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ORIGINAL ARTICLE

Exploring the role and mechanism of sodium benzoate in CUMS-induced depression model of rats

Fudong Guo[1]*, Zhichun Zhang[2]*, Yudiao Liang[3], Rongmei Yang[4], Youguo Tan[3]

1 Department of Neurology, Affiliated Hospital of Chifeng University, Chifeng city, Inner Mongolia autonomous region, 024000, China
2 Department of Psychology, PLA 967 hospital, Dalian, Liaoning, 116011, China
3 Zigong Mental health Centre, Zigong City, Sichuan Province, 643020, China
4 Department of Psychiatry, Hangzhou seventh people's hospital, Hangzhou, Zhejiang, 310013, China

*Contributed equally to this work.

Correspondence to: Dr. Youguo Tan
Zigong Mental health Centre Zigong City Sichuan Province 643020, China
e-mail: tanyouguo2019@sina.com

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Abstract

AIM: This study was aimed to investigate the effects of sodium benzoate in chronic unpredictable mild stress (CUMS)-induced depression model in rats.
MATERIAL AND METHOD: Male rats were exposed to CUMS stress for 6 weeks which includes with multiple unpredictable stressors to induce depression related symptoms and the treatment with sodium benzoate was started at the 4th week of stress protocol (i.e. on the 22nd day) for 21 days during stress protocol .
RESULTS: CUMS significantly increased the immobility period in the forced swimming test and decrease sucrose consumption in the sucrose preference test in rats. In the prefrontal cortex region (PFC) of the brain, a significant decline in the Brain-derived neurotrophic factor (BDNF) levels and Protein kinase A (PKA) was observed in rats. However, sodium benzoate (400 and 800 mg/kg *i.p.*) significantly restored sucrose preference behavior as well as reduced immobility in CUMS-subjected rats in a dose-dependent manner, suggesting the antidepressant potential of sodium benzoate. Also, sodium benzoate treatment significantly increased BDNF levels and PKA activity in the PFC region of the stress subjected rat brain. Moreover, co-administration of H-89, PKA inhibitor (1 and 5 mg/kg) along with sodium benzoate (800 mg/kg) in CUMS subjected rats notably attenuated antidepressant effects of sodium benzoate. H-89 also abolished sodium benzoate-mediated increase in BDNF levels and PKA activity in stress-subjected rats.
CONCLUSION: Sodium benzoate mediated antidepressant actions may be due to a decrease in the d-amino oxidase activity, an increase in BDNF, and PKA levels in PFC region of the brain. Sodium benzoate-mediated modulation of BDNF/PKA signaling may contribute to attenuating depressive-symptoms in unpredictable stress-subjected rats.

INTRODUCTION

Depression is a severe mental disorder characterized by altered mood, loss of pleasure, suicidal thoughts, hopelessness, helplessness, and impaired concentration (Smith 2014; Zhang *et al.* 2018). It is considered as a life-threatening disorder and has been strongly related to suicidal ideation and attempt due to loss of interest in life (Brådvik 2018). Despite the increasing incidences of this disease, it remains untreated due to the lack of effective therapeutic pharmacological agents. Although several antidepressant drugs have been available, still more than 40% of patients fail to respond to antidepressant treatment (Thomas *et al.* 2015). Therefore, there is the utmost need to investigate and identify new effective targets and specific pharmacological agents to effectively treat this major depression disorder. Chronic unpredictable mild stress model (CUMS) is a widely employed model that closely mimics the clinical conditions of depression disorders in rodents (Zhu *et al.* 2014). CUMS model is frequently preferred over other models due to its unpredictable nature of stressors which significantly reduces the development of adaptation.

