ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 8, Issue 1, 2015

ISSN - 0974-2441

Research Article

NON-RECEPTOR MEDIATED ANXIOLYSIS BY AMINO GUANIDINE: A NOVEL ACTIVITY AND SYNERGY WITH DIAZEPAM UNDER STRESSED CONDITIONS

SHALINI YADAV, NEERAJ GILHOTRA*

Department of Pharmaceutical Sciences, Maharshi Dayanand University, Rohtak - 124 001, Haryana, India. Email: neerajmdu@rediffmail.com

Received: 11 November 2014, Revised and Accepted: 22 November 2014

ABSTRACT

Objective: A novel non-receptor mediated anxiolytic activity of amino guanidine (AG; 50 mg/kg, i.p.) was investigated and its possible interaction with diazepam (DZP) (2 mg/kg, i.p.) was explored in mice.

Methods: Elevated plus maze and light/dark box were used to measure anxiety in Swiss albino mice. Plasma nitrite levels were estimated using Griess reagent to observe the biochemical regulation of mice behavior.

Results: AG (50 mg/kg, i.p.) produced a significant antianxiety-like activity and significantly attenuated the plasma nitrite levels in stressed mice. On the other hand, AG (50 mg/kg, i.p.) neither produced a significant antianxiety-like activity nor significantly altered the plasma nitrite levels in unstressed mice. DZP (2 mg/kg, i.p.) produced a significant antianxiety-like activity in unstressed mice but not in stressed mice. DZP (2 mg/kg, i.p.) did not significantly affect plasma nitrite levels in unstressed or stressed mice. AG and DZP, administered together produced a significant antianxiety-like activity in unstressed and stressed mice. However, combination of AG and DZP significantly attenuated the plasma nitrite levels in stressed mice but not in unstressed mice.

Conclusion: These findings suggest a usefulness of a non-GABAergic mechanism of a potential anti-anxiety chemical that may avoid receptor-linked undesired effects as in case of DZP and may be used with DZP under stressed conditions to serve the reduction of dosage of DZP.

Keywords: Amino guanidine, Anxiety, Diazepam, Stress.

INTRODUCTION

Stress has long been observed to play a role in the etiology of neurodegenerative diseases and mental disorders [1]. Immobilization stress induces a generalized increase in the expression of nitric oxide synthase (NOS) and production of nitric oxide (NO), leading to anxious behavior in rodents [2-4]. Exposure to various stressors results in anxiogenic behavior in tests of anxiety in animals [5]. Exploration of open arms in elevated plus maze (EPM) is decreased by different stressors including immobilization [6]. Both (a) exposure to the animal model (normal anxiety) and (b) immobilization (excess anxiety) present two different types of anxiety. Different forms of anxiety have been shown to respond differentially to pharmacological interventions [7].

NO and stress physiology share many common pathways and molecular mechanisms, and NO plays a role in many stress-related diseases [1]. Stress strongly involves activation of NOS and NO-cascade leading to behavioral changes [4]. However, a differential modulation is shown by NO in response to stressful stimuli e.g. intracerebroventricular or peripheral injection of the nonselective NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME), attenuates stress-induced adrenocorticotrophic hormone (ACTH) release [8]. In contrast, pretreatment with L-NAME increases plasma ACTH and corticosterone following peripheral lipopolysaccharide or intravenous injections of cytokines [9]. In stress conditions including physical immobilization for 60 min or 120 min, enhanced anxious behavior has been prevented by L-NAME in rodents [4,10]. Both anxiogenic [11-13] and anxiolytic [14-17] roles have been reported for NO. Contrarily to the neuronal and endothelial NO synthases, inducible nitric oxide synthase (iNOS) is mainly regulated at the transcriptional level [18,19]. iNOS is also expressed in the brain cortex of rats exposed to acute and repeated stress [20,21]. iNOS is a high-output isoform of NOS, which has been implicated in cellular toxicity in many cell systems including the brain [3]. Experimental evidences indicate that stress-induced iNOS expression in brain may mediate the anatomical and clinical features of major depression related neurotoxic damage found in animals and humans after exposure to uncontrollable stress [22,23].

Psychoactive medicines that act through non-receptor mechanisms have been advocated and reported to be devoid of long-term undesired effects like dependence. Therefore, agents working through nonreceptor mechanism are always under investigation for their potential as psychoactive molecules. AG has received much attention as an inhibitor of NOS due to its selectivity towards iNOS, its low acute toxicity and potential clinical usefulness [24]. Amino guanidine (AG) has been observed to show anti-ageing property [25], help alleviate or prevent senile cataracts, thickening of the arteries, kidney failure, thinning bones, and osteoarthritis, improve overall heart and arterial condition [26], stabilize the metabolism of glucose, and helps prevent and treat adult onset diabetes. Recently, AG has been reported to produce an antianxiety – like activity in stressed mice by Gilhotra and Dhingra, 2009 [27].

