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ORIGINAL ARTICLE

Investigating the stress attenuating potential of furosemide in immobilization and electric foot-shock stress models in mice

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Abstract The present study was designed to investigate the antistress effect of furosemide (sodium potassium chloride co-transporter inhibitor) in immobilization and foot-shock stress-induced behavioral alterations in the mice. Acute stress was induced in Swiss albino mice either by applying electric foot shocks of 0.6-mA intensity of 1-s duration with 30-s inter-shock interval for 1 h or immobilizing for 150 min. The acute stress-induced behavioral changes were assessed by using actophotometer, hole board, open-field, and social interaction tests. Biochemically, the corticosterone levels were estimated in the serum as a biomarker of hypothalamus-pituitary-adrenal (HPA) axis. Acute stress resulted in the development of behavioral alterations and elevation of the corticosterone levels. Intraperitoneal administration of furosemide (25 and 50 mg/kg) significantly attenuated immobilization and foot-shock stress-induced behavioral changes along with normalization of the corticosterone levels. It may be concluded that furosemide produces beneficial effects in reestablishing the behavioral and biochemical alterations in immobilization and foot-shock-induced acute stress in mice.

Keywords Stress · Furosemide · Immobilization · Electric foot shock · Corticosterone

Introduction

Stress is a state of threatened homeostasis which produces various physiological and pathological changes (Ravindran et al. 2005; Bali and Jaggi 2013; Bali et al. 2013). The ability to cope with stressor is a crucial determinant of health, and inability to cope up with stressor is associated with the development of various pathophysiological changes. Stress is a predecessor and is a causative factor for the development of anxiety and depression. Both anxiety and depression are the result of an inappropriate adaptation to stress and have been termed as stress-related disorders, with a causal role of the HPA axis (Bali et al. 2014). The changes involved in disturbing the homeostasis of an organism trigger various modifications including alteration in behavior, autonomic function, and over-activation of hypothalamo-pituitary-adrenal (HPA) axis (Bali and Jaggi 2013, 2014). The HPA axis is regulated by a set of hypophysiotrophic neurons present in the medial parvocellular division of the hypothalamic paraventricular nucleus (PVN) (Herman et al. 2005). The activation of the HPA axis quickly process signals in the hypothalamic paraventricular nucleus and increases the release of corticotrophin releasing hormone (CRH) which activates anterior pituitary to cause the secretion of adrenocorticotropin (ACTH), which in turn stimulates the secretion of corticosterone and other glucocorticoids from the adrenal cortex (Pacak et al. 1993). Glucocorticoids are the final effectors of the HPA axis over-activation during stress and may be involved in the generation of various deleterious effects of stress (Herman et al. 2005; Osterlund and Spencer 2011).

Sodium potassium chloride co-transporters (NKCC) belong to the family of cation-dependent chloride co-transporter (CCC) and primarily mediate transport of Na^+, K^+, and Cl^- ions into the cell (Haas and Forbush 2000; Su et al. 2000). NKCC1 and NKCC2 are two members of NKCC family, and NKCC2 is exclusively present on the thick ascending limb of Henle in the kidneys (Geck et al. 1980). However, NKCC1 is widely distributed in various types of tissues, including the stomach, heart, skeletal muscle, lungs, brain, and kidney (Russell 2000; Pedersen et al. 2006).There is extensive distribution of NKCC1 on the components of the HPA axis including the hypothalamus (Nugent et al. 2012),

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anterior pituitary (Zemkova et al. 2008), and adrenal gland (Xie et al. 2003). Furthermore, these channels are also widely distributed in other stress-responsive regions also including the hippocampus, amygdala, and fornix (Okabe et al. 2002; Wang et al. 2002). The neuronal actions of NKCC1 activation are primarily related to the reversal of inhibitory actions of GABA neurotransmitter in the brain regions. Although GABA primarily produces inhibitory actions due to the inward movement of Cl^- ions (hyperpolarization), yet activation of NKCC causes intracellular accumulation of chloride ions to alter the Cl^- gradient. It is followed by net outward movement of Cl^- ions (depolarization) due to opening of GABA-regulated Cl^- channels and reversal of hyperpolarizing (inhibitory) action of GABA to depolarizing (excitatory) actions (Brumback and Staley 2008; Nardou et al. 2009).

The selective inhibitors of NKCC including bumetanide and furosemide are conventionally employed as diuretics. Recent studies have indicated that NKCC1 also plays an important role in various other pathological states, including anxiety, cerebral ischemia, epilepsy, neuropathic pain, and myocardial infarction. The inhibitors of NKCC are shown to produce neuroprotective effects in cerebral ischemia-induced neuronal injury (Beck et al. 2003; Wallace et al. 2012), antiepileptic effects in various models of epilepsy (Brandt et al. 2010), and attenuate neuropathic pain (Pitcher and Cervero 2010). A recent study has shown the anxiolytic effects of bumetanide and furosemide in the conditioned models of anxiety (contextual fear-conditioning and fear-potentiated startle), without altering the behavior in unconditioned models (elevated plus maze and open-field test) of anxiety (Krystal et al. 2012). However, the role of NKCC inhibitors in different experimental stress models has not been investigated yet. Therefore, the present study was designed to investigate the effects of a clinically employed inhibitor of NKCC, i.e., furosemide on behavioral and biochemical alterations in acute immobilization or electric foot-shock models of experimental stress in mice.

