Increase in Citrate and KCI Consumption during Morphine Withdrawal Period is Associated with Reduced Levels of Zinc and Brain-derived Neurotrophic Factor, and Poor Neurogenesis in Male Isolated Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author HF designed the study, wrote the protocol, performed experiments, interpreted the data and wrote the first and final draft of manuscripts with assistance from author MK. Author MA mainly contributed in this study in assessing oxidative-stress status. All authors read and approved the final manuscript.

| *Original Research Article* |

ABSTRACT

Introduction: Sustained remission from substance abuse is often difficult to achieve. However, there are certain factors that may predict the remission from substance dependence during drug withdrawal period. The aim of this study was to assess the possible predictors and patterns associated with drug abuse remission.
Methods and Materials: Fifty-four male Sprague-Dawley rats were divided into four groups 1) socialized group 2) isolated group 3) withdrawal-socialized group 4) withdrawal-isolated group. Rats first received morphine for 7 days and then, were withdrawn from it. BrdU (5-bromo-2'-deoxyuridine) was injected from day 1 to day 14 of the experiment. At the end of the experiment,

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BDNF, MDA and zinc levels were assessed. We used Open-Field Test (OFT) to study drug sensitization and anxiety levels. Furthermore, citrate and KCl consumption, mood state, and neurogenesis were also evaluated.

Results: Zinc and BDNF levels were markedly higher is withdrawal-socialized group. Furthermore, withdrawal-isolated group consumed more citrate and KCl and demonstrated more drug sensitization than the withdrawal-socialized group. In addition, anxiety, MDA, and corticosterone levels were significantly elevated in withdrawal-isolated rats. Both socialized groups had more BrdU-positive cells as compared to isolated groups.

Conclusion: Socialization during the withdrawal period may facilitate remission from drug abuse and improve neurogenesis.

1. INTRODUCTION

Drug addiction is a chronic relapsing disorder characterized by compulsive drug-seeking and drug-taking behavior [1]. However, not all the people exposed to drugs, eventually become addicted. There are certain factors which predispose an individual to develop drug abuse. These include various genetic, environmental, social and biological factors [1].

Neurogenesis is a phenomenon occurring in some areas of the brain like an olfactory bulb and neocortex [2]. Suppressed neurogenesis has been linked numerous disorders like Alzheimer's disease, Parkinson's diseases and depression [3]. Neurogenesis-induced brain plasticity is essential for regulating neural circuits associated with cognition and rewards [4]. Moreover, because of neural connections between the hippocampus and rewarding center, hippocampal neurogenesis may also influence functioning of the rewarding center [5,6].

Personality develops within a society and individuals acquire norms, values, and behaviors from socialization. Socialization may acquire six different forms; primary, secondary, reverse, developmental, anticipatory and re-socialization. In addition, socialization has been associated with improvement in brain functions, whereas social isolation has shown to adversely affect neurogenesis, cognition and behavior [7-10].

Sensitization or augmented psychostimulant effect of drugs that occurs after repeated exposure to drug of abuse, involves the neural events mediating in ventral tegmental area (VTA) and nucleus accumbens (NAc). Sensitization to the drug increases the risk of drug relapse during withdrawal period [1]. It is commonly assessed by monitoring motor activity, such as that during Open-Field Test (OFT) [1,11]. Interestingly, induction of salt appetite alters neuronal morphology in NAc and is associated with sensitization. In addition, behavioral responses induced by morphine abuse and salt depletion cross-sensitize with each other, suggesting the involvement of common neural substrates [11,12].

Mood regulation is important for the well-being of an individual. Mood is regulated by connectivity between various cortical and limbic structures, such as the hippocampus, amygdala, prefrontal cortex and NAc. Although hippocampus is primarily known for memory formation, it also plays a crucial role in regulating stress responses and mood. Disturbances in mood regulation can result in the development of psychopathologies like depression and anxiety [13]. Negative mood states during the withdrawal period compel an individual to perceive a lack of coping skills and thus, makes him/her highly vulnerable to drug relapse.

Zinc (Zn) is an important trace metal in central nervous system. It is most abundant in the cerebral cortex, hilus of the dentate gyrus and mossy fibers (hippocampus). At the distil end of a neuron, Zn is stored in the lumen of synaptic vesicles, and there it co-localizes with glutamate. It is important for brain development and maintenance of brain functions. Furthermore, Zn is actively involved neurotransmitter metabolism and synaptic activity. It also plays an important role in cognitive development and motor functions. Experimental studies indicate that decrease in brain Zn availability can reduce hippocampal neurogenesis [14]. Interestingly, Zn has shown to reduce morphine-dependence intensity, whereas zinc-chelator enhanced withdrawal manifestations [15].

Brain-derived neurotrophic factor (BDNF) is one of the major neurotrophic factors which primarily support neuronal growth and survival. It is highly expressed in brain areas associated with cognition and emotions, such as hippocampus and amygdala [16]. Hippocampal pyramidal neurons in CA3 region are profoundly responsive to changes in BDNF levels. In addition, BDNF plays a critical role in regulating reward associated functions of the mesocorticolimbic dopaminergic system [17].

Oxidative stress status is one of the major determinants of health. Malondialdehyde (MDA) is a naturally occurring organic compound, often used as a marker of oxidative stress. High MDA levels are indicative of lipid peroxidation, which in turn may alter neurogenesis by affecting neuronal membrane lipids [18]. MDA plasma levels have shown to positively correlate with declarative and working memory deficits [19].