Medications that facilitate GABA function are the best medications to alleviate anxiety. Diazepam (DZP), a classical anti-anxiety drug; act on the benzodiazepine receptor as benzodiazepine agonist [28]. DZP has been found to be more effective than other agents in a reduction of symptoms of anxiety [29]. Benzodiazepines are easier to dose, because of their reliable pharmacokinetics, no hepatic auto-induction and metabolism, better dose-response curve and predictable half-lives. Certainly these agents have been the gold standard for alleviating anxiety symptoms both acutely and chronically. However, Clinical problems associated with the benzodiazepines are the tendency to promote tolerance, dependence, and withdrawal. These are relatively addictive substances and DZP, being rapidly absorbed is usually the most abused agent [30].

Here, authors hypothesize 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 AG with that of clinically efficacious anxiolytic; DZP (a benzodiazepine, working through GABAergic agonism) with the purpose of comparing a novel nitriergic mechanism of possible anxiolysis with GABAergic mechanism, which stands as mainstay pathway of anxiety therapeutics. Authors have also employed combination of AG with DZP to explore possible synergy between the two agents.

MATERIALS AND METHODS

Animals

Swiss albino mice (male; 20-30 g) were employed in the present study. Animals were kept in polypropylene boxes covered with sawdust, at controlled temperature, with 12 hrs light/dark cycles. Animals were allowed to habituate to the housing facilities for at least one week before the experiments began. Behavioral studies were conducted in a quiet room. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Drugs

AG was purchased from Sigma Chemical Co. (St. Louis, MO, USA). DZP was obtained in the form of ampoule from Ranbaxy Laboratories Ltd. and suitable diluted. Normal saline (0.9% w/v sodium chloride) was used as vehicle for the drugs [27,31,32].

EPM

The plus-maze apparatus consisted of two open arms, 16 cm × 5 cm, and two closed arms, 16 cm × 5 cm, enclosed by 12 cm high walls with an open roof, arranged so that the two open arms are opposite to each other. The maze was elevated to a height of 25 cm. Each mouse was placed individually at the center of EPM with its head facing toward an open arm. During the 5 min test, time spent in open arms of the maze was recorded. The apparatus was thoroughly cleaned using 5% ethanol before placing each mouse in a cage. In EPM test, percent time spent on the open arms was determined as follows:

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

Light and dark box (LDB)

The apparatus consisted a rectangular box (45 cm × 27 cm × 27 cm), partitioned into two compartments connected by a 7.5 cm × 7.5 cm opening in the wall between compartments. One compartment was painted black from the inside and covered with a roof. The other compartment was open (no roof) and painted white. An animal was placed into the center of the light compartment and was observed for 5 minutes. The observations were made on time spent in open (white/light) compartment. Percent time spent in the light compartment was determined as follows:

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

Biochemical estimation of plasma nitrite

Nitrite is the stable end product of NO in living systems. For nitrite estimation, plasma was collected using cooling centrifuge at 2500 rpm and 4°C for 10 minutes. The plasma was stored in the refrigerator for estimation of nitrite content within 24 hrs. Plasma nitrite was measured by a spectrophotometric assay based on the Griess reaction [33]. Briefly, plasma was mixed with an equal volume of Griess reagent (1% sulfanilamide + 0.1% naphthalene ethylenediamine dihydrochloride + 2.5% orthophosphoric acid) and incubated at room temperature for 10 minutes to yield chromophore. The absorbance was read at 545 nm.

Plasma corticosterone estimation

The quantitative estimation of plasma corticosterone level was performed by the method of Bartos and Pesez [34].

Measurement of spontaneous locomotor activity

Spontaneous horizontal movement of mice was measured using photoactometer (INCO, Ambala). The locomotor activity scores for each animal were recorded for a period of 10 minutes before and after drug treatment.

Experimental design

Experimental animal groups employed in the present study consisted of six mice each (n=6). Unstressed mice were exposed to EPM and LDB for a normal duration (5 minutes), sufficient to assess the anxiety levels in rodents [35]. Stress was produced in mice by immobilizing them for 6 hrs by taping all their four limbs and trunk on a wooden board. 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 that show a significant increase in plasma corticosterone levels were included in the study. Mice subjected to immobilization stress 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 minutes after administration of the drug(s). In the case of stressed mice, behavioral testing was started 10 minutes after setting the animals free from immobilization. Whenever, combinations of drugs were employed, pre-treatments were administered 15 minutes earlier than the subsequent administration. Doses and routes of administration of drugs, employed in the present study, have been adopted from studies, utilizing the selected doses for anxiety assessment, i.e., AG [27,36,37], DZP [38]. 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 [27,39,40]. The experimental protocol was subjected to animal reduction ethics; hence, only stressed mice were treated with drug treatments with a strictly defined objective to answer the research question in hand. Biochemical estimation of nitrite was conducted only in the selected groups that served to substantiate its role in testing the hypothesis of the present study. 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 [3,4,21,40,41]. The experimental protocol was approved by institutional animal ethics committee of Maharshi Dayanand University, Rohtak (date of approval: 14.09.2012; Protocol no.: Pharm. Sci. 1206).

Statistical analysis

The Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test using the software Graph pad In stat; version 3.05 (Graph Pad Software, San Diego, CA, USA). $p<0.05$ was considered as significant.