Material and methods

Animals, drug, and chemical

In the present study, Swiss albino mice, weighing 20–30 g were obtained from Chaudhary Charan Singh, Haryana Agriculture University, Hisar, India. Animals were fed on standard laboratory feed (Ashirwad Industries, Kharar, Distt. Mohali) and watered ad libitum. Animals were housed in the departmental animal house and were exposed to 12-h light/12-h dark cycle (lights on at 0700 hours). The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC), and care of the animals was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Ministry of Environment and Forests, Government of India (Reg. No. 107/1999/CPCSEA). Furosemide was procured from Sanofi Aventis Pharma Limited, J-174, M.I.D.C., Tarapur, Boisar, Thane-401 506, India. All other chemicals and reagents used were purchased from S.D. Fine Chemicals Ltd., Mumbai, India, and were of analytical grade.

Induction of immobilization-induced acute stress

The mice were subjected to acute stress for 150 min by immobilizing in prone position with all their limbs stretched on a board using an adhesive tape. The movement of the head was restricted by keeping the head in a metal loop coiled around the neck (Kvetnansky and Mikulaj 1970; Bhatia et al. 2011; Kumar et al. 2012).

Induction of foot-shock-induced acute stress

Acute electric foot-shock stress was induced by introduction of the mouse in a Plexiglas chamber (26×21×26 cm), with a grid floor made of stainless steel rods (0.3-cm diameter, spaced 1.0 cm apart). Electrical foot shocks of 0.6-mA intensity of 1-s duration with 30-s inter-shock interval were delivered for 1 h (Aoki et al. 2003).

Behavioral assessment

The mice were acclimatized for 5 min on each behavioral test apparatus for 3 days before subjecting to immobilization and foot-shock stress. To avoid potentially confounding results due to novelty of the testing apparatus, it is necessary to acclimatize the animals to the test apparatus in the behavioral studies which in turn reduces the variation in the experimental data. The behavioral tests were performed with the sequence of the actophotometer, the hole board, the open field, and the social interaction with a time gap of 5 min between each successive tests. The experimental procedures were done in between 0830 and 1300 hours, when resting and stress levels of HPA hormones are very stable (Rabasa et al. 2011).

Actophotometer test

The locomotor activity is considered as an index of awareness and was assessed by keeping the mice in the actophotometer individually. The locomotor activity was assessed in terms of counts per 10 min (Agrawal et al. 2011; Kumar et al. 2012). In an actophotometer, the movement of the animal interrupts the beam of light falling on the photocell and a count is recorded digitally. Therefore, the number of counts is directly related to movement of the animal inside the actophotometer chamber.

Hole board test

The hole board test was employed to evaluate the exploratory behavior of animals. The mice were assessed for 10 min in the hole board during which head dips and rearings were recorded (Brown and Nemes 2008; Agrawal et al. 2011; Kumar et al. 2012).

Open-field test

The open-field test has been employed to evaluate the spontaneous activity, general exploration, and ambulation of the rodents. The mice were placed in the center of the open field, and the number of line crossings and rearings was noted for a 10-min period (Manchanda et al. 2011).

Social interaction test

The social interaction test has been widely used as an anxiety-related paradigm. The social interaction test was carried out in the same box in which open-field test was performed. After 5 min of open-field test, the mice were again placed to the same arena for social interaction test. Each experimental animal was tested with a partner mouse, which was socially housed and was not subjected to any stressor. For recognition purpose, the partner mouse of each pair was marked on its back using a nontoxic black marker. During the 10-min test, the following social behaviors of experimental animals were noted: sniffing the partner, contact interaction (physical contact with mutual responses and orientation toward the other), following the partner, climbing over or burrowing under it, and walking around it. Boxing, biting, or threatening the partner mouse, self-grooming, and remaining alone away from the partner were considered as nonsocial behaviors (Kaur et al. 2009; Agrawal et al. 2011).

Blood sampling and serum separation procedure

The blood was withdrawn from retro-orbital sinus and collected in centrifugation tubes. These tubes were allowed to settle at room temperature for 15–20 min for blood clotting. Thereafter, tubes were centrifuged at 2000*g* for 10 min for separation of serum. The blood withdrawal from the retro-orbital sinus has been one of the common methods in rodents as in the hands of a skilled operator retro-orbital bleeding is a gentle procedure that produces minimal and transient pain/distress. Furthermore, this procedure is relatively rapid enabling the collection of blood from a large number of animals within a short period of time.

