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ORIGINAL ARTICLE

Investigating the Relationship between Hippocampal BDNF Gene Expression and Spatial Memory in the Open Field Test in Offspring Born from Rats Treated with Ethanol

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ABSTRACT: Alcohol consumption during pregnancy causes damage to the mother and the developing fetus, especially the central nervous system. Exposure of pregnant mothers to ethanol activates cell death pathways and interferes in the expression of neurotrophic factors and spatial memory of children. The aim of this study is to investigate the relationship between hippocampal BDNF gene expression and spatial memory in the offspring of pregnant rats treated with ethanol, and which period of pregnancy the hippocampus suffers more damage from ethanol consumption. The pregnant rats were randomly divided into five groups (six in each group): the control group (received distilled water only) and four treatment groups that received ethanol (20%, 4.5 g kg^{-1}) by oral gavage, in the first 5 days, the first decade, the second decade and the entire period of pregnancy, respectively. The half of offspring was sacrificed on the 30th day after birth according to ethical principles, and their hippocampal brain tissue was extracted. The other half of the children, who were not victims, were subjected to a behavioral test of spatial memory and cognitive memory using the open field test, 24 hours later. BDNF gene expression was measured by RT-PCR method. The mixture of 1 microgram of RNA and 1 microliter of cDNA synthesis primer was incubated for 5 minutes at 70 degrees. Then 2 microliters of nucleotide mixture, 4 microliters of reverse transcriptase enzyme buffer, 1 microliter of reverse transcriptase enzyme, 1 microliter of RNase inhibitor protein were added and incubated for 10 minutes at 25 degrees, one hour at 42 degrees, and 10 minutes at 70 degrees. For data analysis, SPSS and Excel Softwares and one-way ANOVA and Tukey tests were used. The results showed that ethanol administration during pregnancy induced spatial memory disorder ($p < 0.001$). Alcohol consumption during rat pregnancy led to fetal growth and development disorders and disorders related to learning and spatial and cognitive memory in children, which had the most adverse effects in the treated groups in the second decade and the entire period of pregnancy. It can be concluded that this part of the nervous system during this period of pregnancy suffers more damage from alcohol consumption.

INTRODUCTION

Ethanol is a well-known substance with many medical and industrial uses, which is consumed orally and has dangerous effects on various body parts. The increasing use of alcohol (ethanol) as a beverage in different societies has gradually revealed its harmful effects, and today extensive research is being done on the negative effects of alcohol [1].

Pharmacologically, ethanol is considered a psychoactive

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drug, which has a major depressant and suppressive effect on the central nervous system. Also, this drug has rewarding effects due to its activity on the reward circuit of the brain, which causes addiction and dependence on alcohol. Ethanol easily passes through most of the cell membranes of the body and has no biological barrier in the body, and when consumed by a pregnant mother, it easily passes through the placenta and reaches the fetus [2, 3].

At this stage of development, because the fetus does not have a mature or active ethanol metabolizing enzyme, i.e. alcohol dehydrogenase, the activity and effect of ethanol in the fetus lasts longer than in the mother's body [4].

Worldwide, about 10% of women consume alcohol during pregnancy, and one out of every 67 of these women gives birth to a child with fetal alcohol syndrome (FAS). Children with this syndrome have alcohol-related disorders (FASD) such as developmental delays, facial deformities, nervous system disorders, mental retardation, and cognitive defects [5, 6].

Cognitive defects include learning and memory disorders, social interaction problems, and high mobility and attention disorders. Many of these problems are more prominent during school years and often persist into adulthood [7, 8].

While experimental and clinical researches have largely focused on learning, memory and attention problems, prenatal exposure to alcohol and the prevalence of anxiety-related disorders in youth and adulthood have shown a strong correlation [9].

Ethanol affects different neurotransmitter systems; For example, it inhibits glutamate excitatory receptors and activates GABA inhibitory receptors [10].

Other factors causing mental changes in alcoholics include brain trauma, meningitis, hypoglycemia, liver-induced encephalopathy, alcohol-induced ketoacidosis [11, 12].

The hippocampus, which is involved in the processes of learning and memory, is known as a part of the brain that is very vulnerable and sensitive to the toxic effects of ethanol during the development period, so that the administration of alcohol to rat babies in the first ten days of birth which in terms of the stages of brain development is equivalent to the three-month human fetus, causes an increase in cell death, disruption in synaptogenesis and a permanent defect in neuron plasticity in the hippocampus [13, 14].

These structural changes in different areas of the hippocampus are associated with a decrease in the number of cells and a decrease in neurogenesis in adulthood [15].

Brain-derived growth factor (BDNF) is a neurotrophin that affects cell growth and differentiation and synaptic changes and is expressed in large amounts in the hippocampus of developing fetuses and adults. Alcohol has serious effects on information processing and memory, especially short-term memory and spatial memory related to the hippocampus [16].

The hippocampus plays a vital role in various forms of memory and spatial learning. This part is necessary for processing information related to space recognition and working memory. Since this part plays a vital role in the process of knowing and understanding space, it can be said that exposure to alcohol disrupts the processing of spatial information. The purpose of this study is to investigate the relationship between hippocampal BDNF gene expression and spatial memory in the offspring of pregnant rats treated with ethanol, and which period of pregnancy the hippocampus suffers more damage from ethanol consumption.