RESULTS

Effect of different treatments on mice behavior

Behavior of unstressed mice on EPM and LDB (unstressed control mice group)

Mice, subjected to behavioral paradigms (EPM and LDB) for 5 minutes duration were found to show anxiety-like behavior as characterized by their longer stay in closed arm/dark compartment of EPM ($F_{9,50}$=73.53, $p<0.05$) and LDB ($F_{9,50}$=43.20, $p<0.05$), respectively in total 5 minutes observation time (Figs. 1 and 2).

Effect of vehicle on unstressed mice (Vehicle-treated unstressed mice group)

Administration of normal saline (10 ml/kg of mouse) could not bring any significant change in the behavior of mice on EPM and LDB, as compared to that of unstressed control mice (Figs. 1 and 2).

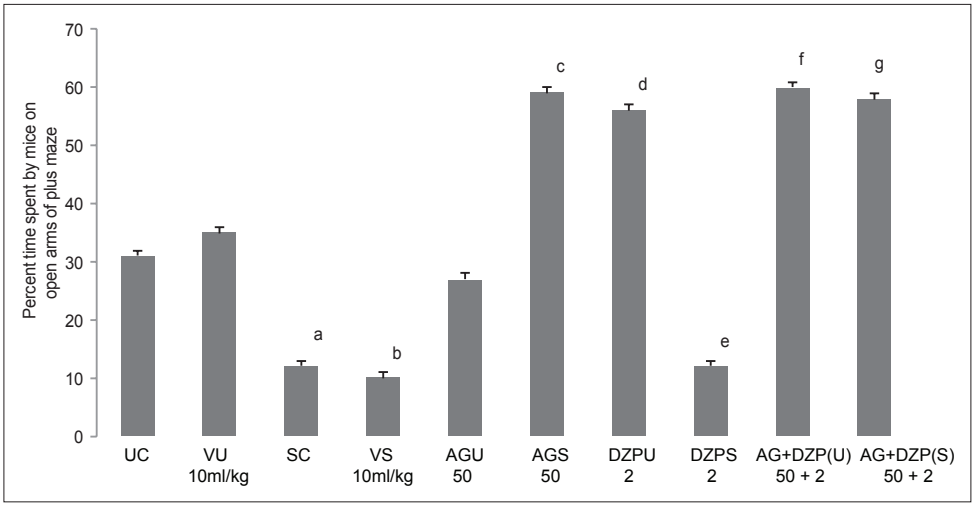

Fig. 1: Effect of different treatments on percentage of time spent by mice on open arms of EPM. n=6 in each group. Values are expressed as mean ± standard error. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test, $F_{9,50}$=73.53; p<0.0001, [a]p<0.05 significant difference from unstressed control mice, [b]p<0.001 significant difference from vehicle-treated unstressed control mice, [c]p<0.001 significant difference from stressed mice, [d]p<0.05 significant difference from unstressed mice, [e]p<0.05 significant difference from DZP-treated unstressed mice, [f]p<0.05 significant difference AG-treated unstressed mice, [g]p<0.05 significant difference from DZP-treated stressed mice. UC: Unstressed control mice, VU: Vehicle-treated unstressed mice, SC: Stressed control mice, VS: Vehicle-treated stressed mice, AGU: Amino guanidine-treated unstressed mice, AGS: Amino guanidine-treated stressed mice, DZPU: Diazepam-treated unstressed mice, DZPS: Diazepam-treated stressed mice, AG+DZP (U): Amino guanidine and diazepam-treated unstressed mice, AG+DZP(S): Amino guanidine and diazepam-treated stressed mice. Values mentioned are doses in mg/kg