Brain-derived neurotrophic factor, (BDNF) performs diverse functions in the brain. BDNF expression or activity is found to be reduced in the stress-sensitive brain regions in stress and depression and upregulation of BDNF significantly alleviates the anxiety and depression (Lee and Kim 2010; Wang *et al.* 2017). It has also been reported that up-regulating cAMP-responsive element-binding protein (CREB), a transcription factor significantly alleviates anxiety and depression (Pandey *et al.* 2003; 2005). It has been documented that CREB phosphorylation at serine 133 residue is mainly regulated via by cAMP-dependent protein kinase A (PKA) (Silva *et al.* 1998). cAMP-PKA signaling is implicated in a different molecular pathway involved in depression and anxiety (Keil *et al.* 2012). Interestingly, studies have demonstrated cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA)-cAMP response element-binding protein (CREB)-brain derived neurotrophic factor (BDNF) signaling pathway in the hippocampus is closely related to depression disorder and the pathogenesis of impairment in cognitive function (Yang *et al.* 2016; Wang *et al.* 2017; Ruan *et al.* 2019).

Sodium benzoate is a widely used food preservative and well known for its hydroxyl-radical scavenging, bacteriostatic and fungistatic properties under acidic conditions (Kim *et al.* 1999). Sodium benzoate drug has been found to be effective against the urea cycle disorder (Scaglia *et al.* 2004), Alzheimer's disease and Parkinson's disease (Barcia *et al.* 2004). Sodium benzoate is a D-amino acid oxidase inhibitor which can increase D-amino acid (DAA) through the inhibition of the activity of D-amino acid oxidase enzyme. It has been documented that the astroglial enzyme, D-amino acid oxidase (DAAO), is abundantly present in the all stress-sensitive brain regions and there have also been number of studies indicating an up-regulation and increased activity of DAAO in different CNS disorders (Madeira *et al.* 2008). A clinical case study also reported that sodium benzoate significantly increased volumes of the thalamus, amygdala, and brainstem in a patient with major depression through the inhibition of DAAO, indicating the beneficial effects of sodium benzoate in relieving the symptoms of depression and anxiety disorder (Lai *et al.* 2013). Considering the potential of DAAO in neuronal functions, the present study shall aim to explore the effects of DAAO inhibitor, sodium benzoate in chronic unpredictable mild stress (CUMS)-induced depression model of rats.

Considering the key role of BDNF/PKA signaling in the pathophysiology of depression along with behavior modulating and DAAO inhibitory potential of sodium benzoate, the study was designed to (a) explore the potential of sodium benzoate in attenuating depression-like behavior in CUMS-subjected rats and (b) to explore the possible contribution of BDNF and PKA in sodium benzoate -mediated antidepressant action in a depression rat model.

MATERIALS AND METHODS

Experimental animals and Drugs

Male Wistar albino rats, weighing 200–250g were employed. All the rats were kept in the animal house under standard laboratory conditions and the study protocol was approved by IAEC of Zigong Mental health Centre Zigong, China. To explore the role of d-amino oxidase and protein kinase A in CUMS, sodium benzoate and PKA inhibitor H-89 were procured from Sigma-Aldrich St. Louis, MO.. Further, the estimation of BDNF was done by using Promega ELISA kits and PKA activity was measured by Enzo Life Sciences International, Inc.

Induction of chronic unpredictable stress

The standard protocol of CUMS with slight modification was employed on the animals to induce depression (Papp *et al.* 1996). Each week consisted of different stressors such as one period (2 h) of paired caging, one period (3 h) of a tilted cage (45°), one period of food deprivation (18 h) immediately followed by 1 h of restricted access to food, two periods of water deprivation (18 h) followed by 1 h exposure to an empty bottle, one 21 h period with wet cage (200 ml water in 100 g sawdust bedding), and one period with 36 h of continuous light. Thus, stressors were presented both during the active (dark) and inactive (light) period of the rats. The stressors were applied randomly to minimize the predictability of the stressors for a week and were continued for 6 weeks to establish the depression model. Control rats were left undisturbed in their home cages. However, in the non-stressed groups, animals were not subjected to any kind of stressful stimulus. The

daily sodium benzoate and H-89 treatment were started at the start of the fourth week of the study protocol.

Behavioral Assessment of Depression

At the end of the protocol, after 6 weeks, different behavioral tests including sucrose consumption and forced swim stress tests were performed in animals. On 3rd, 7th, and 14th day of stress exposure, the freezing response was assessed in response to weekly situational reminders.

