac{\text{Number of seconds spent on open arms}}{\text{300 total seconds of observation}}$$

Light and Dark Box (LDB)

The light and dark box (LDB) employed in the present study consisted 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:

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

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 by 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 6h 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, not 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 doc 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 paradigms (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. 1and 2].

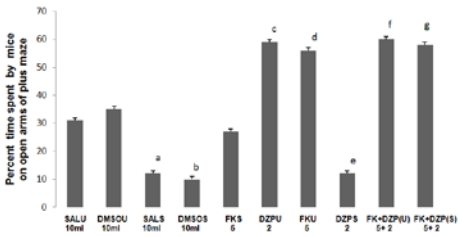

Fig. 1: Effect of different treatments on percentage of time spent by mice on open arms of elevated plus maze. n=6 in each group.

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<0.001significant 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 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

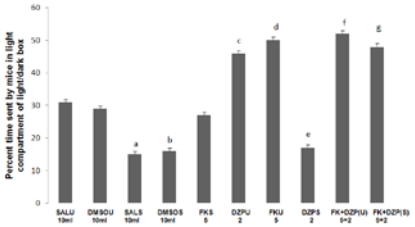

Fig. 2: Effect of different treatments on percentage of time spent by mice in light compartment of light/dark box. n=6 in each group.

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

mice, b = p<0.001significant 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 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 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 but not in stressed 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].

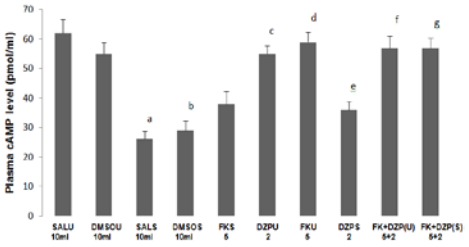

Fig. 3: Effect of different treatments on plasma cAMP level (pmol/ml). n=6 in each group.

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) = 3.4; p<0.0001, a = p<0.05 significant difference from unstressed control mice, b = p<0.001significant 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 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 unstressed or stressed controls (results not shown).

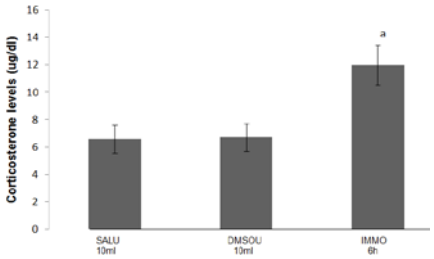

Fig. 4: Effect of different treatments on plasma corticosterone levels (ug/dl). n = 6 in each group.

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. SALU: normal saline- treated unstressed mice; DMSOU: dimethyl sulfoxide-treated unstressed mice; IMMO: immobilization stress; 6h: six hours. Values mentioned are doses of vehicles

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) dibutyryl 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 newer 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

