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Original Article

Paradoxical Regulation of Copper and Zinc and Changes in Neurogenesis, Alcohol Preference and Salt Appetite in Isolated Male Rats

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Background and Aims: Alcohol abuse is an important concern of many societies. Hippocampal neurogenesis regulates abusing drugs in a positive manner. The aim of this study was to identify factors that regulate neurogenesis in isolation period that increase preference for alcohol and salt.

Materials and Methods: In this study sixteen rats were randomly divided into two groups: Pair (social) and isolation. Rats in the isolation group were isolated for 14 days plus one week for acclimatization. Rats in pair group also were kept in the same condition for 14 days. In this period BrdU (50 mg/kg/day/i.p.) was injected. At the end of the experiment, rats examined for copper, zinc, malondialdehyde (MDA), neurogenesis, salt consumption and alcohol preference.

Results: Zinc in serum reduced in isolated rats, but copper in serum paradoxically increased in isolated rats. Neurogenesis reduced in isolated rats. Also, MDA in serum, salt consumption, and alcohol consumption increased in the isolated group.

Conclusions: Social isolation with reduction of neurogenesis predisposes rats to consume more alcohol and also salt. The reduction in neurogenesis is associated with paradoxical balance of zinc and copper and increase in MDA in serum. So, regulation of copper and zinc may have beneficial effects on neurogenesis, sensitization and alcohol preference.

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Introduction

Substance abuse is one of the most serious concerns of human society. After poverty, atomic crisis, and environment degradation, addiction is the first issue that consumes most afford and energy, both for controlling addiction and its adverse effects such as period of withdrawal. So, identifying behaviors that favor abusing more drugs and deteriorating their adverse effects is mandatory. Alcohol dependence is a serious health concern among adolescents. It is the most prevalent substance abused in many countries such as the United States. The progression to binge drinking occurs in some abusers. Social isolation is a maladaptive behavior that occurs for some reasons. It can occur through improper health, misappropriate personality and social disturbances. In the previous studies, social isolation has been associated with impaired brain functions such as memory and mood balance. Social isolation also deregulates dopamine balances in the brain [1].

Neurogenesis occurs in two brain regions: sub ventricular zone and dentate gyrus [2]. It has been suggested that it is involved in learning, memory, recovery after stroke and depression [3]. Neurogenesis is also affected by drugs such as morphine and cocaine [4]. Development of occasional abuse to everyday abuse occurs with unknown mechanisms. Finding new mechanism is of great help for curing substance abuse. Hippocampal neurogenesis can be a center for regulation of drug abuse. In a study for three reasons hippocampal neurogenesis has been postulated to have a role in drug addiction development: 1) chronic abuse inhibits neurogenesis 2) drug abuse does not affect sub ventricular zone 3) the alternation of neurogenesis is not dependent on corticotropin-releasing factor as it occurs in some conditions such as stress, age, and exercise [5].

Zinc is an important element that is necessary for the function of many enzymes in the brain. It is more concentrated in the hippocampus, amygdala and neocortex. Zinc deficiency affects cognitive function [6]. Zinc deficiency decreases neurogenesis [7]. Copper also is an important element essential for many bodily functions [8]. Neurogenesis also is affected by its alternations [9]. Copper is highly regulated very sensitive in the body because deregulation of copper level can have neurodegenerative effects. It also involves in the synthesis of neurotransmitters [8].

Salt consumption (as a marker of sensitization) is a predictive sign of poor prognosis in drug abuse [10]. A condition that favors its development is an important issue that is to be delineated for avoiding binge drinking. Salt consumption development is unknown phenomenon that has been examined differently in several studies, but frequent abuse is more associated with this behavior development [11]. In this study, another hypothesis is examined, without using drugs before testing it has been evaluated. In this way, reduced neurogenesis is a major factor for developing salt consumption and isolation favors its development.

Stress-oxidative stress is assessed in different ways. In this study, malondialdehyde (MDA) level was assessed. Zinc and copper help antioxidant defense enzymes [12]. It also causes lipid peroxidation.