We designed this study to assess if KCl and citrate consumption can be used as indices for prognosis of drug relapse. Furthermore, we aimed to evaluate the effect of socialization during withdrawal period on KCl and citrate consumption. We also studied isolation-induced disturbances in neurogenesis, zinc levels and BDNF levels.

2. METHODS AND MATERIALS

2.1 Animal Care

The experimental protocols followed in this study were conformed to the Guidelines for the Care and Use of Laboratory Animals published by National Institutes of Health (NIH Publication No.85-23, revised 1996) and were approved by the institutional ethical committee of Tehran University of Medical Sciences (Tehran, Iran).

2.2 Animals

Fifty-four adult male Sprague-Dawley rats aged between 8 to 9 weeks (weighing 200-250 g) were used in this study. The rats were divided into four experimental groups (n=9 per group); socialized group, isolated group, withdrawal-socialized group and withdrawal-isolated group. Eighteen rats were used for modeling socialization (Fig. 1).

2.3 Morphine Addiction and Withdrawal (Group C and D)

For adaptation, all animals received 0.75 mg/rat, i.p. of morphine sulfate for three days. Rats were rendered morphine-dependent by intraperitoneal injections of increasing doses of morphine (baseline dose: 5 mg/kg/day) for 7 days. Next, Naltrexone was injected (3 mg/kg, i.p) in day 8 (Fig. 2). For treatment groups, twenty-seven rats were used (9 rats were used for the socialization of animals in group C).

2.4 Isolation and Socialization of Animals

Animals in the isolated group were housed individually in cages with walls covered with black plastic. Isolated animals were housed in separate rooms in order to attain true isolation. The rooms were well-ventilated and kept quiet. In socialized group, rats were housed in pairs and the cages were left transparent. Animals were caged for 1-week adaptation period followed by two weeks of the experimental period [9,10]. For control groups, twenty-seven rats were used (9 rats were used for the socialization of animals in group A) (Fig. 3).

2.5 Experimental Protocol

Animals in both control groups (socialized and isolated groups) received normal saline. After an adaptation period, six animals from each group received Bromo–deoxyuridine (BrdU; 50 mg/kg, i.p.) (Sigma-Aldrich Co.) for 14 days. At the end of 14th day, Open-Field Test (OFT) was performed. Furthermore, from day 15 to day 19, KCl and citrate consumption were assessed. On day 20, tail suspension test (TST) was performed. Following this, the animals were euthanized and serum was obtained for evaluating zinc and MDA levels, and CSF was collected for BDNF assessment. The heads were decapitated and the brains were sectioned to study neurogenesis by counting BrdU positive cells.

2.6 Open-Field Test (OFT)

This assay was used to evaluate anxiety and locomotor activity in experimental animals. The base of OFT apparatus was divided into 16 equally spaced squares bordered with opaque and 70 cm high walls. The whole apparatus was painted black except for the 6 mm broad white lines that divided the ground into 16 squares. This apparatus was illuminated using a 100 W bulb which focused on the field from a height of about 110 cm. Except for the open field; the entire room was kept in dark during the experiment. In order to observe subsequent behaviors for evaluating anxiety and locomotor activity, each animal was brought to the center of

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the setup for about 5 min. Four typical behaviors in OFT were assessed and scored [20,21].

2.6.1 Ambulation distance

Total distance of the grid lines crossed by each rat.

2.6.2 Center square entries

Number of times each rat enters the central red square lines with all four paws.

2.6.3 Time spent in the center square

It defined as the time spent by each rat in the central square.

2.6.4 Rearing

It defined as the frequency with which each rat stands on its hind legs in the field.
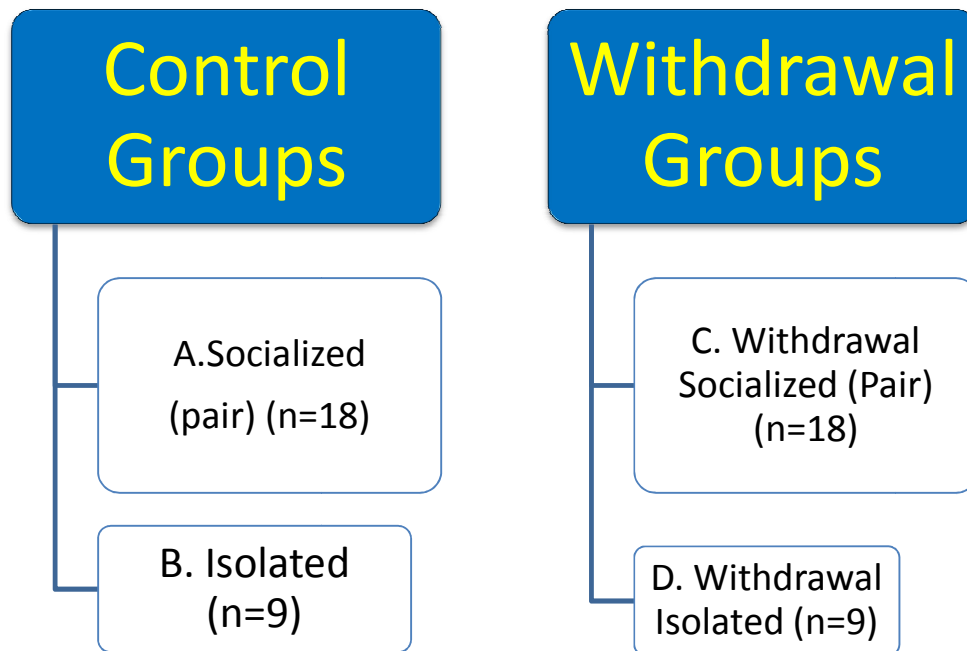
Control Groups