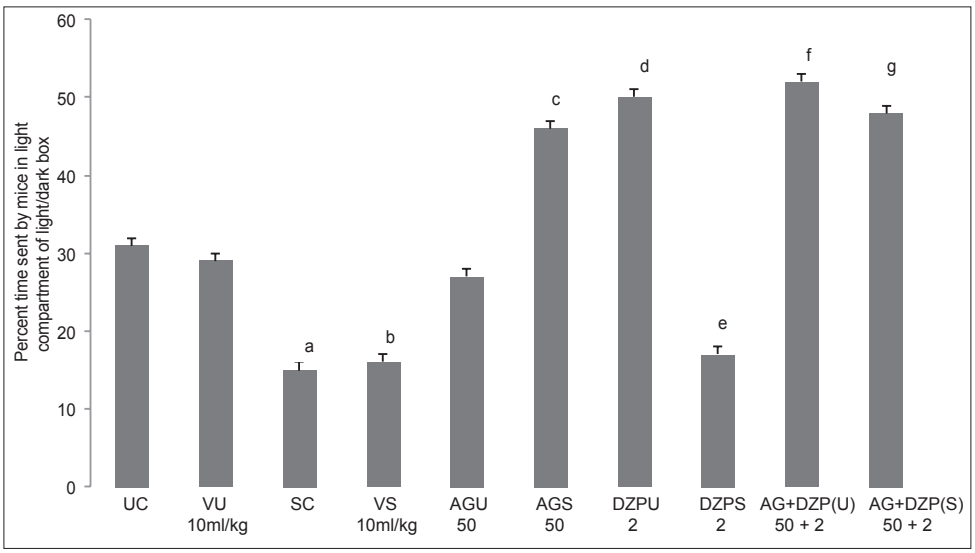

Fig. 2: Effect of different treatments on percentage of time spent by mice in the light compartment of light/dark box. n=6 in each group. Values are expressed as mean ± standard error. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test, $F_{9,50}$=43.20, p<0.0001, [a]p<0.05 significant difference from unstressed control mice, [b]p<0.001 significant difference from vehicle-treated unstressed control mice, [c]p<0.001 significant difference from stressed mice, [d]p<0.05 significant difference from unstressed mice, [e]p<0.05 significant difference from DZP-treated unstressed mice; [f]p<0.05 significant difference AG-treated unstressed mice; [g]p<0.05 significant difference from DZP-treated stressed mice. UC: Unstressed control mice, VU: Vehicle treated unstressed mice, SC: Stressed control mice, VS: Vehicle-treated stressed mice, AGU: Amino guanidine-treated unstressed mice, AGS: Amino guanidine-treated stressed mice, DZPU: Diazepam-treated unstressed mice, DZPS: Diazepam-treated stressed mice, AG+DZP (U): Amino guanidine and diazepam-treated unstressed mice, AG+DZP(S): Amino guanidine and diazepam-treated stressed mice. Values mentioned are doses in mg/kg

Effect of 6h immobilization on behavior of mice (stressed control mice group)

6h immobilization served to significantly enhance an anxiety-like behavior of mice on EPM ($F_{9,50}$=73.53, p<0.05) and LDB ($F_{9,50}$=43.20, p<0.05) as compared to that of unstressed control mice (Figs. 1 and 2).

Effect of vehicle on behavior of stressed mice (Vehicle-treated stressed mice group)

Administration of normal saline (10 ml/kg of mouse) could not bring any significant change in the behavior of stressed mice on EPM and LDB, as compared to that of stressed control mice (Figs. 1 and 2).

Effect of AG on behavior of unstressed control mice
Administration of AG (50 mg/kg, i.p.) did not produce a significant change in anxiety-like behavior of unstressed control mice as compared to that of vehicle-treated unstressed mice (Figs. 1 and 2).

Effect of AG on behavior of stressed control mice
Administration of AG (50 mg/kg, i.p.) produced a significant antianxiety-like activity in stressed control mice on EPM ($F_{9,50}$=73.53, p<0.001) and LDB ($F_{9,50}$=43.20, p<0.001) as compared to that of vehicle-treated stressed mice (Figs. 1 and 2).

Effect of DZP on behavior of unstressed control mice
Administration of DZP (2 mg/kg, i.p.) produced a significant antianxiety-like behavior of unstressed control mice on EPM ($F_{9,50}$=73.53, p<0.05) and LDB ($F_{9,50}$=43.20, p<0.05) as compared to that of vehicle-treated unstressed mice (Figs. 1 and 2).

Effect of DZP on behavior of stressed control mice
Administration of DZP (2 mg/kg, i.p.) did not produce a significant antianxiety-like activity stressed control mice as compared to that of vehicle-treated stressed mice (Figs. 1 and 2).

Effect of AG and DZP on behavior of unstressed control mice
Administration of AG and DZP further produced a significant antianxiety-like behavior in unstressed control mice, similar to that in DZP-treated unstressed mice and significantly different from AG-treated unstressed mice (Figs. 1 and 2).

Effect of AG and DZP behavior of stressed control mice
Administration of AG and DZP further produced a significant antianxiety-like behavior in stressed control mice similar to that in AG-treated stressed mice, and significantly different from DZP-treated stressed mice (Figs. 1 and 2).

Effect of selected treatments on plasma corticosterone levels
Six hours immobilization stress produced a significant increase in plasma corticosterone levels in mice, as compared to that of unstressed control mice (Fig. 3).

Effect of selected treatments on plasma nitrite levels
6h immobilization stress produced a significant increase in plasma nitrite levels in mice, as compared to that of unstressed control mice. AG (50 mg/kg, i.p.) significantly attenuated the increased plasma nitrite levels in stressed mice (Fig. 4). DZP (2 mg/kg, i.p.) could not bring any significant change in plasma nitrite levels of stressed mice. Administration of DZP (2 mg/kg, i.p.) in combination with AG (50 mg/kg, i.p.) also could not bring any significant change in plasma nitrite levels of stressed mice, as compared to that of stressed mice, treated with AG (50 mg/kg, i.p.) alone.