Zinc and copper are two elements that are involved in regulation of neurogenesis and cognitive function [8, 13]. It is postulated that alternation of copper and zinc levels in turn alter neurogenesis. Neurogenesis in hippocampus regulates drug abuse, but the exact mechanism that neurogenesis in hippocampus favors abusing drugs is not well understood. So, in this study, salt appetite was examined to find out if social isolation can induce sensitization by decreasing neurogenesis. In this way, a new mechanism for inducing abusing alcohol would be identified.

The aim of this study was to discover how preference to alcohol abuse changes along with neurogenesis changes and along with it other signs of worse prognosis such as sensitization develop. Among these questions, the role of other factors such as copper and zinc is important because good treatment can be established. So, this study was performed for finding a way for controlling the development and establishing good treatment for alcohol abuse development.

Materials and Methods

Animal care

The experimental protocols followed in this study were conformed to the guidelines for the care and use of laboratory animals published by the national institution of health (NIH Publication No. 85-23, revised 1996) and was further approved by the Institutional Ethical Committee at Tehran university of medical science (Tehran, Iran).

Animals

In this study, 16 male Sprague-Dawley rats weighing 200 to 250 grams were used in two separate groups: pair (social) and isolation. One rat was used for modeling socialization. So, 8 rats were added to the experiment (overall 24 rats were used). For isolating rats in this study, rats were put in a separate small cage (27×15×21) covered with black plastic [14]. For modeling pair (social) state two rats were put in a large cage (42×15×21). It should be noted that in the modeling social state, there is a controversy if two or three rats is satisfactory. In this study, two rats were put in a cage [15].

Experimental design

In this study, sixteen rats were divided into two groups: Pair (social) and isolation. Rats in isolation group were isolated for 14 days plus one week for acclimatization. Rats in pair group also were kept in the same condition for 14 days. In this period, BrdU (50 mg/kg/i.p.) was injected. At the end of experiment, rats examined for copper, zinc, MDA, neurogenesis, sensitization and alcohol preference. Between behavioral tasks, one day gap was considered for the avoidance of behavioral interference.

Food consumption

Conventional chokes (food pellet) were offered for 24 hours. It was weighted before offering. At the end of 24 hours, they were again weighted. After subtracting it from total

with digital balance total food consumption in 24 hours calculated.

Salt appetite

To evaluate salt appetite, animals were placed on food and water restriction plan for 24 hour. Animals were offered NaCl 3% (Sigma-Aldrich) for one hour. Total NaCl consumption was calculated [16].

Alcohol preference test

At the end of 14 days of the experiment for 24-hour, rats were introduced alcohols 10% for assessing the preference for alcohol consumption. Briefly, in this experiment water is removed and rats just introduced alcohol 10% for 24-hour. The rats were placed in individual cages for this experiment. The alcohol 10% solution was made by mixing alcohol with tap water. Rats were freely accessed to food. In this protocol, alcohol consumption in 24-hour was corrected based on water intake. Lower consumption of water in 24-hour was correlated with taking more alcohol in this experiment. Water consumption was measured the day before alcohol preference test. This formula was used to measure the preference for alcohol: (Alcohol consumption / Alcohol consumption + Water consumption)×100 [17].

Copper and zinc assessment

For obtaining plasma, after thoracotomy before paraformaldehyde perfusion, five milliliter blood was taken from left heart. After coagulation and centrifugation, plasma was collected in micro tubes and stored in -70ºC. For preparing plasma for analysis of copper and zinc level, at first they were incubated with 65% citric acid for 2 hours. Then, for one hour they were incubated with 65% perchloric acid. The final solution was examined with atomic spectroscopy (Varian-220-FS-aa). After obtaining absorbed wavelength, it was adjusted with calibration curve and expressed as p.p.m [6].