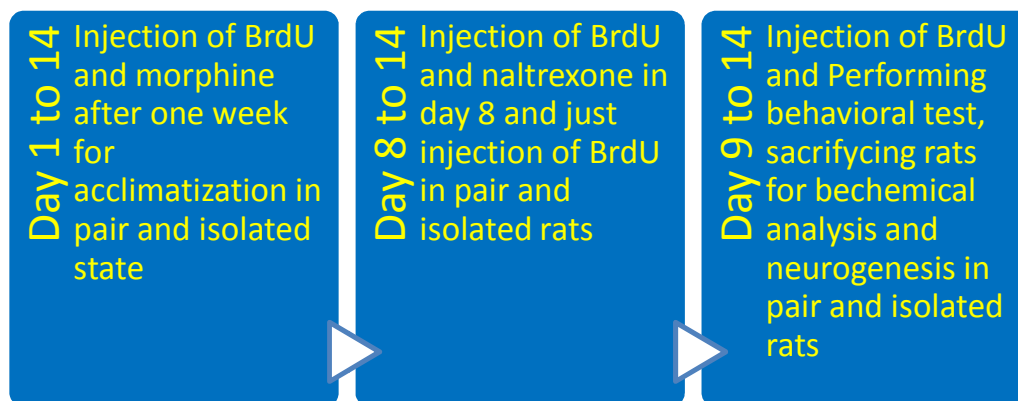A.Socialized (pair) (n=18)

B. Isolated (n=9)

Withdrawal Groups

C. Withdrawal Socialized (Pair) (n=18)

D. Withdrawal Isolated (n=9)

Fig. 1. Experimental groups

Day 1 to 14 Injection of BrdU and morphine after one week for acclimatization in pair and isolated state

Day 8 to 14 Injection of BrdU and naltrexone in day 8 and just injection of BrdU in pair and isolated rats

Day 9 to 14 Injection of BrdU and Performing behavioral test, sacrifycing rats for bechemical analysis and neurogenesis in pair and isolated rats

Fig. 2. Treatment groups (group C and D)

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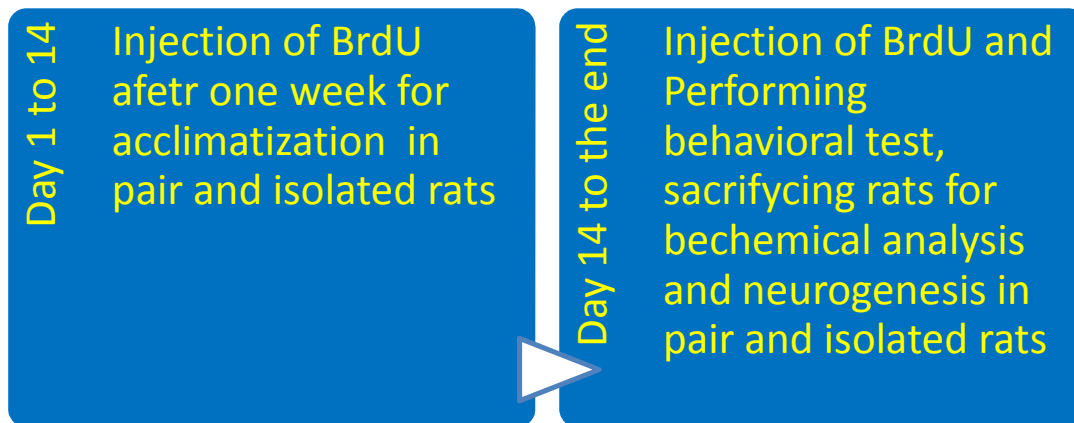| Day 1 to 14 | Injection of BrdU afetr one week for acclimatization in pair and isolated rats | Day 14 to the end | Injection of BrdU and Performing behavioral test, sacrifycing rats for bechemical analysis and neurogenesis in pair and isolated rats |

Fig. 3. Control groups (Group A and B)

2.7 Citrate Preference Test

For this experiment, 50 mM of Citrate (300 ml/rat) (Sigma-Aldrich) was prepared and the two-bottle test was performed for 48 hour. To prevent potential location preference of drinking, the position of the bottles was changed every 24 hr. Food and water were made available prior to the citrate preference test. At the end of the experiment, the preference for the citrate was determined as the percentage of citrate solution ingested relative to the total intake [22].

2.8 Assessment of KCl Consumption

We measured overnight KCl intake to gain insight about the rats' appetite for KCl. In this experiment, rats were given a 24-hr two-bottle choice test with dH_2O and 200 mM (300 ml/rat) KCl (Merck). The relative positions of the two bottles were counterbalanced across rats. Intake of KCl relative to total intake was calculated [23].

2.9 Tail suspension Test (TST)

In TST, each rat was suspended by its tail (50 cm above the ground) against a fixed metal rod with its body facing downwards. Normally, the rat tried to escape from this stressful state by trying to climb up the metal rod. However, depressed ones gave up and remained immobile. Therefore, we recorded the duration of immobility in a 5-min period which was indicative of depression-like behavior [24].