Serum corticosterone measurement

The corticosterone levels in the mice serum were assessed by spectrofluorometric assay (Glick et al. 1964; Lim et al. 1981). Twenty-five microliters of serum was mixed with 25 μl of distilled water, 0.6 ml of ethylene dichloride, and 0.1 ml of 1 N sodium hydroxide. The mixture was shaken by vortexing for 2 min and centrifuged at 550 g for 5 min. The top alkaline layer was pipetted off carefully. Then, 0.1 ml of distilled water and 1.2 ml of carbon tetrachloride were added to the remaining corticosterone precipitate and solvent layer. This mixture was again shaken vigorously for 2 min by vortexing and followed by centrifugation at 550*g* for 5 min. After centrifugation, the top aqueous layer was discarded, and 0.4 ml of freshly prepared acid-alcohol mixture (3:1 of H_2SO_4-absolute ethanol) was added to the remaining solution to induce fluorescence development. The final mixture was again shaken by vortexing for 2 min and then centrifuged at 550*g* for 5 min. The mixture was allowed to settle for 30 min at room temperature. Thereafter, the top solvent layer was discarded, and the bottom acid layer was used for determining fluorescence production at 475-nm excitation and 525-nm emissions on a spectrofluorometer (Bhatia et al. 2011; Kumar et al. 2012).

Experimental protocol

Eight groups, each comprising of six Swiss albino mice, were employed in the present study.

Group I: nonstress control Mice were not subjected to any stressor, and different behavioral tests including actophotometer, hole board, open-field, and social interaction tests were performed. At the end, the blood was withdrawn from retro-orbital sinus for the estimation of corticosterone.

Group II: immobilization stress Mice were subjected to immobilization stress for 150 min, and thereafter, different behavioral tests such as actophotometer, the hole board, the open-field, and the social interaction tests were performed. At the end, the blood was withdrawn from retro-orbital sinus for the estimation of corticosterone.

Group III: furosemide (25 mg/kg, i.p.) in immobilization stress A single dose of furosemide (25 mg/kg, *i.p.*) was administered 30 min prior to immobilization stress. Thereafter, the behavioral and the biochemical parameters were assessed as described in group II.

Group IV: furosemide (50 mg/kg, i.p.) in immobilization stress A single dose of furosemide (50 mg/kg, *i.p.*) was administered 30 min prior to immobilization stress. Thereafter, the behavioral and the biochemical parameters were assessed as described in group II.

Group V: electric foot-shock stress Mice were subjected to foot-shock stress for 60 min, and thereafter, different behavioral tests such as actophotometer, the hole board, the open-field, and the social interaction tests were performed. At the end, the blood was withdrawn from retro-orbital sinus for the estimation of corticosterone.

Group VI: furosemide (25 mg/kg, i.p.) in foot-shock stress A single dose of furosemide (25 mg/kg, *i.p.*) was administered 30 min prior to foot-shock stress. Thereafter, the behavioral and the biochemical parameters were assessed as described in group V.

Group VII: furosemide (50 mg/kg, i.p.) in foot-shock stress A single dose of furosemide (50 mg/kg, *i.p.*) was administered 30 min prior to foot-shock stress. Thereafter, the behavioral and the biochemical parameters were assessed as described in group V.

Group VIII: furosemide (50 mg/kg, i.p.) per se A single dose of furosemide (50 mg/kg, *i.p.*) was administered, and thereafter, the behavioral and the biochemical parameters were assessed as described in group II.

Statistical analysis

The results were expressed as mean±standard error of means (S.E.M.). Data were statistically analyzed by using one-way ANOVA followed by Tukey's multiple range test. The value of $p<0.05$ was considered to be statistically significant.

Results

Effect of furosemide on the locomotor activity in immobilized or foot-shock-stress-subjected mice in actophotometer test

In immobilization or foot-shock-stressed mice, the locomotor activity was decreased significantly as compared to the nonstressed mice. Administration of furosemide (25 mg/kg, *i.p.*) and (50 mg/kg, *i.p.*) 30 min before subjecting to immobilization or foot-shock stress significantly attenuated immobilization or foot-shock stress-induced decrease in locomotor activity. Furthermore, per se administration of furosemide (50 mg/kg, *i.p.*) in the nonstressed mice did not modulate locomotor activity in a significant manner (Fig. 1).

Effect of furosemide on head dips and rearings in immobilized and foot-shock stress-subjected mice in hole board test

The head dips are considered to be an index of exploration or curiosity, while the frequency of rearings reflects the exploration of novel surrounding. In immobilization and foot-shock-stressed mice, the frequency of head dips and rearings was decreased significantly as compared to the nonstressed mice. Administration of furosemide (25 mg/kg, *i.p.*) and (50 mg/kg, *i.p.*) 30 min before subjecting to immobilization or foot-shock stress resulted in significant attenuation of immobilization or foot-shock stress-induced decrease in frequency of head dips and rearings. Furthermore, per se administration of furosemide (50 mg/kg, *i.p.*) in the nonstressed mice did not modulate exploratory behavior in a significant manner (Figs. 2 and 3).

Effect of furosemide on rearings and line crossings in immobilized and foot-shock stress-subjected mice in open-field test

The line crossings are taken as an indicator of motor activity, and the frequency of rearings reflects the exploration of novel surroundings. In immobilization or foot-shock-stressed mice, the number of line crossings was decreased significantly as compared to the nonstressed mice. Administration of furosemide (25 mg/kg, *i.p.*) and (50 mg/kg, *i.p.*) 30 min before subjecting to immobilization or foot-shock stress resulted in significant attenuation of immobilization or foot-shock stress-induced decrease in line crossings and frequency of rearings. Furthermore, per se administration of furosemide (50 mg/kg, *i.p.*) in the nonstressed mice did not modulate the motor activity and exploratory behavior in a significant manner (Figs. 4 and 5).