Sucrose Preference test

The sucrose consumption test is indicative of the extent of anhedonia. One day before the conduct of test, the rats were trained for 1% (m/V) sucrose solution. At the end of the protocol, during the behavioral testing phase, mice were kept to access both tap water as well as a 1% sucrose solution for 24 h. The bottle's position was changed randomly at the left or right sides of the cage to avoid the place preference. The value of sucrose preference was calculated as a percentage of the volume of the consumed 1% sucrose solution relative to the total volume of liquid intake (Papp *et al.* 1996; Wu *et al.* 2017).

Forced swim stress Test

In the forced swimming test (FST), the mice were placed in clear glass cylinders (40 cm tall × 18 cm diameter) filled with water (25 °C), approximately 23 cm deep, to prevent their tails from touching the bottom. Immobility was recorded during the total 4 min testing period. Rats were considered to be immobile when they made only small movements necessary to float and keep their heads above the water surface. Briefly, climbing behavior (defined as upward-directed movements of the forepaws usually along the side of the swim chamber), swimming behavior (defined as horizontal movement throughout the swim chamber which includes crossing across quadrants of the cylinder), and immobility (measured when no additional activity was observed other than that required to keep the rat's head above the water) were measured in the modified FST (Detke *et al.* 1995).

Brain tissue sample preparation

At the end of the behavioral test, the animals were sacrificed and the brain region frontal cortex was isolated from the rat brain on ice, weighed and frozen in liquid nitrogen, and transported to –80 °C until assays were performed.

Enzyme-linked immunoassay (ELISA)

The brain samples were homogenized in 1 ml of lysis buffer containing protease and phosphatase inhibitors and were used for ELISA-based estimation of BDNF and PKA activity using San microplate reader and the procedure was conducted per the instructions of the manufacturer.

Determination of BDNF and PKA

The PFC region of the brain was removed after completion of behavioral tests and it was homogenized in the lysis buffer which contains protease and phosphatase inhibitors. These inhibitors are added to the lysis reagents to avoid further degradation of extracted proteins and also to obtain the best possible yield. The homogenate was subjected to high-speed centrifugation at 10000×g for 10 min at 4°C. Thereafter, the supernatants were removed and employed for ELISA-based estimation of BDNF and PKA levels at 450 nm. The procedure for ELISA was followed as per the instructions of the manufacturer. The biochemical results were expressed as nanograms/mg of protein. The protein levels in the homogenate were quantified using the Folin-Lowery method (Elfving *et al.* 2010; Liu *et al.* 2012).

Experimental Design

In this study, total of nine groups with seven rats per group were employed in the present study.

Group I: Normal Control

The animals were placed in a cage for six weeks and were not disturbed except during the cleaning of cages. All mentioned behavioral tests were performed on the 42nd day. After that, the animals were sacrificed to measure the levels of BDNF and PKA in the frontal cortex.

Group II: Chronic unpredictable stress

Rats were exposed to chronic unpredictable stress for six weeks as described above. Thereafter, the behavioral tests were conducted on the 42nd day and the animals were sacrificed to measure the levels of BDNF and PKA in the frontal cortex.

Groups III: Sodium Benzoate (400 mg/kg) in CUMS subjected rats

Sodium benzoate (400 mg/kg) was administered for the last 21 days (starting from 4th week) to stress-subjected animals. Thereafter, animals were subjected to behavioral and biochemical tests as described in group II.

Groups IV: Sodium Benzoate (800 mg/kg) in CUMS subjected rats

Sodium benzoate (800 mg/kg) was administered for the last 21 days (starting from 4th week) to stress-subjected animals. Thereafter, animals were subjected to behavioral and biochemical tests as described in group II.

Groups V: H-89, PKA inhibitor (1 mg/kg) and sodium benzoate (800 mg/kg) in CUMS subjected rats

PKA inhibitor i.e. H-89 (1 mg/kg) was co-administered with sodium benzoate for 21 days and the effects were observed on the behavior and biochemical parameters as described in group II.