MDA measurement in serum

For assessment of the level of MDA in serum, firstly, we made a solution composed of 1 milliliter of trichloroacetic acid 20% (TCA 20%) and thiobarbituric acid 1% (TBA 1%) to the total volume of 2 ml. Then, 100 milliliter of biologic solution (serum) was added to the above solution and was placed in boiling bath for 90 minutes. After cooling to room temperature it was centrifuged at 1000 gravidity for 10 minutes for removing insoluble particles. Then, the final solution was assessed for the absorbance of UV wavelength of 532 nanometers with a spectrophotometer.

Immunohistochemistry

At the end of 14th-day, the animals were sedated and anesthetized with xylazine (10 mg/kg) and ketamine (100 mg/kg), respectively. The brain was first perfused by 100 ml normal saline and then fixed by 100 ml paraformaldehyde 4% via cardiac infusion. Then, the brain removed from the skull. For 48 hours brain was kept in Phosphate-buffer saline (PBS)+paraformaldehyde 4% and then kept in the third day in sucrose 10%+paraformaldehyde 4%+PBS, in the forth day sucrose 20%+paraformaldehyde 4%+PBS and in the rest in sucrose 30%+paraformaldehyde 4%+PBS. The cryosections (30 µm) were prepared from the hippocampal region (dentate gyrus). Five sections per animal were stained for BrdU-positive neurons with the kit (Roche) for histochemistry. Briefly after diluting primary antibody and secondary antibody solutions and

adjusting PH, the sections were rinsed by them. Then, color solution was made and added for giving color to newly proliferated BrdU-incorporated neurons.

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, hilus, granular cell layer and molecular layer were assessed using a light microscope (Zeiss, Germany) were counted in a blinded manner bilaterally. BrdU-positive cells were counted in the dentate gyrus in rostrocaudal fashion. As shown in Fig. 9, regions that were counted in the hippocampus were whole dentate gyrus. BrdU-positive neurons appeared much bigger than usual and appeared as singles or cluster cells. The Mean was estimated for every five sections in this study and neurons were not multiplied by each section count [18].

Statistical analysis

Data were analyzed using SPSS version 22 and Graph pad Prism 5. An independent sample two-tailed t-test was performed for all experiments. Data were represented as mean±SEM and $P<0.05$ considered significant.

Result

Level of zinc in isolation period reduced compared to pair state (1.715±0.015 vs 1.54±0.01 p.p.m, $P<0.01$) (Fig. 1).

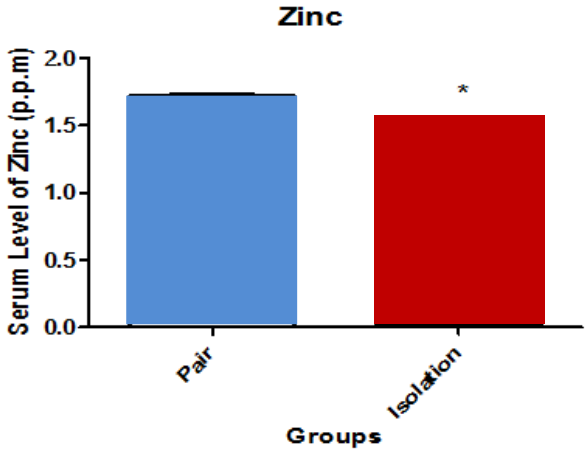

Fig. 1. Level of zinc in serum reduced in rats in isolation. Isolation has increased the need for this substance (N=6). Data are as represented as mean±SEM. *p<0.01

Level of copper in the isolation period increased compared to the pair state (0.47±0.02 vs 0.69±0.025 p.p.m, $p<0.02$). Copper indirectly affected cognitive function and neurogenesis. The copper level correlated negatively with prognosis (Fig. 2).