Effect of furosemide on following and avoidance in immobilized or foot-shock stress-subjected mice in the social interaction test

In immobilization or foot-shock-stressed mice, avoidance (nonsocial behavior) time was significantly increased, and following time (social behavior) was significantly decreased as compared to the nonstressed mice, which exhibited social behavior (following the partner). Treatment with furosemide (25 mg/kg, *i.p.*) and (50 mg/kg, *i.p.*) 30 min before subjecting to immobilization or foot-shock stress resulted in significant attenuation of immobilization and foot-shock stress-induced nonsocial behavior. Furthermore, per se treatment with furosemide (50 mg/kg*i.p.*) did not modulate the social behavior in normal mice (Figs. 6 and 7).

Effect of furosemide on serum corticosterone levels in immobilized or foot-shock stress-subjected mice

In immobilization or foot-shock-stressed mice, there was significant rise in the serum corticosterone levels as

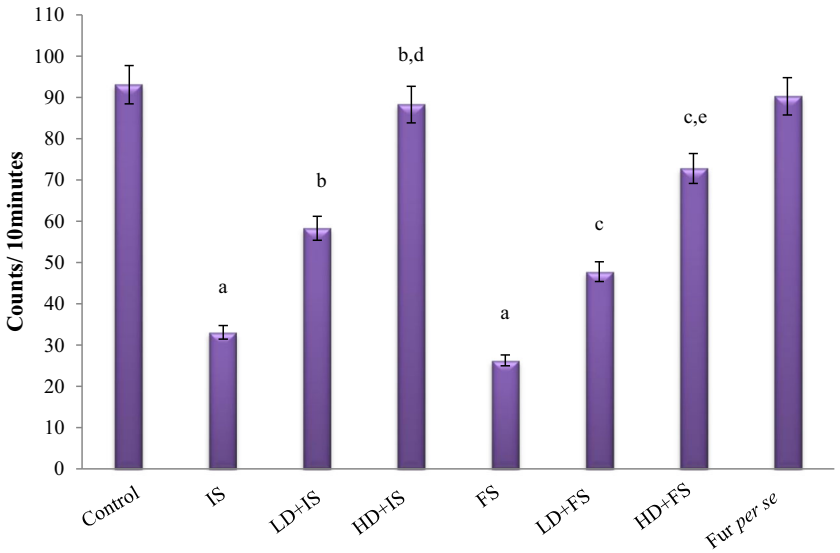

Fig. 1 Effect of furosemide on locomotor activity in immobilized or foot-shock stress-subjected mice in terms of counts in 10-min time interval in the actophotometer test. Data were statistically analyzed by using one-way ANOVA followed by Tukey's multiple range test, $n=6$, [$F(7,40)=35.571$; $p<0.001$], $^{a}p<0.05$ versus control group; $^{b}p<0.05$ versus immobilization stress group; $^{c}p<0.05$ versus foot-shock stress group; $^{d}p<0.05$ versus furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group; $^{e}p<0.05$ versus furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group. *IS* immobilization stress group, *LD+IS* furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group, *HD+IS* furosemide high dose (50 mg/kg, *i.p.*) in immobilization stress group, *FS* foot-shock stress group, *LD+FS* furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group, *HD+FS* furosemide high dose (50 mg/kg, *i.p.*) in foot-shock stress group, *Fur per se* furosemide (50 mg/kg, *i.p.*) per se

compared to the nonstressed mice. Treatment with furosemide (25 mg/kg, *i.p.*) and (50 mg/kg, *i.p.*) 30 min before subjecting to immobilization or foot-shock stress resulted in significant attenuation of immobilization and foot-shock stress-induced increase in the corticosterone levels. Furthermore, per se treatment with furosemide (50 mg/kg *i.p.*) did not modulate corticosterone levels in normal mice (Fig. 8).

Discussion

In the present study, induction of acute stress by subjecting mice to immobilization stress for 150 min produced significant alterations in behavior of animals including a decrease in locomotor activity in actophotometer test, decrease in head dips, and rearings in the hole board test along with decrease in total line crossings and rearings in the open-field test and