2.10 Assessment of Zinc

For obtaining plasma, 5 ml blood was collected from the heart in heparinized tubes and centrifuged at 6000 rpm for 20 min. The plasma was collected and stored at -70 ºC. For analysis of zinc levels, first, the plasma was incubated with 65% citric acid for 2 hr and then, with 65% perchloric acid for 1 hr. The final solution was examined using an atomic spectrometer (Varian-220-FS-aa). The obtained wavelength was adjusted using calibration curve and expressed as p.p.m [25].

2.11 Assessment of BDNF levels

After obtaining CSF (0.4- 1 µl/rat), BDNF levels were assessed using ELISA kit (Promega, USA) according to the manufacturer's instructions.

2.12 Assessment of MDA Levels

Serum sample (100 µl) was mixed with 1 mL 30% trichloroacetic acid (TCA, Sigma-Aldrich Co.) and 1 mL 0.375% thiobarbituric acid (TBA, Sigma-Aldrich Co.). The mixture was heated at 90ºC for 60 min and centrifuged at 12000 g for 5 minutes. The final product was measured at 532 nm using UV-visible spectrophotometer. It was assessed using the standard curve and was expressed as µmol/L [26].

2.13 Immunohistochemistry

Following adaptation of animals, BrdU (50 mg/kg; i.p., Sigma-Aldrich Co.) was injected for 14 days. BrdU is an analogue of thymine base which is incorporated in DNA of newly proliferated neurons in the dentate gyrus of the hippocampus. At the end of 14th day, animals were euthanized using ketamine (100 mg/kg) and xylazine (10 mg/kg). The brains were perfused with 100 ml normal saline and then,

fixed with 100 ml paraformaldehyde 4% via intra - cardial infusion. After fixation, the brains were removed from the skull. For the first 2 days, the brains were kept in PBS + paraformaldehyde 4% and then at day 3, in sucrose 10% + paraformaldehyde 4% + PBS. Throughout day 4, the brains were kept in sucrose 20% + paraformaldehyde 4% + PBS and for the rest of the days, they were kept in sucrose 30% + paraformaldehyde 4% + PBS. The cryosections (30 μm) were prepared from the hippocampal region and five sections per animal were selected and stained for BrdU-positive neurons using a commercially available anti-BrdU antibody kit (5-Bromo-2-dU Labeling and Detection Kit II; Roche). BrdU-positive cells in the dentate gyrus (Fig. 9) were counted directly under light microscope (Zeiss Co.) at 400X magnification. BrdU-positive neurons appeared colored brown were observed as single cells or in clusters [8,27].

2.14 Quantification of BrdU Positive Cells

Every fifth section throughout the hippocampus (total 10 sections for each rat) was processed for BrdU immunohistochemistry. All BrdU-positive cells in the sub granular zone (SGZ), hilus, granular cell layer (GCL) and molecular layer were counted under light microscope (Zeiss, Germany) in a blinded manner. The BrdU positive cells in dentate gyrus were counted in a rostrocaudal fashion. BrdU-positive neurons appeared much bigger than usual and appeared singly or in clusters. A mean - value was calculated for every five sections [28].

2.15 Statistical Analysis

Data analysis was performed using SPSS version 22 and Graphpad Prism. Data were presented as mean ± SEM. Statistical comparisons between the means of different groups were performed using Two-way ANOVA followed by Post-hoc Tukey. P<0.05 was considered statistically significant.

3. RESULTS

3.1 Behaviors in OFT

Rearing and locomotor activity: Frequency of rearing and locomotor activity were higher in withdrawal-isolated rats (D) as compared to withdrawal-socialized rats (C). Animals in the withdrawal-isolated group (C) were more immobile than isolated (B), socialized (A) and
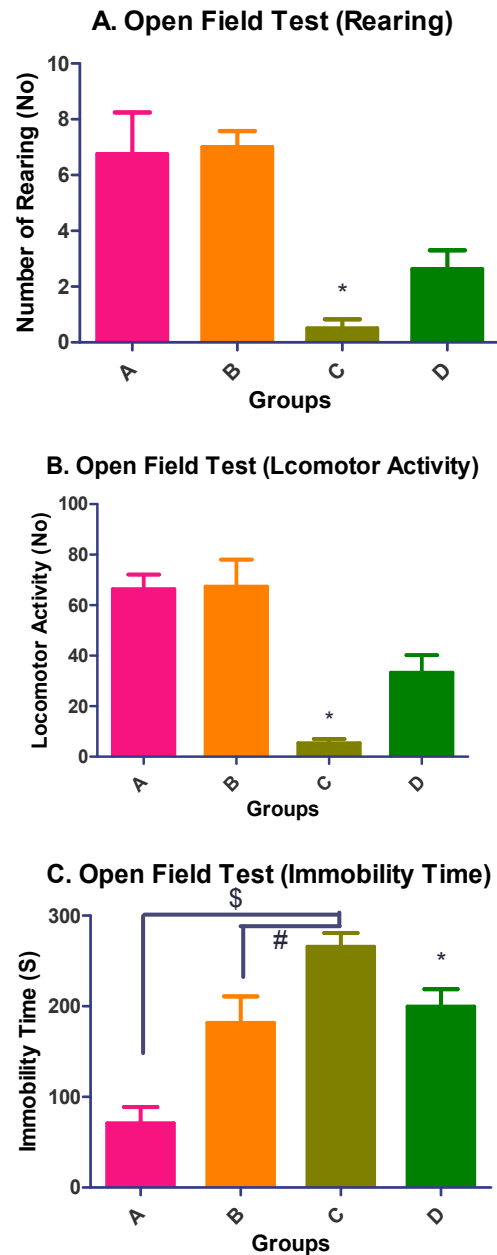
withdrawal-socialized rats (D) (Fig. 4 A, B, and C).