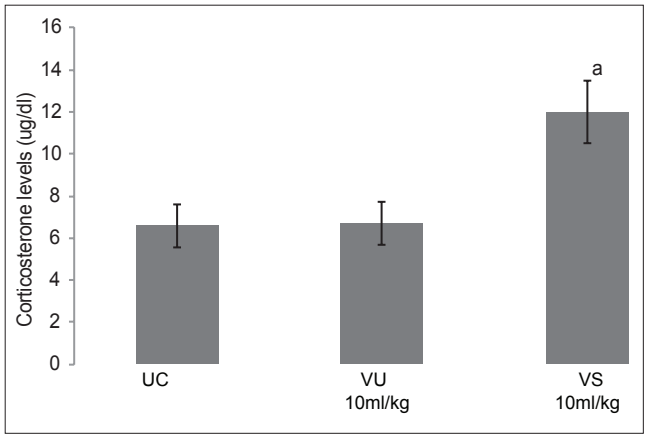

Fig. 3: Effect of different treatments on plasma corticosterone levels (ug/dl). n=6 in each group. Values are expressed as mean ± standard error. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test, $F_{2,15}$=6.76; p<0.0001, [a]p<0.05 significant difference from unstressed control mice and vehicle-treated unstressed mice. UC: Unstressed control mice, VU: Vehicle-treated unstressed mice, VS: Vehicle-treated stressed mice. Values mentioned are doses of vehicle

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.

DISCUSSION

Forced immobilization is one of the best-explored models of stress in rodents. This model combines emotional stress (escape reaction) and physiological stress (muscle work), resulting in both restricted mobility and aggression. As painful stimuli are not directly involved in restraint stress, this form of stress is probably more akin to physiological stress [42]. Stress-potentiated behavior serves to be a better measure of anxiety because it is the relevant and physiologically close pathological feature of anxiety in humans [43]. Stress initiates a series of underlying mechanisms and cascades and one of the several implicated chemicals that is elevated in stress, is NO [1]. Acute restraint stress for as short as 2 hrs has been reported to activate NOS and is known to increase the anxiety behavior in rodents [4]. Restraint used in the present study (6 hrs) has also produced the similar behavior of anxiety in mice.

Both EPM and LDT are behaviorally and pharmacologically validated animal models of anxiety used for years to test anxiety. Moreover, EPM and light and dark test offer a similar environment of light and dark transition, against which the animals have been tested for behavioral responses. So this combination of tests in the series may serve to enhance the robustness of the procedure. We have found that stress-exposed mice were more anxious in their behavior, when tested on EPM and LDT as compared to unstressed mice. Belzung and Griebel, 2001 [7] have discussed that continuous exposure to different stressors results in the enhancement of normal anxiety.

In the present study, AG (50 mg/kg) showed significant anti-anxiety effect in immobilization-induced stressed mice; employing well-known behavioral models of anxiety, such as EPM and light/dark test. AG (50 mg/kg and 100 mg/kg) used in the present study has decreased immobilization stress-induced anxiogenic behavior in EPM and LDT. These observed effects of AG support the involvement of iNOS in immobilization stress-induced damage in rodents [20], an experimental procedure accepted relevant as a model of stress disorders in humans [44].

DZP (2 mg/kg) produced a significant anxiolytic-like effect in unstressed mice but could not exert significant anxiolysis in stressed mice. In this study, immobilization induced stress-induced a significant attenuation in the anxiolytic effect of DZP in well-known behavioral models of anxiety, such as the EPM and LDT. This is the second report showing the insignificant antianxiety effect of DZP at a dose of 2 mg/kg in immobilization (6 hrs) induced stressed mice after report of Sharma *et al*., 2011 [45]. Reduction of GABAergic tone was found in stressed animals [46,47]. The above reports are further supported by studies on DZP viz. L-Arginine (100 mg/kg, i.p.), assumed to increase the synthesis of NO, has been reported to abolish the anxiolytic-like effect of DZP (2 mg/kg, i.p.) [38]. In the present study, DZP failed to bring any change in nitrite levels in both unstressed and stressed mice. 6h immobilization stress produces a significant increase in nitrite levels and exaggeration of anxiety with persistent changes in GABA-benzodiazepine-barbiturate complex in brains of stressed animals [48]. This inability of DZP to modify the stress-induced increased nitriergic influence may be responsible for the compromised effect of DZP in stressed mice.