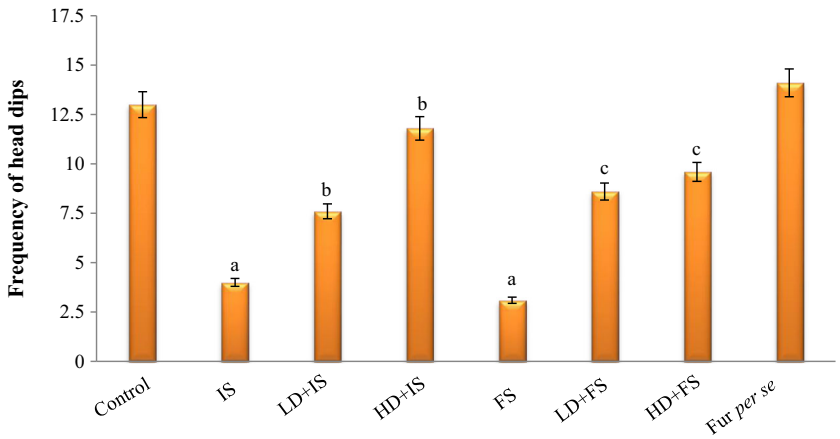

Fig. 2 Effect of furosemide on exploratory behavior in immobilized or foot-shock stress-subjected mice in terms of frequency of head dips in 10-min time interval in the hole board test. Data were statistically analyzed by using one-way ANOVA followed by Tukey's multiple range test, $n=6$, [$F(7,40)=12.376$; $p<0.001$], $^{a}p<0.05$ versus control group; $^{b}p<0.05$ versus immobilization stress group; $^{c}p<0.05$ versus foot-shock stress group. *IS* immobilization stress group, *LD+IS* furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group, *HD+IS* furosemide high dose (50 mg/kg, *i.p.*) in immobilization stress group, *FS* foot-shock stress group, *LD+FS* furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group, *HD+FS* furosemide high dose (50 mg/kg, *i.p.*) in foot-shock stress group, *Fur per se* furosemide (50 mg/kg, *i.p.*) per se

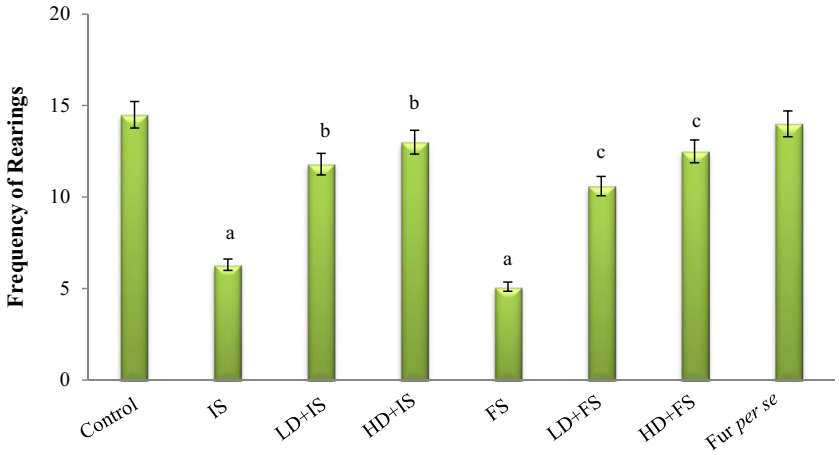

Fig. 3 Effect of furosemide on exploratory behavior in immobilized or foot-shock stress-subjected mice in terms of frequency of rearings in 10-min time interval in the hole board test. Data were statistically analyzed by using one-way ANOVA followed by Tukey's multiple range test, $n=6$, [$F(7,40)=9.880$; $p<0.001$], $^{a}p<0.05$ versus control group; $^{b}p<0.05$ versus immobilization stress group; $^{c}p<0.05$ versus foot-shock stress group. *IS* immobilization stress group, *LD+IS* furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group, *HD+IS* furosemide high dose (50 mg/kg, *i.p.*) in immobilization stress group, *FS* foot-shock stress group, *LD+FS* furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group, *HD+FS* furosemide high dose (50 mg/kg, *i.p.*) in foot-shock stress group, *Fur per se* furosemide (50 mg/kg, *i.p.*) per se

decrease in time of following and an increase in time of avoidance in the social-interaction test. Immobilization stress-induced behavioral alterations including decrease in locomotor activity, exploratory behavior, and social behavior have also been reported in our earlier study (Kumar et al. 2012). Immobilization is an intense stressor associated with struggling and muscular exertions that constitute a physical stress along with induction of psychological stress due to limited movement and exposure in an open area (Kvetnansky et al. 1979). Immobilization-induced stress has been one of more commonly employed model for the induction of acute stress because it produces an unavoidable physical and psychological stress in laboratory animals (Kasuga et al. 1999; Kumar et al. 2012; Jaggi et al. 2011). Furthermore, decrease in locomotor activity, exploratory, and social behavior was also observed in mice subjected to foot-shock stress by applying series of electric shocks of 0.6-mA intensity of 1-s duration with inter-shock interval of 30 s for 1 h. Electric foot-shock is also a very commonly employed stressor to induce acute stress in rodents, and it reliably produces both physical as well as psychological stress (Jaggi et al. 2011; Bali et al. 2013; Haj-Mirzaian et al. 2014).