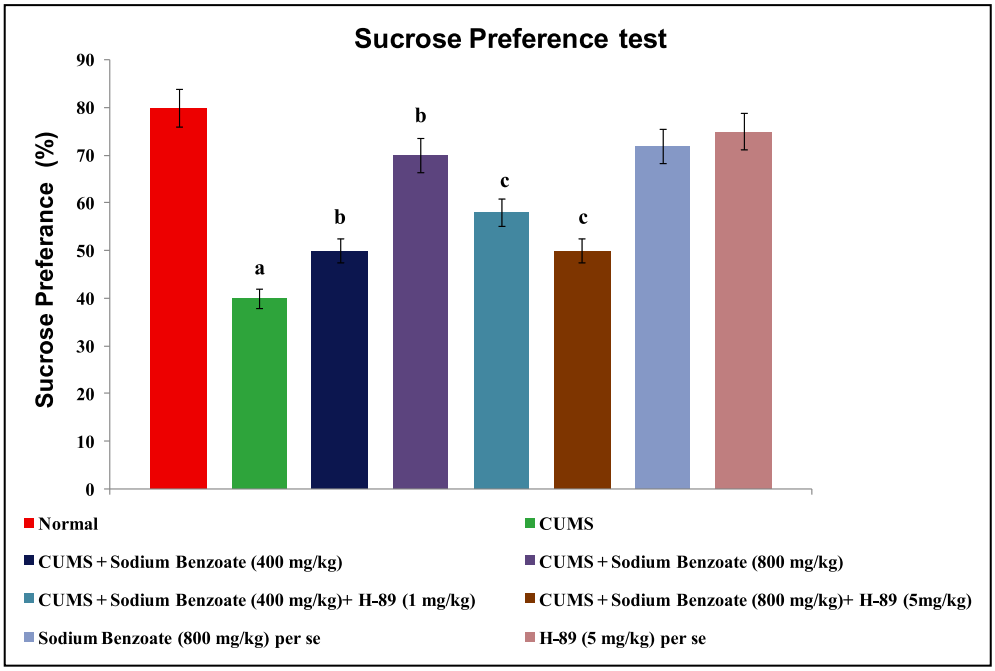

Fig. 1. Effects of chronic unpredictable mild stress and pharmacological interventions on the sucrose consumption in sucrose preference test: Values are expressed as mean ± S.D. with n = 8 $^{a}p< 0.05$ as compared to non-stress control; $^{b}p< 0.05$ as compared to CUMS control; $^{c}p< 0.05$ as compared to sodium benzoate in CUMS.

Groups VI: H-89, PKA inhibitor (5 mg/kg), and sodium benzoate (800 mg/kg) in CUMS subjected rats
PKA inhibitor i.e. H-89 (5 mg/kg) was co-administered with sodium benzoate for 21 days and the effects were observed on the behavior and biochemical parameters as described in group II.

Group VIII: Sodium benzoate (800 mg/kg) per se:
Sodium benzoate was administered in normal rats through *i.p.* for the last 21 days. All mentioned behavioral tests were performed on the 42nd day. After that, the animals were sacrificed to measure the levels of BDNF and PKA in the frontal cortex.