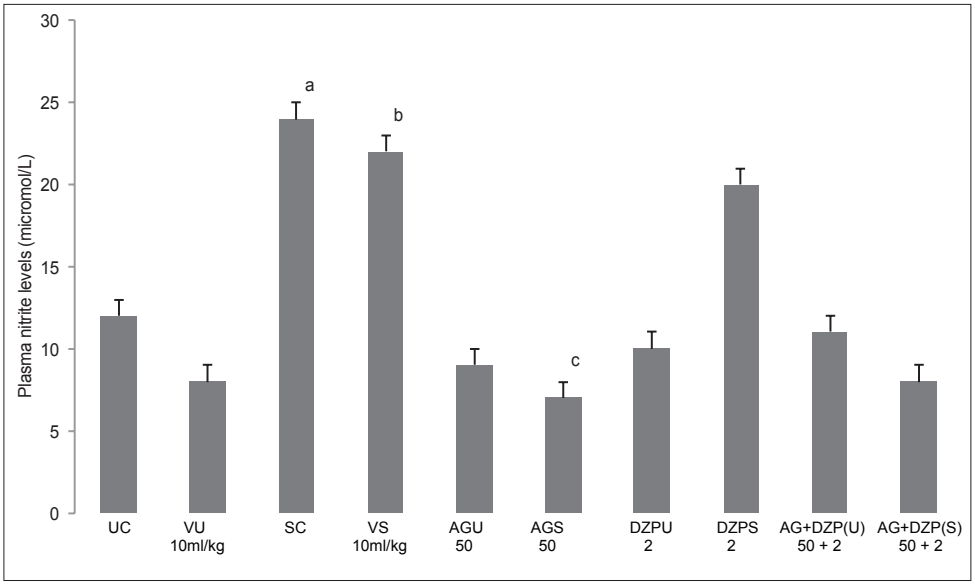

Fig. 4: Effect of different treatments on plasma nitrite levels (micromole/liter). n=6 in each group. Values are expressed as mean ± standard error. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test, $F_{9,50}$=3.4, p<0.0001, [a]p<0.05 significant difference from unstressed control mice, [b]p<0.001 significant difference from vehicle-treated unstressed control mice, [c]p<0.001 significant difference from stressed mice. UC: Unstressed control mice, VU: Vehicle treated unstressed mice, SC: Stressed control mice, VS: Vehicle-treated stressed mice, AGU: Amino guanidine-treated unstressed mice, AGS: Amino guanidine-treated stressed mice, DZPU: Diazepam-treated unstressed mice, DZPS: Diazepam-treated stressed mice AG+DZP (U): Amino guanidine and diazepam-treated unstressed mice, AG+DZP(S): Amino guanidine and diazepam-treated stressed mice. Values mentioned are doses in mg/kg

Stronger effect of AG in stressed mice, even more than that of DZP may be attributed to the inhibitory effect of AG on iNOS. On the other hand, DZP has no effect on NO in stressed conditions, because of which, the effect of DZP is suppressed in stressed conditions. This observation is supported by the earlier observation from our own laboratory [45]. Similar observations are recorded in studies, where GABA levels are decreased in stressed animals, and GABA dependent anti-anxiety activity of DZP is suppressed [37,45,49]. These observations strengthen the results of experiments, that, pharmacological treatments may behave differently in different forms of anxiety as documented by Belzung and Griebel, 2001 [7] that even standard treatments like benzodiazepines and serotonin receptor agonists behaved differently in different forms of anxiety and also supported by similar earlier observation on DZP [37,45]. Further, the study fulfills the author's objectives and answers the study questions in a point wise manner that forms the conclusion of the present study.

The insignificant difference by drug treatments in locomotor activity of unstressed and stressed mice is supported by earlier reports on DZP [50,51] and AG [27,37].

AG, a selective inhibitor of iNOS, reduced the plasma nitrite levels in mice previously subjected to immobilization stress, which further supports the observed anti-anxiety effect of AG. Our results are in accordance with the earlier reported role of NO as anxiogenic mediator [14,52]. The inability of DZP to bring any change in plasma nitrite levels may be supported by the earlier indications that Moreover, it is shown that benzodiazepine anxiolytics do not protect against various emotional changes produced by stress stimuli in mice [53].

Of course, the advent of more non-addictive and safer anxiety medications led to underuse 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. It is quite obvious to be tempted when a molecule with marketed status like AG indicates a possibility of use in combination with another clinically efficacious agent (DZP). 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 [54]. Similar reports of GABAergic receptor system disturbances by immobilization stress indicate possibility of similar compromise in effect of DZP in stressed conditions and AG (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 nitriergic mechanism of enzymatic inhibition (iNOS inhibition), thus avoiding receptor-mediated adverse effect profile of the conventional anxiolytics. These findings are even important in light of reports that under stressed conditions, psychopathological symptoms appear to precipitate and manifest to even more extent. Therefore, the present findings help to provide a clue to the possible utility of AG in stress-related anxiety alone or co-administration with DZP. The findings of the present study may further be strengthened by future studies on AG in combination with other drugs in the benzodiazepine and non-benzodiazepine category.

REFERENCES

1. Esch T, Stefano GB, Fricchione GL, Benson H. The role of stress in neurodegenerative diseases and mental disorders. Neuro Endocrinol Lett 2002;23(3):199-208.
2. Tsuchiya T, Kishimoto J, Koyama J, Ozawa T. Modulatory effect of L-NAME, a specific nitric oxide synthase (NOS) inhibitor, on stress-induced changes in plasma adrenocorticotropic hormone (ACTH) and corticosterone levels in rats: Physiological significance of stress-induced NOS activation in hypothalamic-pituitary-adrenal axis. Brain Res 1997;776(1-2):68-74.
3. Madrigal JL, Hurtado O, Moro MA, Lizasoain I, Lorenzo P, Castrillo A, *et al*. The increase in TNF-alpha levels is implicated in NF-kappaB activation and inducible nitric oxide synthase expression in brain cortex after immobilization stress. Neuropsychopharmacology 2002;26(2):155-63.
4. Sevgi S, Ozek M, Eroglu L. L-NAME prevents anxiety-like and depression-like behavior in rats exposed to restraint stress. Methods Find Exp Clin Pharmacol 2006;28(2):95-9.
5. Hata T, Nishikawa H, Itoh E, Funakami Y. Anxiety-like behavior in elevated plus-maze tests in repeatedly cold-stressed mice. Jpn J Pharmacol 2001;85(2):189-96.