Fig. 4. A. Frequency of rearing in OFT (n=9). B. Score of locomotor activity in OFT (n=9). C. Immobility time in OFT (n=9) D. Data are represented as mean ± SEM
*Symbol * indicates a significant difference between adjacent groups and, symbols $ and # indicate significant differences between groups apart from each other. A. socialized group, B. isolated group, C. withdrawal-socialized group and D. withdrawal-isolated group.*

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3.2 Sour and Bitter Appetite

Citrate and KCl consumption was higher in withdrawal-isolated rats (D) as compared to withdrawal-socialized rats (C). In addition, rats in the withdrawal-socialized group (C) consumed more KCl than rats in the control groups (A and B) (Figs. 5 A and B).
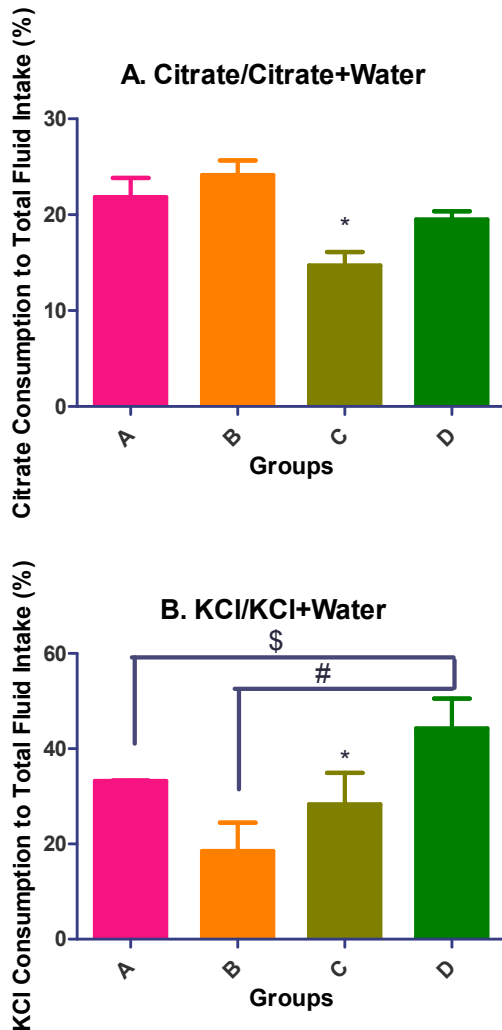
A. Citrate/Citrate+Water

B. KCl/KCl+Water

Fig. 5. A. Percentage of citrate consumption relative to the total fluid intake (n=9). B. Percentage of KCl consumption relative to total fluid intake (n=9). Data are represented as mean ± SEM

*Symbol * indicates a significant difference between adjacent groups and, symbols $ and # indicate a significant difference between groups apart from each other. A. socialized group, B. isolated group, C. withdrawal-socialized group and D. withdrawal-isolated group.*

3.3 Assessment of Mood State with Tail suspension Test

Duration of immobility was higher in withdrawal-isolated rats (D) compared to withdrawal socialized rats (C). In addition, socialized rats (A) were more mobile than withdrawal-isolated (D)and withdrawal-socialized(C) rats (Fig. 6).

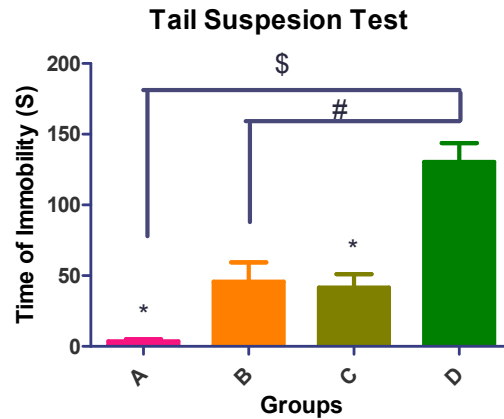### Tail Suspesion Test

Fig. 6. Time of immobility (sec) in tail suspension test (n=9). Data are represented as mean ± SEM

*Symbol * indicates a significant difference between adjacent groups and, symbols $ and # indicate a significant difference between groups apart from each other. A. socialized group, B. isolated group, C. withdrawal-socialized group and D. withdrawal-isolated group*

3.4 BDNF Levels

CSF BDNF levels were markedly elevated in socialized rats (A) as compared to isolated (B) and withdrawal-isolated (D) rats. Furthermore, withdrawal- socialized rats (C) had higher BDNF levels than isolated (B) and withdrawal-isolated (D) rats (Fig. 7).

3.5 Assessment of Plasma Zinc Levels

Zinc levels were markedly reduced in withdrawal-isolated rats (D) as compared to socialized (A) and withdrawal-socialized (C) rats. Socialized rats (A) had significantly higher zinc levels as compared to isolated rats (B). Furthermore, withdrawal-isolated rats (D) had lower zinc levels than isolated rats (B) (Fig. 8).