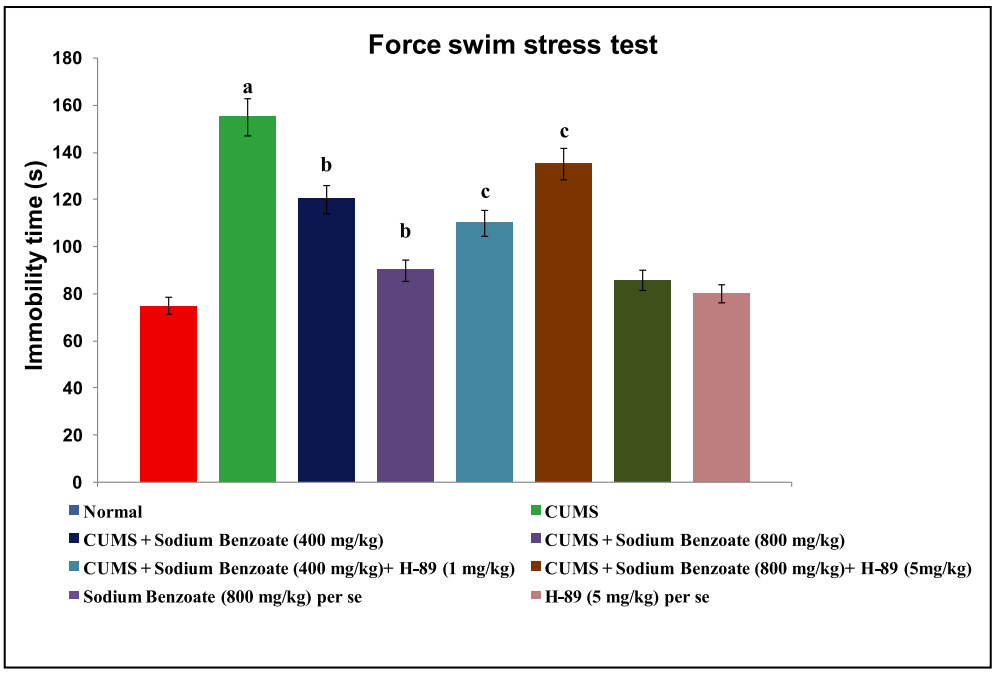

Fig. 2. Effects of chronic unpredictable mild stress and pharmacological interventions on the immobility period in force swim stress test: Values are expressed as mean ± S.D. with n = 8 $^{a}p< 0.05$ as compared to non-stress control; $^{b}p< 0.05$ as compared to CUMS control; $^{c}p< 0.05$ as compared to sodium benzoate in CUMS.

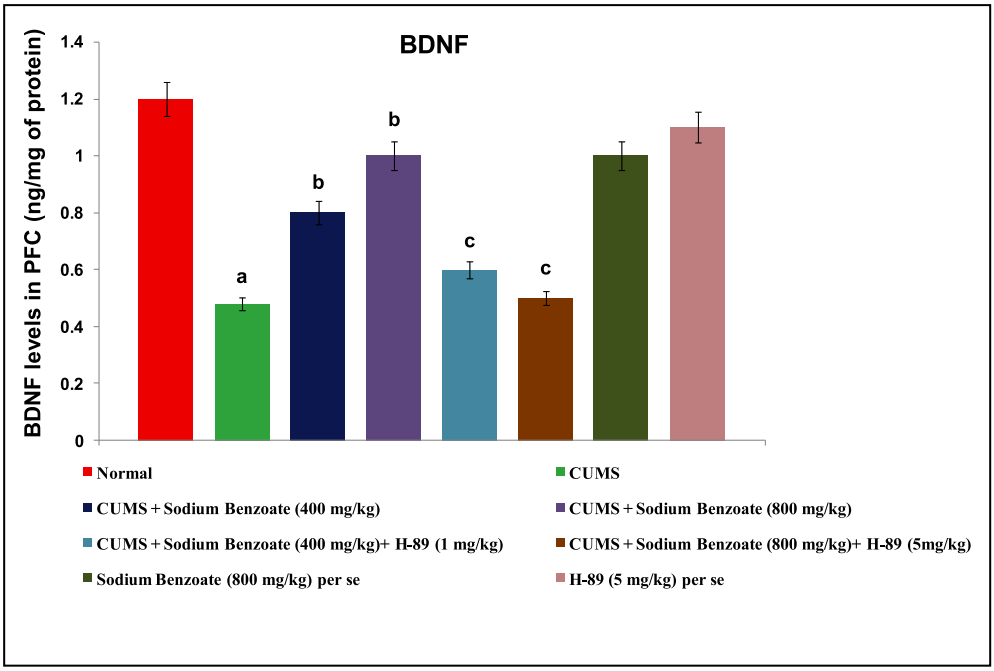

Fig. 3. Effects of chronic unpredictable mild stress and pharmacological interventions on the BDNF levels in prefrontal cortex: Values are expressed as mean ± S.D. with n = 8 in each group $^{a}p< 0.05$ as compared to non-stress control; $^{b}p< 0.05$ as compared to CUMS control; $^{c}p< 0.05$ as compared to sodium benzoate in CUMS.