6. Albonetti ME, Farabollini F. Behavioural responses to single and repeated restraint in male and female rats. Behav Processes 1992;28(1-2):97-109.
7. Belzung C, Griebel G. Measuring normal and pathological anxiety-like behaviour in mice: A review. Behav Brain Res 2001;125(1-2):141-9.
8. Kim CK, Rivier CL. Nitric oxide and carbon monoxide have a stimulatory role in the hypothalamic-pituitary-adrenal response to physico-emotional stressors in rats. Endocrinology 2000;141(6):2244-53.
9. Harada S, Imaki T, Chikada N, Naruse M, Demura H. Distinct distribution and time-course changes in neuronal nitric oxide synthase and inducible NOS in the paraventricular nucleus following lipopolysaccharide injection. Brain Res 1999;821(2):322-32.
10. Heinrichs SC, Pich EM, Miczek KA, Britton KT, Koob GF. Corticotropin-releasing factor antagonist reduces emotionality in socially defeated rats via direct neurotropic action. Brain Res 1992;581(2):190-7.
11. Quock RM, Nguyen E. Possible involvement of nitric oxide in chlordiazepoxide-induced anxiolysis in mice. Life Sci 1992;51(25):PL255-60.
12. Monzón ME, Varas MM, De Barioglio SR. Anxiogenesis induced by nitric oxide synthase inhibition and anxiolytic effect of melanin-concentrating hormone (MCH) in rat brain. Peptides 2001;22(7):1043-7.
13. Roohbakhsh A, Moghaddam AH, Massoudi R, Zarrindast MR. Role of dorsal hippocampal cannabinoid receptors and nitric oxide in anxiety like behaviours in rats using the elevated plus-maze test. Clin Exp Pharmacol Physiol 2007;34(3):223-9.
14. Volke V, Soosaar A, Kõks S, Bourin M, Männistö PT, Vasar E. 7-Nitroindazole, a nitric oxide synthase inhibitor, has anxiolytic-like properties in exploratory models of anxiety. Psychopharmacology (Berl) 1997;131(4):399-405.
15. Yildiz F, Ulak G, Erden BF, Gacar N. Anxiolytic-like effects of 7-nitroindazole in the rat plus-maze test. Pharmacol Biochem Behav 2000;65(2):199-202.
16. Forestiero D, Manfrim CM, Guimarães FS, de Oliveira RM. Anxiolytic-like effects induced by nitric oxide synthase inhibitors microinjected into the medial amygdala of rats. Psychopharmacology (Berl) 2006;184(2):166-72.
17. Spolidório PC, Echeverry MB, Iyomasa M, Guimarães FS, Del Bel EA. Anxiolytic effects induced by inhibition of the nitric oxide-cGMP pathway in the rat dorsal hippocampus. Psychopharmacology (Berl) 2007;195(2):183-92.
18. Stuehr DJ, Marletta MA. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma. J Immunol 1987;139(2):518-25.
19. Förstermann U, Kleinert H. Nitric oxide synthase: Expression and expressional control of the three isoforms. Naunyn Schmiedebergs Arch Pharmacol 1995;352(4):351-64.
20. Olivenza R, Moro MA, Lizasoain I, Lorenzo P, Fernández AP, Rodrigo J, *et al*. Chronic stress induces the expression of inducible nitric oxide synthase in rat brain cortex. J Neurochem 2000;74(2):785-91.
21. Madrigal JL, Moro MA, Lizasoain I, Lorenzo P, Castrillo A, Boscá L, *et al*. Inducible nitric oxide synthase expression in brain cortex after acute restraint stress is regulated by nuclear factor kappaB-mediated mechanisms. J Neurochem 2001;76(2):532-8.
22. Sapolsky RM, Uno H, Rebert CS, Finch CE. Hippocampal damage associated with prolonged glucocorticoid exposure in primates. J Neurosci 1990;10(9):2897-902.
23. Sheline YI, Wang PW, Gado MH, Csernansky JG, Vannier MW. Hippocampal atrophy in recurrent major depression. Proc Natl Acad Sci U S A 1996;93(9):3908-13.
24. Southan GJ, Szabó C. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. Biochem Pharmacol 1996;51(4):383-94.
25. Klandorf H, Zhou Q, Sams AR. Inhibition by aminoguanidine of glucose-derived collagen cross-linking in skeletal muscle of broiler breeder hens. Poult Sci 1996;75(3):432-7.
26. Lee FY, Wang SS, Tsai YT, Lin HJ, Lin HC, Chu CJ, *et al*. Aminoguanidine corrects hyperdynamic circulation without ameliorating portal hypertension and portal hypertensive gastropathy in anesthetized portal hypertensive rats. J Hepatol 1997;26(3):687-93.
27. Gilhotra N, Dhingra D. Involvement of NO-cGMP pathway in anti-anxiety effect of aminoguanidine in stressed mice. Prog Neuropsychopharmacol Biol Psychiatry 2009;33(8):1502-7.
28. Schwartz TL, Nihalani N, Simionescu M, Hopkins G. History repeats itself: Pharmacodynamic trends in the treatment of anxiety disorders. Curr Pharm Des 2005;11(2):255-63.