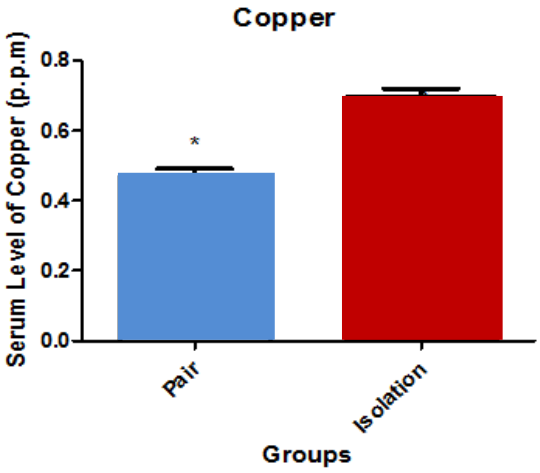

Fig. 2. Level of Copper in serum increased in rats in isolation. Isolation has caused this phenomenon. This is a pathologic condition (N=6). Data are as represented as mean±SEM. *p<0.02

Food intake increased in the pair state compared to isolation state (21.83 ± 0.7326 vs 17.39±0.94 gr, p<0.03). In the pair state, the rewarding system seems to work more properly (Fig. 3).

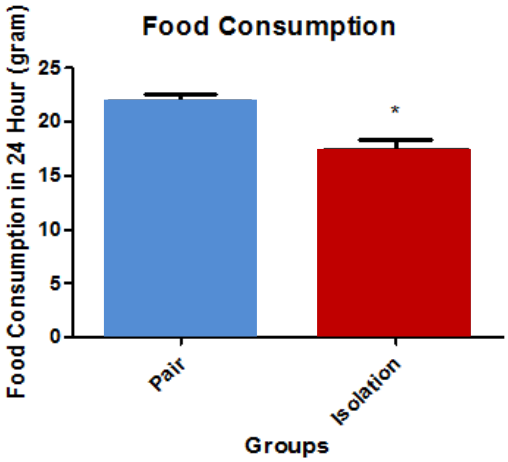

Fig. 3. Food consumption in a 24-hour reduced in rats in isolation. Pair state has positive effects in the establishment of positive behavior (N=8). Data are as represented as mean ±SEM. *P<0.03

Alcohol consumption increased in the isolation state compared to the pair state (72.08 ± 0.9671 vs 77.53 ± 1.234%, p<0.02). This test was used for assessing the probability of dependence to alcohol. Ingestion of more alcohol correlates positively with dependence (Fig. 4).

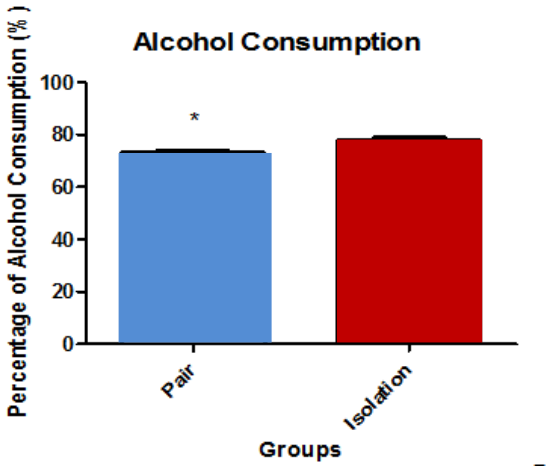

Fig. 4. Percentage of alcohol consumption in a 24-hour increased in rats in isolation. Isolation has caused this phenomenon. It is indicative of dependence to alcohol (N=4). Data are as represented as mean ± SEM. *P<0.02

Salt consumption increased in the isolation state compared to the pair state (0.25±0.25 vs 1.833±0.441 ml, P<0.02). Ingestion of more salt was associated with sensitization, a sign for poor prognosis. Drinking more is associated with uncontrollable alcohol; drinking. Epigenetic factors were also suspected for this phenomenon (Fig. 5).