Group IX

H-89 (5 mg/kg) per se: H-89 was administered in normal rats for the last 21 days. All mentioned behavioral tests were performed on the 42nd day. After that, the animals were sacrificed to measure the levels of BDNF and PKA in the frontal cortex.

Statistical Analysis

The results were analyzed using Graph Pad Prism (GraphPad Software Inc., La Jolla, CA, USA). One-way ANOVA followed by *post hoc* Tukey's test was used for comparing the statistical differences among different groups. The results were expressed in the form

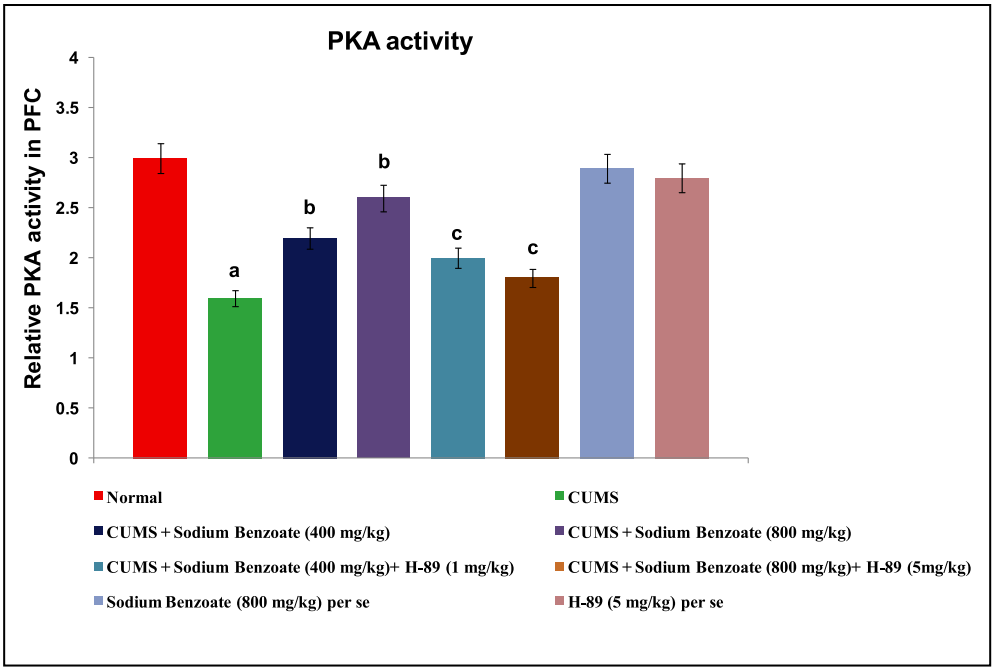

Fig. 4. Effects of chronic unpredictable mild stress and pharmacological interventions on the d-amino oxidase activity in the prefrontal cortex: Values are expressed as mean ± S.D. with n = 8. $^{a}p< 0.05$ as compared to non-stress control; $^{b}p< 0.05$ as compared to CUMS control; $^{c}p< 0.05$ as compared to sodium benzoate in CUMS.

of mean± S.D. $p<0.05$ was considered as statistically significant.

RESULTS

Effects of CUMS on the behavioral and biochemical parameters in rats

A six-week chronic unpredictable mild stress (CUMS) exposure led to significant behavioral alterations, assessed on the 42nd day i.e. at the end of the protocol. A decrease in sucrose consumption was observed in the sucrose preference test on the 42nd day as compared to the normal group, suggesting the significant development of anhedonia (Figure 1). Further, a significant increase in the immobility period was also noted as compared to normal rats in a forced swim test (Figure 2). Biochemically, a decrease in the BDNF and PKA activity was observed in CUMS-subjected rats, assessed on the 42nd day of the protocol (Figures 3 and 4).