3.6 MDA Levels

Serum MDA levels were markedly elevated in isolated rats (B) as compared to socialized (A)

and withdrawal-socialized (C) rats. Furthermore, MDA levels were higher in withdrawal-isolated rats (D) as compared to socialized (A) and withdrawal-socialized (C) rats (Fig. 9).
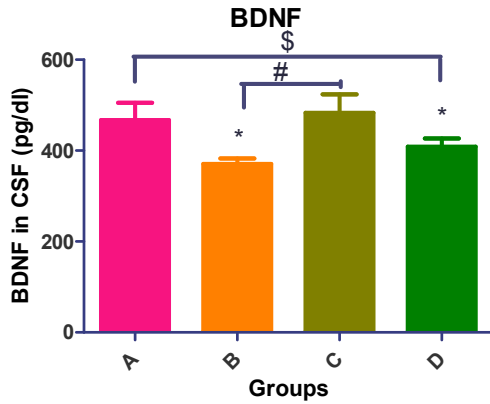
than withdrawal-isolated rats (D). Interestingly, rats in withdrawal- socialized group (C) had more BrdU-positive cells than socialized rats (A), possibly due to reactive neurogenesis (Figs. 10, 11 and 12).

Fig. 7. BDNF levels in CSF (pg/ml) (n=6). Data are represented as mean ± SEM
*Symbol * indicates a significant difference between adjacent groups and, symbols $ and # indicate a significant difference between groups apart from each other. A. socialized group, B. isolated group, C. withdrawal-socialized group and D. withdrawal-isolated group.*
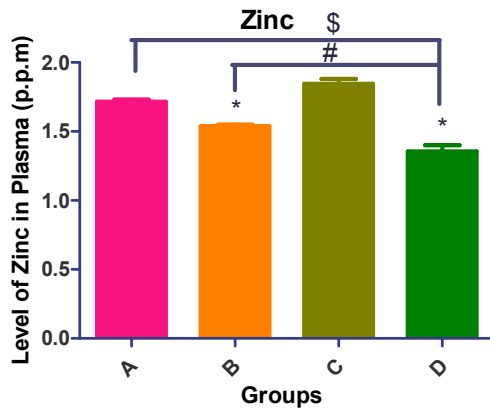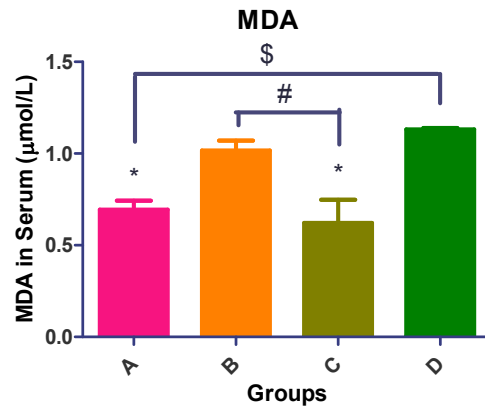

Fig. 9. Serum MDA levels (µmol/L) (n=6). Data are represented as mean ± SEM
*Symbol * indicates significant difference between adjacent groups and, symbols $ and # indicate significant difference between groups apart from each other. A. socialized group, B. isolated group, C. withdrawal-socialized group and D. withdrawal-isolated group*

Fig. 8. Plasma Zinc levels (p.p.m) (n=6). Data are represented as mean ± SEM.
*Symbol * indicates significant difference between adjacent groups and, symbols $ and # indicate significant difference between groups apart from each other. A. socialized group, B. isolated group, C. withdrawal-socialized group and D. withdrawal-isolated group*

3.7 Neurogenesis (*Number of BrdU-positive cells*)

Withdrawal-isolated rats (D) had fewer BrdU-positive cells as compared to socialized (A) and withdrawal-socialized (C) rats. In addition, isolated rats (B) had fewer BrdU-positive cells

Fig. 10. Number of BrdU-positive cells in dentate gyrus of hippocampus (n=6). Data are represented as mean ± SEM
*Symbol * indicates a significant difference between adjacent groups and, symbols $, &, @ and # indicate a significant difference between groups apart from each other. A. socialized group, B. isolated group, C. withdrawal-socialized group and D. withdrawal-isolated group.*
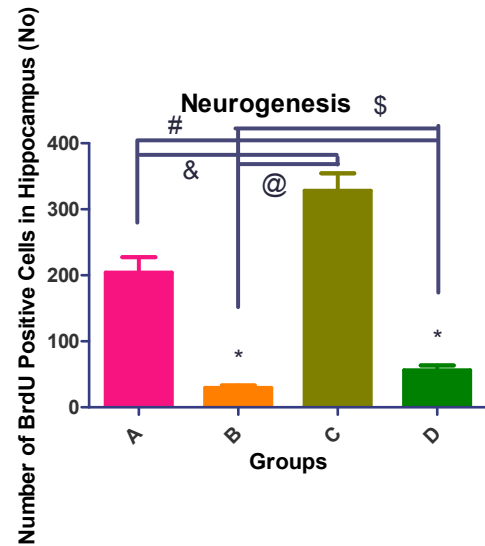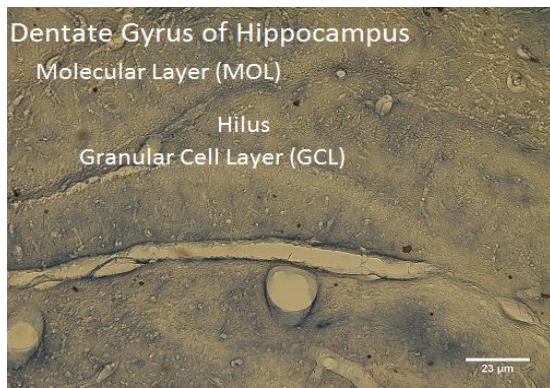
Fig. 11. Different layers of the dentate gyrus of the hippocampus are shown in this picture. BrdU-positive cells were counted in these areas; molecular layer (outer (OML), middle (MML) and inner (IML)), granular cell layer and hilus (sub granular zone (SGZ) and deep hilus)

4. DISCUSSION

The current study shows that socialization during withdrawal period reduces morphine sensitization, Citrate and KCl consumption, oxidative stress, and anxiety-like behavior. Furthermore, socialization improves BDNF levels, Zn levels, and neurogenesis.