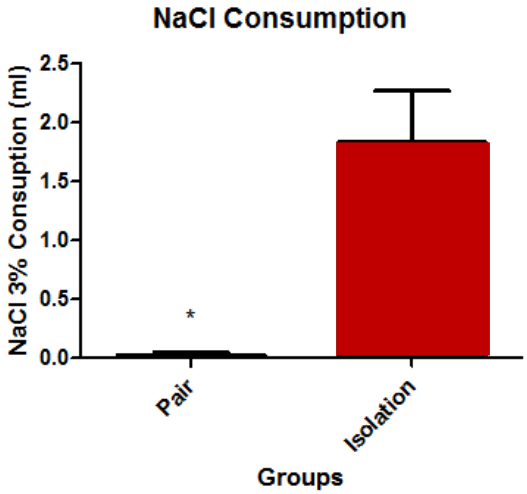

Fig. 5. Amount of NaCl 3% consumption in 1 hour increased in rats in isolation. Isolation has caused this phenomenon. As the figure shows there is a significant difference (N=8). Data are as represented as mean ±SEM. *P<0.02

Level of MDA was higher in the isolated group than the pair group (0.6575±0.050 vs 1.016±0.05390 µmol/l, p<0.003). An elevated level of MDA as the result of isolation interfered with biological enzymes and impaired brain function (Fig. 6).

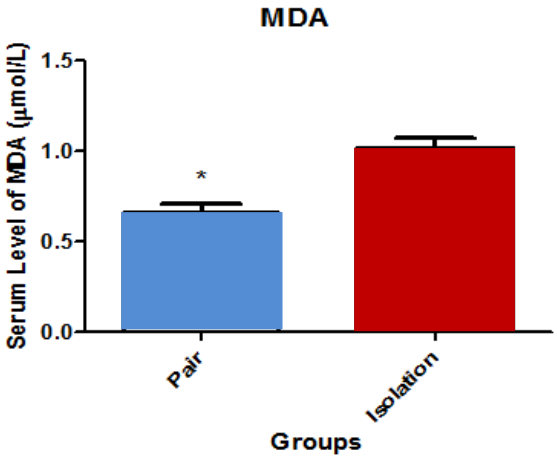

Fig. 6. MDA level increased in isolated state and it impairs cell signaling. Zinc and copper improve antioxidant effect and this elevation also predicts impair zinc and copper level (N=6). Data are as represented as mean ±SEM. *p<0.003

Proliferation of new neurons significantly reduced in isolation compared to the pair state (204±23.42 vs 27.6±3.709 No, p<0.001) (Figs. 7, 8 & 9).

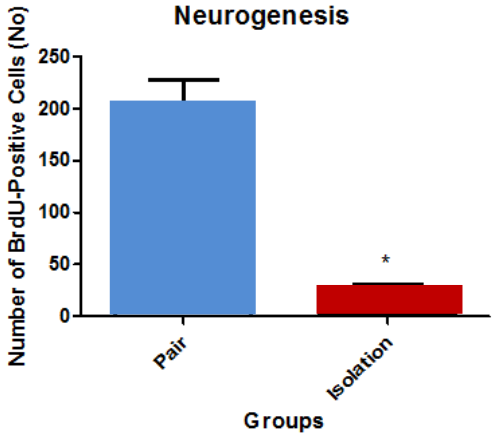

Fig. 7. Number of BrdU positive cells indicative of new neurons reduced in rats in isolation. Isolation has caused this phenomenon directly or indirectly. As the figure shows there is a significant difference (N=6). Data are as represented as mean ± SEM. *P<0.001

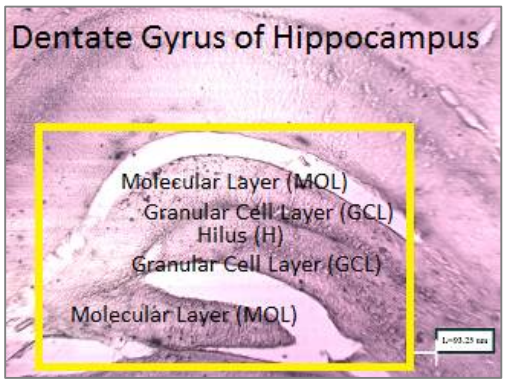

Fig. 8. Different parts of the dentate gyrus of Hippocampus have been marked in the picture. Counting of the BrdU-positive cell has been done in these areas. It has three parts: molecular layer (MOL) (outer (OML), middle (MML) and inner (IML)), granular cell layer (GCL) and hilus (sub granular zone (SGZ) and deep hilus).