Effects of sodium benzoate on behavioral alterations in the sucrose preference and forced swim stress tests in CUMS-subjected rats

Treatment of chronic unpredictable stress subjected rats with sodium benzoate (400 and 800 mg/kg) for three weeks exhibited a significant increase in sucrose consumption in the sucrose preference test at the end of the protocol as compared to the CUMS control group (Figure 1). In a swim test, a significant decrease in the immobility period on the 42nd day in sodium benzoate-treated rats was also observed as compared to the CUMS control group (Figure 2). The effects of sodium benzoate at a high dose i.e. 800 mg/kg were significantly higher than the sodium benzoate at a low dose i.e. 400 mg/kg. Four weeks of treatment with sodium benzoate led to a significant increase in the expression of BDNF and PKA in CUMS-subjected rats (Figures 3 and 4).

Effects of H-89, PKA inhibitor on sodium benzoate-mediated behavioral and biochemical alterations in CUMS-subjected rats

In this study, co-administration of *PKA inhibitor* i.e. H-89 (1 and 5 mg/kg) with sodium benzoate (800 mg/kg) for three weeks abolished sodium benzoate-mediated restoration of sucrose consumption behavior and immobility period in CUMS-subjected rats (Figure 1). There was a marked decrease in the sucrose preference and increase in the immobility period on 42nd, respectively in response to co-administration of H-89 and sodium benzoate in chronic unpredictable stress-subjected rats (Figure 2). The effects of H-89 at a high dose of 5 mg/kg were significantly higher than at the low dose of 1 mg/kg. Co-administration of H-89 abolished the sodium benzoate-mediated increase in the BDNF levels and PKA activity in stress-subjected rats (Figures 3 and 4).

DISCUSSION

In the present study, 42 days or 6-week stress exposure of chronic unpredictable mild stress led to significant behavioral changes assessed in terms of increase in immobile period in force swim stress and decrease in the sucrose preference in the sucrose preference test. Chronic unpredictable stress model is a commonly employed model to induce depression in rodents. The duration and methodology of the chronic unpredictable mild stress model employed in the present study were based on the published literature (Zhang *et al.* 2014; Hu *et al.* 2017). The protocol was consists of random and unpredictable exposure to a variety of stressors to mimic the clinical conditions of major depressive disorder (Papp *et al.* 1996). Furthermore, this model is more advantageous among other models as no adaptation is observed following random and unpredictable stress exposure. Sucrose consumption test is a widely employed behavioral test to assess the anhedonic behavior in rodents (Willner *et al.* 1987) and is based on the natural preference for a sweet solution. It indicates the the development of depression like behavior in chronic unpredictable mild stress-subjected rats. Further, a significant increase in the immobility period in the forced swim stress test, indicates hopelessness again suggests the existence of depression like behavior (Huang *et al.* 2017). The absence of sucrose consumption and increased immobility period noted in the present study in the sucrose preference and forced swim tests, suggest the significant development of depression in response to chronic unpredictable stress in rats.

Pharmacological treatment with sodium benzoate (400 and 800 mg/kg) for three weeks i.e. significantly decreased sucrose consumption and increased immobility period as compared to the stress control group, signifying the attenuation of CUMS-induced depressed behaviour. The doses of sodium benzoate in the present study were selected on the basis of previously published literatute (Walia *et al.* 2019). An increase in sucrose preference and a decrease in the immobility period on 42nd day in sodium benzoate treated rats also suggesting the antidepressant potential of sodium benzoate. Sodium benzoate, a D-amino acid oxidase inhibitor, is widely employed as a food preservative and well known for its hydroxyl-radical scavenging, bacteriostatic and fungistatic properties (Kim *et al.* 1999). Sodium benzoate drug has been found to be effective against the urea cycle disorder (Scaglia *et al.* 2004), Alzheimer's disease and Parkinson's disease (Barcia *et al.* 2004). It has been reported that sodium benzoate can increase D-amino acid (DAA) through the inhibition of the activity of D-amino acid oxidase enzyme (Lai *et al.* 2012; Lai *et al.* 2013). DAAO is an astroglial enzyme present in the different stress-sensitive brain regions including the prefrontal cortex. There have been many studies indicating upregulation and increased activity of DAAO in

different CNS disorders (Madeira *et al.* 2008). Clinical case studies have demonstrated that sodium benzoate significantly increased volumes of thalamus, amygdala, and brainstem in a patient with major depression through the inhibition of DAAO, indicating the beneficial effects of sodium benzoate in relieving the symptoms of depression and anxiety disorder (Lai *et al.* 2013). However, several preclinical studies have shown its therapeutic potential in Alzheimer's and Schizophrenia (Madeira *et al.* 2008). This study is the first preclinical study demonstrating the antidepressant action of sodium benzoate in chronic unpredictable mild stress-subjected rats.