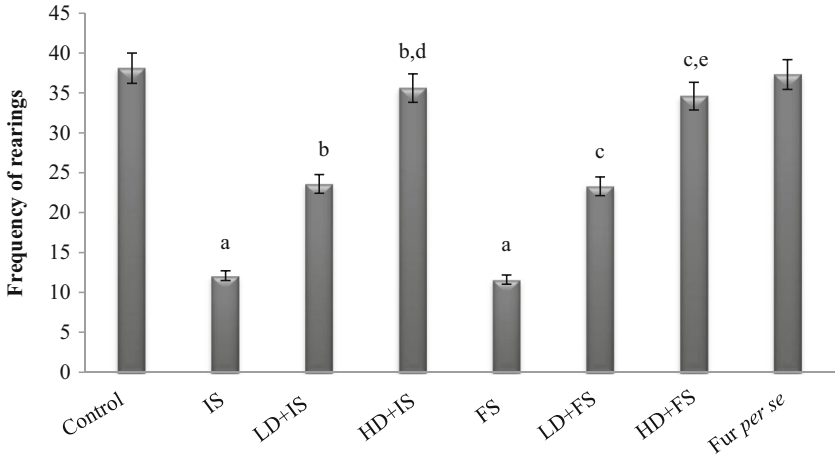

Fig. 4 Effect of furosemide on exploratory behavior in immobilized or foot-shock stress-subjected mice in terms of frequency of rearings in 10-min time interval in the open-field test. Data were statistically analyzed by using one-way ANOVA followed by Tukey's multiple range test, $n=6$, [$F(7,40)=19.268$; $p<0.001$], $^{a}p<0.05$ versus control group; $^{b}p<0.05$ versus immobilization stress group; $^{c}p<0.05$ versus foot-shock stress group; $^{d}p<0.05$ versus furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group; $^{e}p<0.05$ versus furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group. *IS* immobilization stress group, *LD+IS* furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group, *HD+IS* furosemide high dose (50 mg/kg, *i.p.*) in immobilization stress group, *FS* foot-shock stress group, *LD+FS* furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group, *HD+FS* furosemide high dose (50 mg/kg, *i.p.*) in foot-shock stress group, *Fur per se* furosemide (50 mg/kg, *i.p.*) per se

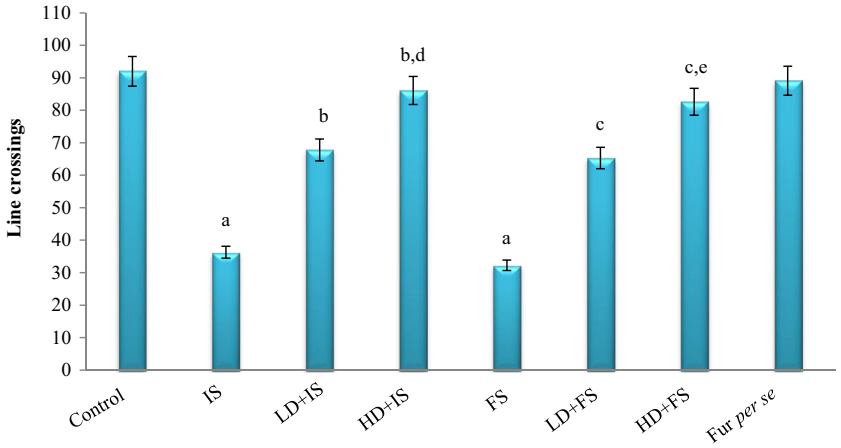

Fig. 5 Effect of furosemide on motor activity in immobilized or foot-shock stress-subjected mice in terms of line crossings in 10-min time interval in the open-field test. Data were statistically analyzed by using one-way ANOVA followed by Tukey's multiple range test, $n=6$, [$F(7,40)=42.872$; $p<0.001$], $^{a}p<0.05$ versus control group; $^{b}p<0.05$ versus immobilization stress group; $^{c}p<0.05$ versus foot-shock stress group; $^{d}p<0.05$ versus furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group; $^{e}p<0.05$ versus furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group. *IS* immobilization stress group, *LD+IS* furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group, *HD+IS* furosemide high dose (50 mg/kg, *i.p.*) in immobilization stress group, *FS* foot-shock stress group, *LD+FS* furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group, *HD+FS* furosemide high dose (50 mg/kg, *i.p.*) in foot-shock stress group, *Fur per se* furosemide (50 mg/kg, *i.p.*) per se

In the present study, pretreatment with furosemide (25 and 50 mg/kg) produced significant reductions in acute immobilization or foot-shock stress-induced behavioral alterations in mice. Furosemide is a selective inhibitor of NKCC and is clinically employed in patients with mild to severe congestive heart failure (Francis et al. 1985) and portal hypertension (Thapaliya et al. 2013) due to its diuretic effect. Apart from the well documented diuretic actions of NKCC blockers, the preclinical studies have also suggested their important role in attenuating cerebral ischemia-induced neuronal injury (Roberts et al. 1987), epilepsy (Oriaifo et al. 2012), neuropathic pain (Granados-Soto et al. 2005), and myocardial infarction (Ternacle et al. 2013). Recently, Krystal et al. reported that the administration of furosemide exerts significant anxiolytic effects in rats in conditioned models of anxiety (contextual fear-conditioning and fear-potentiated startle), but not in the