29. Elie R, Lamontagne Y. Alprazolam and diazepam in the treatment of generalized anxiety. J Clin Psychopharmacol 1984;4(3):125-9.
30. Saddock BJ, Saddock VA. Kaplan & Saddock's Pocket Handbook of Psychiatric Drug Treatment. 3rd ed. USA: Lippincoat, Williams, & Wilkins; 2001.
31. Talarek S, Listos J, Fidecka S. Role of nitric oxide in the development of tolerance to diazepam-induced motor impairment in mice. Pharmacol Rep 2008;60(4):475-82.
32. Talarek S, Orzelska J, Listos J, Fidecka S. Effects of sildenafil treatment on the development of tolerance to diazepam-induced motor impairment and sedation in mice. Pharmacol Rep 2010;62(4):627-34.
33. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem 1982;126(1):131-8.
34. Bartos J, Pesez M. Colorimetric and fluorimetric determination of steroids. Pure Appl Chem 1979;51:2157-69.
35. 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(2):195-206.
36. Harvey BH, Oosthuizen F, Brand L, Wegener G, Stein DJ. Stress-restress evokes sustained iNOS activity and altered GABA levels and NMDA receptors in rat hippocampus. Psychopharmacology (Berl) 2004;175(4):494-502.
37. Gilhotra N, Dhingra D. GABAergic and nitriergic modulation by curcumin for its antianxiety-like activity in mice. Brain Res 2010;1352:167-75.
38. Volke V, Soosaar A, Kõks S, Vasar E, Männistö PT. L-Arginine abolishes the anxiolytic-like effect of diazepam in the elevated plus-maze test in rats. Eur J Pharmacol 1998;351(3):287-90.
39. Goyal R, Anil K. Protective effect of alprazolam in acute immobilization stress-induced certain behavioral and biochemical alterations in mice. Pharmacol Rep 2007;59(2):284-90.
40. Kumari B, Kumar A, Dhir A. Protective effect of non-selective and selective COX-2-inhibitors in acute immobilization stress-induced behavioral and biochemical alterations. Pharmacol Rep 2007;59(6):699-707.
41. Gilhotra N, Jain H, Dhingra D. Differential effects of nitric oxide synthase inhibitors on anxiety in unstressed and stressed mice. Indian J Exp Biol 2010;48(4):365-72.
42. Bhattacharya SK, Bhattachatyya D. Effect of restraint stress on rat brain serotonin. J Biosci 1982;4:269-74.
43. 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(1-3):163-75.
44. Bremner JD. Animal models for the neurobiology of trauma. PTSD Res Quart 1991;2:1.
45. Sharma V, Gilhotra R, Dhingra D, Gilhotra N. Possible underlying influence of p38MAPK and NF-κB in the diminished anti-anxiety effect of diazepam in stressed mice. J Pharmacol Sci 2011;116(3):257-63.
46. Concas A, Mele S, Biggio G. Foot shock stress decreases chloride efflux from rat brain synaptoneurosomes. Eur J Pharmacol 1987;135(3):423-7.
47. Drugan RC, Morrow AL, Weizman R, Weizman A, Deutsch SI, Crawley JN, *et al*. Stress-induced behavioral depression in the rat is associated with a decrease in GABA receptor-mediated chloride ion flux and brain benzodiazepine receptor occupancy. Brain Res 1989;487(1):45-51.
48. Weizman A, Bidder M, Fares F, Gavish M. Food deprivation modulates gamma-aminobutyric acid receptors and peripheral benzodiazepine binding sites in rats. Brain Res 1990;535(1):96-100.
49. Gilhotra N, Dhingra D. Thymoquinone produced antianxiety-like effects in mice through modulation of GABA and NO levels. Pharmacol Rep 2011;63(3):660-9.
50. Krsiak M. Timid singly-housed mice: Their value in prediction of psychotropic activity of drugs. Br J Pharmacol 1975;55:141-50.
51. Kurt M, Bilge SS, Aksoz E, Kukula O, Celik S, Kesim Y. Effect of sildenafil on anxiety in the plus-maze test in mice. Pol J Pharmacol 2004;56(3):353-7.
52. Faria MS, Muscará MN, Moreno Júnior H, Teixeira SA, Dias HB, De Oliveira B, *et al*. Acute inhibition of nitric oxide synthesis induces anxiolysis in the plus maze test. Eur J Pharmacol 1997;323(1):37-43.
53. Tsuji M, Takeda H, Matsumiya T. Different effects of 5-HT1A receptor agonists and benzodiazepine anxiolytics on the emotional state of naive and stressed mice: A study using the hole-board test. Psychopharmacology (Berl) 2000;152(2):157-66.
54. Cowen PJ, Nutt DJ. Abstinence symptoms after withdrawal of tranquillising drugs: Is there a common neurochemical mechanism? Lancet 1982;2(8294):360-2.