Further, to explore the underlying possible mechanisms involved in sodium benzoate-mediated antidepressant actions, the levels of BDNF and PKA were assessed in CUMS-subjected rats. A significant decrease in the BDNF and PKA levels were observed in the PFC region of the brain in chronic stress-subjected rats on the 42nd day of the stress protocol. BDNF has been described to perform number of functions in the CNS and also implicated in many CNS associated disorders. Further, several studies have shown a significant downregulation of BDNF in major depressive disorders and antidepressant effects in response to BDNF infusion in animal models of depression (Shirayama *et al.* 2002; Nasrallah *et al.* 2019). Furthermore, studies have shown the downregulation of PKA activity in anxiety and depression and it has been postulated that activation of PKA may modulate anxiety and depression (Yang *et al.* 2016; Wang *et al.* 2017; Ruan *et al.* 2019). Interestingly, a relationship has been found between BDNF and PKA in the Pathophysiology of depression (Yang *et al.* 2016; Ruan *et al.* 2019). It can be proposed that a significant decrease in the BDNF and PKA following unpredictable stress may results in depressive like-behavior in rats.

In the present study, three weeks of sodium benzoate treatment led to a significant increase in the expression of BDNF and PKA in the PFC cortex of the brain of chronic unpredictable stress-subjected rats. It also suggests that an increase in the expression of BDNF and PKA are responsible for sodium benzoate-mediated antidepressant actions. Further, it is plausible to suggest that sodium benzoate notably modulates BDNF/PKA signaling to produce antidepressant effects stress-subjected rats. This is the first study to delineate the increase in the expression of BDNF and PKA in the stress-sensitive PFC region of the brain following sodium benzoate treatment.

To further determine the contribution of PKA in the depression, H-89 i.e. PKA inhibitor was co-administered with sodium benzoate in chronic stress-subjected animals. It has also been observed that co-administration of H-89, PKA inhibitor (1 and 5 mg/kg) along with sodium benzoate (800 mg/kg) for three weeks attenuated the sodium benzoate-mediated restoration of depression behavior. The doses of H-89, PKA inhibitor were selected on the basis of published literatute (Seyedi *et al.* 2014). On the 42nd day, a significant decrease in the sucrose preference and increase in the immobility period was observed in response to H-89 and sodium benzoate treatment in stress-subjected rats. PKA signaling has been implicated in the multiple neuronal functions including release of neurotransmitter and post-synaptic responses which are related to the depression-like behavior development. Other reports also suggests that the regulation of PKA-CREB-BDNF signaling pathways produces anti-depressant effects (Ruan *et al.* 2019). The selective PKA inhibitor, H-89 was applied to confirm the critical role of PKA in the sodium benzoate mediated anti-depressive effects. The results obtained from the present study suggest that the suppression of PKA with selective inhibitor markedly abolished the sodium benzoate induced increase in sucrose preference and immobile period duration and BDNF. It can be plausible to suggest that the anti-depressant potential of sodium benzoate may be attributed to the regulation of PKA/CREB/BDNF signaling pathway.

CONCLUSION

Sodium benzoate significantly attenuated the depression-like behavior in chronic unpredictable mild stress-induced depression model in rats. The sodium benzoate- mediated anti-depressive actions may be due to an increase in the BDNF, PKA, and decrease in d-amino oxidase activity in the stress-sensitive PFC region of the brain. In other words, sodium benzoate-mediated modulation of PKA/CREB/BDNF signaling may contribute to attenuating depression-like behavior in chronic unpredictable mild stress-subjected rats.

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DECLARATION OF CONFLICT OF INTEREST

None.

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