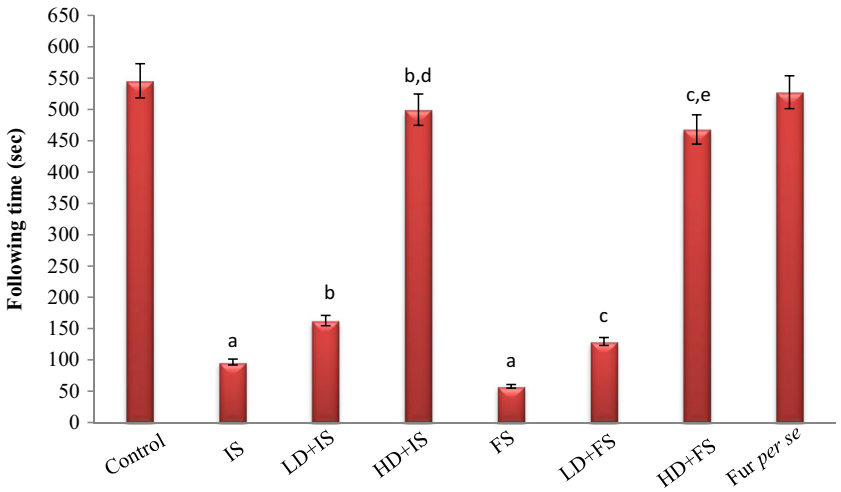

Fig. 6 Effect of furosemide on following time in immobilized or foot-shock stress-subjected mice in 10-min time interval in social interaction test. Data were statistically analyzed by using one-way ANOVA followed by Tukey's multiple range test, $n=6$, [$F(7,40)=1219.994$; $p<0.001$], $^{a}p<0.05$ versus control group; $^{b}p<0.05$ versus immobilization stress group; $^{c}p<0.05$ versus foot-shock stress group; $^{d}p<0.05$ versus furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group; $^{e}p<0.05$ versus furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group. *IS* immobilization stress group, *LD+IS* furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group, *HD+IS* furosemide high dose (50 mg/kg, *i.p.*) in immobilization stress group, *FS* foot-shock stress group, *LD+FS* furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group, *HD+FS* furosemide high dose (50 mg/kg, *i.p.*) in foot-shock stress group, *Fur per se* furosemide (50 mg/kg, *i.p.*) per se

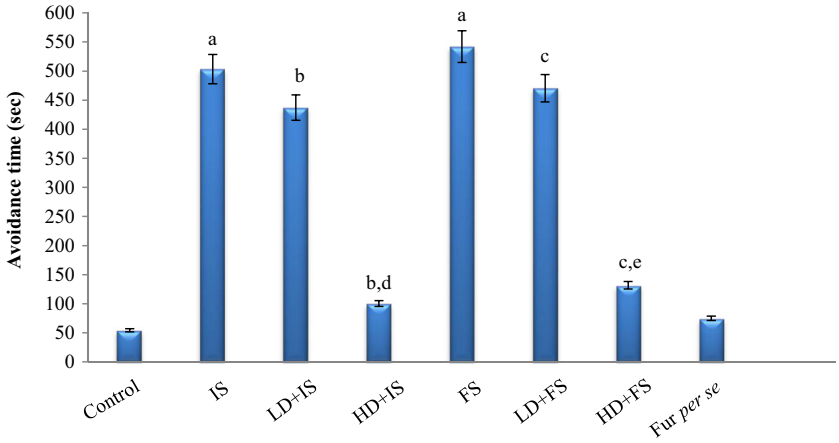

Fig. 7 Effect of furosemide on avoidance time in immobilized or foot-shock stress-subjected mice in 10-min time interval in social interaction test. Data were statistically analyzed by using one-way ANOVA followed by Tukey's multiple range test, $n=6$, [$F(7,40)=1069.0$; $p<0.001$], [a]$p<0.05$ versus control group; [b]$p<0.05$ versus immobilization stress group; [c]$p<0.05$ versus foot-shock stress group; [d]$p<0.05$ versus furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group; [e]$p<0.05$ versus furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group. *IS* immobilization stress group, *LD+IS* furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group, *HD+IS* furosemide high dose (50 mg/kg, *i.p.*) in immobilization stress group, *FS* foot-shock stress group, *LD+FS* furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group, *HD+FS* furosemide high dose (50 mg/kg, *i.p.*) in foot-shock stress group, *Fur per se* furosemide (50 mg/kg, *i.p.*) per se

unconditioned models of anxiety (elevated plus maze and open-field test) (Krystal et al. 2012). However, it is the first report suggesting the antistress effects of furosemide in acute immobilization and electric foot-shock models of experimental stress in mice. Studies have documented the widespread presence of NKCC1 in different stress-responsive brain regions, including the hypothalamus, pituitary, hippocampus, amygdala, and fornix (Okabe et al. 2002; Wang et al. 2002; Nugent et al. 2012; Xie et al. 2003; Zemkova et al. 2008). Furosemide is a nonselective blocker of NKCC and inhibits both NKCC1 and NKCC2. However, due to exclusive distribution of NKCC1 in the central nervous system, the central actions of furosemide may be attributed to activation of NKCC1. It may be possible that stress activates NKCC1 in these stress-responsive areas to initiate stress-related behavioral alterations. The neuronal actions of NKCC activation in the brain have been mainly linked to the reversal of GABA's actions, i.e., reversal of GABA-mediated hyperpolarization to GABA-mediated depolarization (Brumback and Staley 2008; Nardou et al. 2009).