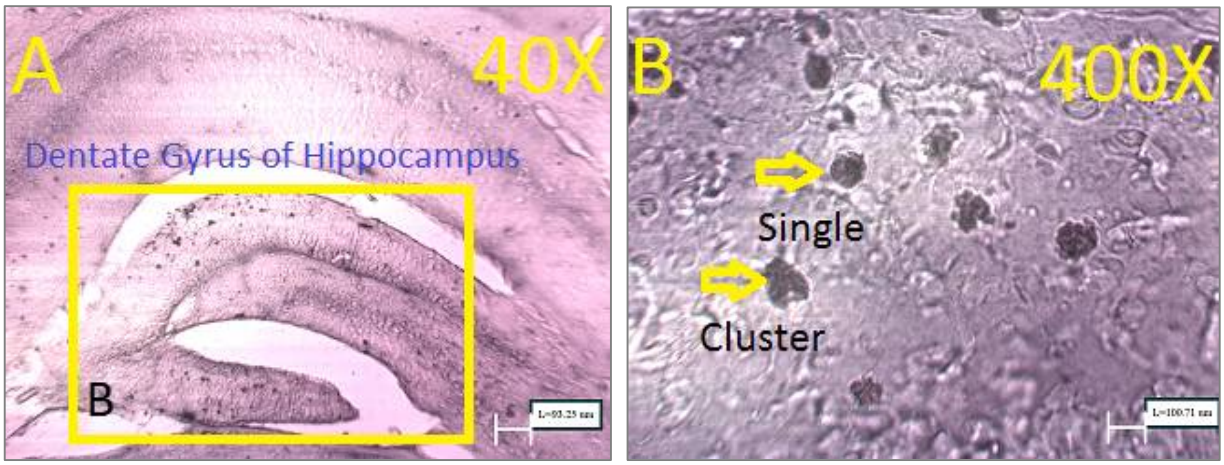

Fig. 9. A) In the region constricted by lines the BrdU-positive cells have been counted from rostral to caudal under light microscopy. B) BrdU-positive cells have been colored brown. They may be in cluster forms (more than two neurons) or single forms. It should be noted that all BrdU-positive neurons have not been shown by arrows.

Discussion

In this study, isolation alters alcohol preference test and salt appetite with reduction of neurogenesis and paradoxical regulation of zinc and copper. Progression of substance abuse from occasional abuse to addiction is a multistage process that begins with acquisition, maintenance, detoxification, abstinence and relapse to drug abuse [19]. In this regard, neurogenesis in hippocampus also is a multistage process, which is believed that every stage of alternation in shape, maturation, and dendritization are involved in a specific pattern of relapse [20].

There is some evidence that supports the regulation of rewarding behavior by hippocampus neurogenesis. Because neurogenesis is regulated by environmental modalities such as stress, environmental enrichment, and physical exercise, it is believed that neurogenesis in the hippocampus can be a center that can influence rewarding center independently [20]. In this study, isolation has also changed the neurogenesis and may be rewarding nature of hippocampus neurogenesis. The rewarding behavior that was measured is alcohol consumption and salt consumption. In this study, isolation affects proliferation. In this regard, there is a positive correlation between the number of new neurons and function of the hippocampus.

The hippocampus is known the region of the brain that is related to addictive behaviors. In previous studies, stimulation of hippocampus has caused relapsed [21]. Also, the hippocampus can stimulate a region of the brain to involve in drug-seeking and drug-taking behavior such as ventral tegmental area ventral tegmental area (VTA) [22] and Nucleus accumbens [23]. Neurogenesis in hippocampus thus can regulate addictive behavior.

In the previous studies, alcohol preference test was influenced by gender and strains of rats

[24]. Type A gamma-aminobutyric acid knock-out rats also had altered alcohol consumption [25]. In this study, isolation shifted alcohol consumption to more consumption. This is indicative of the influence of environment on this behavior. Along with the reduction of neurogenesis, this can be concluded that rewarding center can be in the hippocampus and also because of vicinity to the rewarding center can modulate the rewarding –dependent behavior.