Psychostimulants, like cocaine, heroin and morphine enhance the efflux dopamine in the reward circuitry- neuronal projections from the midbrain ventral tegmental area (VTA) to the nucleus accumbens (NAc) and frontal cortex [29,30]. In addition, the hippocampus is anatomically well-positioned to influence the reward center. Current Literature suggests that addictive drugs reduce the hippocampal neurogenesis [6,29]. However, it is still unclear if reduced neurogenesis is the cause or consequence of addiction [31]

Previous research from our laboratory has demonstrated that neurogenesis increases with socialization [32]. However, in this study, we evaluated hippocampal neurogenesis as it influences other variables, like mood state, drug sensitization, and Zn levels. Neurogenesis in the dentate gyrus of hippocampus takes place several stages. The neuronal precursors first differentiate into neuronal stem cells niches. For this stage of neurogenesis, the prominent biomarkers are nestin, sox2, and Ki67. During the phase of neuronal maturation, markers like

NeuN and calbindin are expressed by neurons. The development of neuronal precursors into mature neurons requires about three weeks. Thus, the neurogenesis observed in the current study does not implicate the presence of mature neurons [33].

Substance abuse hijacks the rewarding center of the brain and when the drug of abuse is withdrawn, the reward pathways are 'dysregulated'-a phenomenon termed as functional neurotoxicity. It has been postulated that persistent functional neurotoxicity could be responsible for the long-lasting vulnerability to relapse [34]. Moreover, the body's desire to achieve stability (allostasis) against the 'dysregulated' reward pathways may contribute to drug-relapse. Allostatic load increases markedly during chronic stress and may contribute to the development of numerous psychiatric disorders [35].

We accessed anxiety levels in animals by evaluating the immobility duration in OFT and TST. We found low anxiety levels in withdrawal-socialized rats as compared to withdrawal-isolated rats. Mood disorders like anxiety and depression have been strongly linked to impairments in serotonin (5-HT) neurotransmission [4].

Hippocampus - the hub of learning and memory is connected with the brain structures implicated in regulating affect and mood. Various stressors have known to precipitate mood disorders in individuals. The stress-related rise in glucocorticoids cause the arrest of cell-cycle and depresses BDNF levels in the hippocampus. Consequently, mood disturbances and co-morbid psychiatric conditions are associated with poor neurogenesis [36,37]. As hippocampal neurogenesis plays an essential role in regulating stress responses by exerting negative feedback on the hypothalamic–pituitary–adrenal (HPA) axis, hippocampal neuronal damage causes a loss of this inhibitory control and leads to a further rise in glucocorticoids. Accumulating evidence suggests that stress and depression increase the vulnerability of drug abuse in susceptible individuals and the risk of drug relapse in addicts [38].

With respect to our study, social isolation can be considered as a stressor which induces the state of depression, reduces the hippocampal neurogenesis and increases the risk of drug
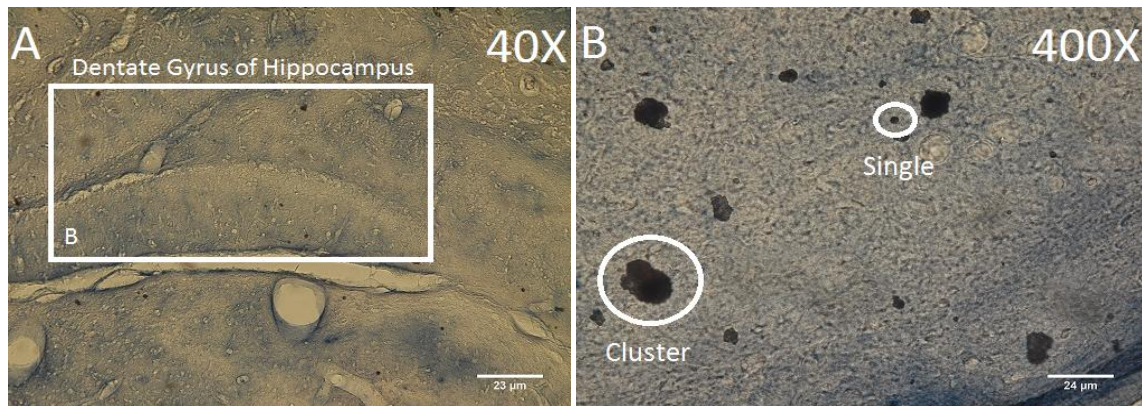
Fig. 12. A. Dentate gyrus of the hippocampus B. BrdU-positive cells (dark-brown) occurring singly and in clusters

relapse. The unpleasant experience of isolation may have induced pleasure-seeking behavior in rats and the memorable pleasure of the initial drug consumption can cause relapse to drug-seeking [34]. On the other hand, socialization promotes oxytocin and prolactin secretion - hormones involved in facilitating the process of neurogenesis [39,40]. Furthermore, socialization-induced neurogenesis can also be attributed to improved serotonin levels in response to social interaction [41].