1. Korte SM, De Boer SF. A robust animal model of state anxiety: fear potentiated behaviour in the elevated plus maze. Eur J Pharmacol 2003;463:163-75.
2. Beer B, Chasin M, Clody DE, Voge JR, Horovitz ZR. Cyclic adenosine monophosphate phosphodiesterase in brain: effect on anxiety. Sci 1972;176:428–30.
3. Nibuya M, Nestler EJ, Duman SR. Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. J Neurosci 1996;16:2365–72.
4. Robello M, Amico C, Bucossi G, Cupello A, Rapallino MV, Thellung S. Nitric oxide and GABAA receptor function in the rat cerebral cortex and cerebellar granule cells. Neurosci 1996;74:99-105.
5. Skinner JE, Welch KMA, Reed JC, Nell JH. Psychological stress reduces cyclic 3',5'-adenosine monophosphate levels in the cerebral cortex of conscious rats, as determined by a new cryogenic method of rapid tissue fixation. J Neurochem 1978;30:691-8.
6. Chen C-C, Yang C-H, Huang C-C, Hsu K-S. Acute stress impairs hippocampal mossy fiber-CA3 long-term potentiation by enhancing cAMP-specific phosphodiesterase 4 activity. Neuropsychopharmacol 2010;235:1605-17.
7. Silvestre JS, Fernandez AG, Palacios JM. Effects of rolipram on the elevated plus-maze test in rats: a preliminary study. J Psychopharmacol 1999;13:274–7.
8. Bersudsky Y, Kotler M, Shifrin M, Belmaker RH. A preliminary study of possible psychoactive effects of intravenous forskolin in depressed and schizophrenic patients. J Neural Transm 1996;103:1463–7.
9. Shindou T, Nonaka H, Richardson PJ, Mori A, Kase H, Ichimura M. Presynaptic adenosine A2A receptors enhance GABAergic synaptic transmission via a cyclic AMP dependent mechanism in the rat globus pallidus. Br J Pharmacol 2002;136:296–302.
10. Schwartz TL, Nihalani N, Simionescu M, Hopkins G. History repeats itself: pharmacodynamic trends in the treatment of anxiety disorders. Curr Pharm Des 2005;11:255–63.
11. Cherry JA, Thompson BE, Pho V. Diazepam and rolipram differentially inhibit cyclic AMP-specific phosphodiesterases PDE4A1 and PDE4B3 in the mouse. Biochim Biophys Acta-Gene Struct Expression 2001;1518:27–35.
12. Carceles MD, Ribó AR, Davalos R, Martinez T, Hernandez J. Effect of diazepam on adenosine 3′,5′-cyclic monophosphate (cAMP) plasma levels in anesthetized patients. Clin Ther 2004;26:737-43.
13. Kelley DJ, Bhattacharya A, Lahvis GP, Yin JC, Malter J, Davidson RJ. The cyclic AMP phenotype of fragile X and autism. Neurosci Biobehav Rev 2008;32:1533–43.
14. Saddock BJ, Saddock VA. In Kaplan and Saddock's (eds): Pocket handbook of psychiatric drug treatment. Lippincott, USA: Williams and Wilkins; 2001.
15. Bartos J, Pesez M. Colorimetric and fluorimetric determination of steroids. Pure Appl Chem 1979;51:2157–69.
16. Calatayud F, Belzung C, Aubert A. Ethological validation and the assessment of anxiety-like behaviours: methodological comparison of classical analyses and structural approaches. Behav Processes 2004;67:195–206.
17. Madrigal JLM, Olivia HO, Moro MA, Lizasoain I, Lorenzo P, Castrillo A, *et al*. The increase in TNF-α Levels is implicated in NF-κB activation and inducible nitric oxide synthase expression in brain cortex after immobilization stress. Neuropsychopharmacol 2002;26:155–63.
18. Sevgi S, Ozek M, Eroglu L. L-NAME prevents anxiety like and depression-like behaviour in rats exposed to restraint stress. Methods Find Exp Clin Pharmacol 2006;28:95–9.
19. Goyal R, Kumar A. Protective effect of alprazolam in acute immobilization stress-induced certain behavioral and biochemical alterations in mice. Pharmacol Rep 2007;59:284–90.
20. Kumari B, Kumar A, Dhir A. Protective effects of non-selective and selective COX-2 inhibitors in acute immobilization stress induced behavioral and biochemical alterations. Pharmacol Rep 2007;59:699.
21. Gilhotra N, Dhingra D. Involvement of NO-cGMP pathway in anti-anxiety effect of aminoguanidine in stressed mice. Prog Neuropsychopharmacol Biol Psychiatry 2009;33:1502–7.
22. Gilhotra N, Dhingra D. GABAergic and nitriergic modulation by curcumin for its antianxiety-like activity in mice. Brain Res 2010;1352:167–75.
23. Bhattacharya SK, Bhattacharya D. Effect of restraint stress on rat brain serotonin. J Biosci 1982;4:269–74.
24. Koyama S, Kubo C, Rhee JS, Akaike N. Presynaptic serotonergic inhibition of GABAergic synaptic transmission in mechanically dissociated rat basolateral amygdala neurons. J Physiol 1999;518:525-38.
25. Sharma V, Gilhotra R, Dhingra D, Gilhotra N. Possible underlying influence of p38MAPKinase and NF-kappaB in compromised effect of diazepam in stressed mice. J Pharmacol Sci 2011;116:257-63.
26. Sun AY, Li DX, Wang YL. Stress-induced changes of central GABAergic function in rats. Zhongguo Yao Li Xue Bao 1991;12:387–90.