Salt consumption is an ominous sign that is predicative of sudden drug seeking and taking behavior [26]. For the development of salt consumption long term and repeated drug taking is necessary. In this study, it was proved that isolation can precipitate the occurrence of salt consumption. It can be characterized in the open field with increased number of rearing and loco motor activity [27]. In other experiments, it is characterized by an increase in the consumption of salt as it occurred in this study. In the previous studies, alternations in neuroplasticity and the number of neurons in the nucleus accumbens and changes in the number of NMDA receptors in dorsomedial striatum have associated with consuming more salt.

Low level of zinc in isolation period seems to decrease proliferation of neurons in the dentate gyrus of the hippocampus. Low level of zinc in isolation period can be the result of low appetite in isolation period. In this study, rats in the isolated group consumed less food than the rats in the pair group. Copper paradoxically increased isolation period. This can be interpreted that new proliferative neurons are more prone to a level of zinc than copper. This is because of local storage of copper in the hippocampus; in the first stage of the disease has been enough. On the other hand, it seems that zinc consumption has been increased dramatically and this has accounted for low zinc level. The mechanism by which zinc affect neurogenesis and central nervous system function has been elucidated in the previous studies. Functions of many enzymes are dependent on zinc. Many of these enzymes are involved in proliferation such as DNA polymerase, RNA polymerase, histone deacetylase and DNA ligase. Other enzymes are metalloproteinases and dehydrogenases and zinc –fingers [28]. Copper also is involved in many vital enzymes such as metalloproteinases. Zinc besides proliferation of new neurons is necessary for maturation of them and the expression of nestin [13]. The mechanism that is responsible for low proliferation rate is involvement of transcriptional factors. In the previous studies, zinc deficiency has reduced neurogenesis [7]. Also, copper can influence neurogenesis by modulation of manganese level in the subventricular zone [9]. The increase in the copper level has been attributed to the pathologic state [8]. Copper increase differentiation of new neurons to mature cells by expression of transcriptional factors.

Appetite in isolation group was reduced. This can interpret as the decline in the flexibility of behavior that is related to the proper function of rewarding behaviors. Hippocampal neurogenesis in this regard can be considered as a center for regulation feeding behavior directly or indirectly. This

has relation to the development of unwanted behavior.

The advantage of being in the social or pair state can be attributed to the hormonal factors. In several studies, the hormone that is responsible for positive effects of social interaction has been known as oxytocin [29]. In other studies, prolactin also has been had positive effects [30]. These positive effects partly have been attributed to increase neurogenesis and partly to the other factors.

Gene-environment interaction plays an important role in alcohol dependence [31]. It is well-known that epigenetic changes occur in some forms such as methylation and acetylation. Areas of brain responsible drug dependence are prone to epigenetic changes. In this study, it is postulated that epigenetic changes in neurogenesis is responsible for both low proliferation and may be responsiveness of the other brain areas for drugs such as VTA and nucleus accumbens [32].

Increasing in MDA production impairs signaling for both maturation and proliferation of young neurons for enough neurogenesis [33]. As it was stated, copper and zinc balance is necessary for antioxidant defense. Treatment for normalizing MDA level alleviates symptoms and improves neurogenesis. Recently MDA has been proposed as a biomarker for assessing the severity of drug abuse vulnerability [34].

Conclusion

In this study, we saw that hippocampal neurogenesis involves in behavioral flexibility. The rewarding aspects of behaviors can be regulated directly or indirectly by hippocampal neurogenesis. The relation is positive, so with increasing neurogenesis, the behaviors are regulated in a positive manner. In this sense, copper and zinc regulation plays a vital role. Thus restoration of neurogenesis and balance of minerals such as zinc and copper can help flexibility of behaviors and proper habits.

Conflict of Interest

None to declare.

Acknowledgment

There is no acknowledgement to declare.

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