Brain-derived neurotrophic factor (BDNF) is an important nerve growth factor which facilitates neuronal growth and survival. This potent growth factor also regulates hippocampal neurogenesis. Previous studies have reported that reduced levels of BDNF are associated with neurodegeneration and cognitive deficits [17,42]. We observed a significant reduction in BDNF levels and a subsequent decrease in hippocampal neurogenesis in isolated rats. Besides hippocampus, neurogenesis occurs in nucleus accumbens [2], striatum [43], limbic system [44] and substantia nigra [45]. Future investigations are needed to assess the role of neurogenesis in these areas in addictive behaviors.

We assessed drug sensitization in isolated and socialized animals by observing the frequency of rearing and locomotor activity. In withdrawal-isolated animals, the locomotor activity and frequency of rearing were lesser than that of socialized rats. These results indicate isolation-induced drug-sensitization. Behavioral sensitization to addictive drugs can be studied in rodents by evaluating the increase in ambulatory activity and frequency of more focused, non-ambulatory behaviors, such as sniffing, rearing, licking, and gnawing [1]. Yamaguchi et al. demonstrated that repeated dose of cocaine reduces hippocampal neurogenesis which may be involved in cocaine-induced behavioral sensitization and cognitive deficits [46]. It has been shown that alcohol consumption increases the activity of NMDA receptors within the dorsal striatum and contributes to dysfunctional synaptic changes which may be responsible for excessive alcohol intake, and relapse during the withdrawal period [47]. Abrahao et al. demonstrated that ethanol-sensitized mice had more responsive dopamine receptors and they exhibited more responsiveness to the administration of dopamine agonist [48]. In addition, there exists an age-dependent difference in the sensitivity of various drugs [49]. In our study, socialization of animals has attenuated behavioral sensitization to morphine by increasing neurogenesis.

For the first time, the current study used citrate (sour) and KCl (bitter) consumption as the indices of sensitization. Previous studies support this hypothesis that salt appetite is associated with sensitization. In addition, cross-sensitization has been reported between various drugs of abuse, like amphetamine and cocaine [50], and also between drugs and naturally rewarding substances like morphine and salt [11]. Thus, increase appetite for citrate and KCl can also represent high drug-sensitization in isolated rats. Repeated administration of drugs needed for development of sensitization (in an open field test observed as increased locomotion), but if the same mechanisms cause increased salt consumption (another sign of sensitization) there is a lack of studies [51]. However, the mechanism behind the socialization-induced

reduction in drug-sensitization is not well-understood. In one study, the adverse effects of amphetamine were reversed by oxytocin (a peptide which reinforces social bonding) [52]. Keeping in view the role of the hippocampus in taste learning, socialization-induced neurogenesis can be speculated in corrective taste-related learning [53].

For the probable role of adult neurogenesis in tastant sensation and drug sensitization that both were assessed in the open field test and salt-like consumption, there are not many studies, however in this study higher rate of consumption was associated with low rate of neurogenesis. Addiction decreases neurogenesis [54] and salt consumption is associated with poor prognosis in addiction period [55]. However, addiction is associated with poor neurogenesis and enhanced salt-craving. These factors contribute to the poor prognosis of drug abuse. Since neurogenesis is a form of neuroplasticity, addiction-induced maladaptive synaptic plasticity can affect the functions of the hippocampus and peri-hippocampal areas like rewarding center. Nuclues accumbens - a chief component of brain reward system, contains neurons which respond to the rewarding and aversive effects of taste stimuli. Thus, insufficient neurogenesis can not only affect the cognitive functions of the brain but may also affect rewards related to taste sensation such as citrate and KCl [56].

Zinc is important element involved in regulating addictive behaviors directly or indirectly via regulation of thiamin, vitamin C, folate and Vitamin E [57]. The neurons in cortex and amygdala are rich in zinc content [15]. A balanced zinc homeostasis is required for sufficient neurogenesis [58] and Zn deficiency is implicated in mood disorders like depression, anxiety, and anorexia [59,60]. We found that socialization improved the Zn levels which in turn assist the brain in regulating positive behaviors essential for remission from substance abuse.

Malondialdehyde (MDA) is a naturally occurring organic compound, often used as a marker of lipid peroxidation. Increase levels of MDA interfere with the neuronal signaling involved in proliferation and maturation of neurons. Sordi et al. demonstrated that MDA and BDNF levels can be the markers of the severity of cocaine use during withdrawal [61]. We observed high MDA levels in isolated and withdrawal-isolated rats. These findings indicate isolation-induced oxidative, which in turn may reduce

neurogenesis and may increase the risk of drug-relapse during withdrawal.

Drug addiction is a disease associated with compulsive drug-seeking and drug-taking behaviors. There are certain genetic, biological and social factors which predispose an individual to turn into an addict. Among them, social factors are of key importance as they can be controlled. The current study suggests that socialization during withdrawal period can facilitate remission from the drug of abuse.

5. CONCLUSION

During the withdrawal period, socialization reduces anxiety, oxidative stress, and drug-sensitization, while improving BDNF levels and neurogenesis. Thus, social interactions during withdrawal period can reduce the risk of drug relapse.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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