Furthermore, in the present investigation, there was a significant rise in the serum corticosterone levels in response to

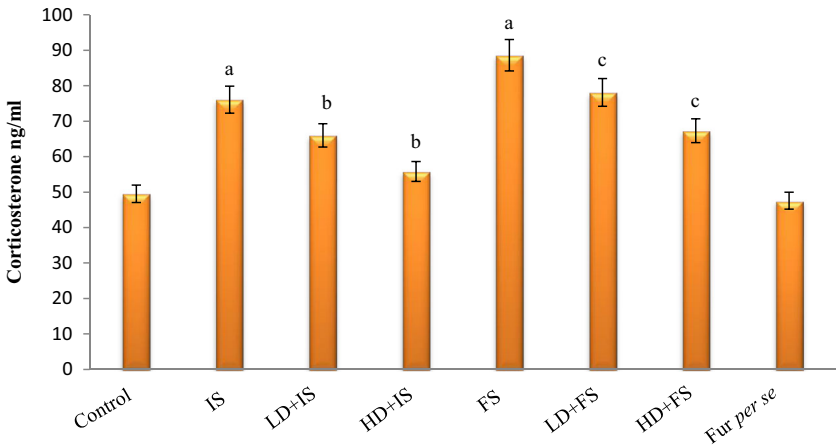

Fig. 8 Effect of furosemide on serum corticosterone level in immobilized or foot-shock stress-subjected mice in corticosterone estimation. Data were statistically analyzed by using one-way ANOVA followed by Tukey's multiple range test, $n=6$, [$F(7,40)=43.296$; $p<0.001$], [a]$p<0.05$ versus control group; [b]$p<0.05$ versus immobilization stress group; [c]$p<0.05$ versus foot-shock stress group. *IS* immobilization stress group, *LD+IS* furosemide low dose (25 mg/kg, *i.p.*) in immobilization stress group, *HD+IS* furosemide high dose (50 mg/kg, *i.p.*) inimmobilization stress group, *FS* foot-shock stress group, *LD+FS* furosemide low dose (25 mg/kg, *i.p.*) in foot-shock stress group, *HD+FS* furosemide high dose (50 mg/kg, *i.p.*) in foot-shock stress group, *Fur per se* furosemide (50 mg/kg, *i.p.*) per se

acute immobilization or foot-shock stress suggesting the hyper-activation of the HPA axis. Studies have well documented the hyper-activation of the HPA axis in response to acute stress, and an increase in the corticosterone levels in response to over-activation of the HPA axis has been correlated with stress-induced behavioral alterations, including fear, anxiety, and other psychological changes (Busquet et al. 2010; Doron et al. 2014). In contrast to well-documented reports regarding the key role of corticosterone in producing behavioral alterations during stress, some studies also report that the HPA axis activation is not involved in stress-induced behavioral deficits. It is described that some of the deleterious effects of predatory stress are mainly mediated by changes in foraging patterns that carry nutritional costs than by changes in glucocorticoid concentrations (Creel et al. 2009).

Stress is regulated by more than one brain regions at different levels, and the major brain areas involved in controlling behavior during stress are the amygdala, the cingulate gyrus, the fornix, the hippocampus, the hypothalamus, and anterior pituitary (as HPA axis) (Roozendaal et al. 2009; Bali and Jaggi 2013). Studies have reported that these stress-responsive areas of the brain contain more corticosteroid receptors as compared to any other brain region; therefore, these are highly susceptible to the effects of stress. It is important to note that the hippocampus, amygdala, and prefrontal cortex are also implicated in the HPA axis to regulate the release of CRH from the CRH neurons into the hypophyseal portal system, which produces signals in the pituitary gland to stimulate the release of ACTH. ACTH stimulates the release of cortisol from the adrenal cortex in humans but releases corticosterone in rodents (Pacak et al. 1993). Thus, corticosterone is the end product due to the activation of hypothalamic-pituitary-adrenal axis; therefore, stress conditions are associated with an increase in plasma corticosterone level (Osterlund and Spencer 2011). Corticosterone and other glucocorticoids possess wide spectrum of actions affecting gene expression and regulation throughout the body, and rise in levels of corticosterone produces various deleterious effects on behavior such as memory deficits, fear, and anxiety (Akil and Morano 1995; Busquet et al. 2010; Kumar et al. 2012). Furthermore, in the present investigation, pretreatment with furosemide (25 and 50 mg/kg) significantly attenuated acute immobilization or foot-shock stress-induced rise in serum corticosterone levels in a significant manner. It has been reported that NKCC are localized on hypothalamic paraventricular nucleus which regulates the activity of the HPA axis (Maguire and Salpekar 2013; Ye et al. 2012). These two behavioral and biochemical measures have been observed to be in parallel relationship, but still, there is need of more direct evidence on the functional relationship.

Therefore, it may be suggested that acute immobilization or foot-shock stress-induced behavioral alterations observed in the present study are due to increased corticosterone levels and furosemide-mediated normalization of corticosterone levels may be responsible for restoration of behavioral alterations in experimental models of stress. Based on these, it may be concluded that furosemide produces beneficial effects in restoring acute immobilization or foot-shock stress-induced behavioral deficits that may be due to its direct actions on stress-responsive brain regions or indirectly through reduction of the corticosterone release.

Conclusion

The present study concluded that immobilization and foot shock induce behavioral and biochemical alteration along with significant elevation of the corticosterone levels. Furosemide mediates restoration of acute immobilization or foot-shock stress-induced behavioral deficit. Therefore, it may be suggested that sodium potassium chloride co-transporter is predominantly responsible for immobilization and foot-shock stress-induced behavioral and biochemical changes.

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