MATERIALS AND METHODS

Ttreatment of animals

Adult male and female Wistar rats weighing 180-200 grams were prepared (5male mice and 30 female mice) and transferred to the animal house of Islamic Azad University, Damghan Branch, following animal welfare guidelines. Six rats per cage were kept in 12 hours of darkness/12 hours of light and temperature of 20-22˚C and had free access to food and water.

One week after acclimatization of the animals to the new environment, the experiments began. Male and female mice were kept in separate cages overnight for mating. Pregnant rats were randomly divided into 5 groups of 6 from the day zero of pregnancy as follows: 1- Control group: normal pregnancy receiving distilled water by gavage. Treatment groups a 20% ethanol solution in distilled water at a dose of 4.5 g kg^{-1} by oral gavage once

a day. 2- The group that was treated with ethanol during the first five days of pregnancy. 3- The group who were treated with ethanol in the first ten days of pregnancy. 4- The group who were treated with ethanol in the second ten days of pregnancy (10th day to. 5- The group that was treated with ethanol during the entire pregnancy. Male and female rats were kept in separate cages overnight for mating. A number of children were sacrificed on the 30th day after birth, and the hippocampal tissue of the brain was extracted, and the other half of the children who were not sacrificed, were subjected to behavioral tests of spatial memory and cognitive memory using the open field test 24 hours later.

Molecular method

RT-PCR method was used to measure gene expression. For cDNA synthesis, the mixture of 1µg of RNA and 1 microliter of cDNA synthesis primer was incubated for 5 minutes at 70 degrees. Then 2 microliters of nucleotide mixture, 4 microliters of reverse transcriptase enzyme buffer, 1 microliter of reverse transcriptase enzyme, 1 microliter of RNase inhibitor protein were added and incubated for 10 minutes at 25 degrees, one hour at 42 degrees, and 10 minutes at 70 degrees. The reaction mixture includes2 microliters cDNA, 0.75 microliters MgCl2, 2.5 microliters DNA polymerase enzyme buffer, 0.125 microliters DNA polymerase enzyme, 0.5 microliters nucleotide mixture, and sense and antisense primers for each gene with a final concentration of 0.5 µM. After initial denaturation at 94 degrees for two minutes, PCR was performed according to the following schedule: denaturation at 94 degrees for 15 seconds, annealing at 60 degrees for 1 minute, and synthesis at 72 degrees for 1 minute. The program was completed with a total of 29 cycles. After completing the cycles, the final synthesis reaction was performed at 72 degrees for 2 minutes. Table 1 shows the primer specifications.

PCR method

The necessary materials for PCR: cDNA, Tag DNA polymerase enzyme (U μl^{-1}), C8108TA, C7507CG DNA polymerase enzyme buffer, Mixture of nucleotides (10 mM) C7604DN, GAPDH, GLT1 sense and antisense primers, Mgcl2 (50 mM) C7506TP, DNA (10 mg ml^{-1}) 881603 PR, DNA Ladder (bp100), Electrophoresis buffer (0.5 X TBE). Table 1 shows the primers used for the reaction.

PCR program for BDNF gene: 1- Denaturation at 94°C for 15 seconds, 2-Annealing at 60 degrees for 1 minute, 3-Synthesis at 72 degrees for 1 minute, 4-This program was done with a total of 29 cycles, 5-After completing the cycles, the final synthesis reaction was performed at 72 degrees for 2 minutes (Primer code: Accession Number BDNF NM-012513).

PCR program for GAPDH gene: 1-Denaturation at 94 degrees for 15 seconds, 2-Annealing at 55 degrees for 30 seconds, 3-Synthesis at 72 degrees for 1 minute, 4-This program was done with a total of 30 cycles. 5-After completing the cycles, the final synthesis reaction was performed at 72 degrees for 2 minutes (Primer code: Gapdh mw 342530).

Table 1. The primers used for PCR

Primer	Sequence	Primer size (bp)	Product size (bp)
GAPDH-F	5'-TGACATCAAGAAGGTGGTGAA-3'	21	203
GAPDH-R	5'-CCCTGTTGCTGTAGGCGTATT-3'	21	203
BDNF-F[57]	5'-GCCCAACGAAGAAAACCATA-3'	20	405
BDNF-R	5'-GATTGGGTAGTTCGGCATTG-3'	20	405

Electrophoresis

A mixture of 10 microliters of the PCR product with 2 microliters of loading buffer was electrophoresed on a 1.5% agarose gel (TBE buffer, final concentration 0.5X, voltage V100, time 30 minutes). The gel was observed with a gel display device (UVI doc). In this research, GAPDH was used as a control gene. The relative amplification rate of each gene was measured by Image J software. To determine the relative amount of gene

duplication, the band intensity of each gene was measured relative to the GAPDH gene band intensity.

Open field habituation test

This test is done to check spatial memory. An animal was placed in an open field in a new environment without any stimuli and the animal was allowed to explore the field freely for a certain period of time. The dimensions of rectangular open field chamber were 50 cm high, 60 cm long and 40 cm wide. At the beginning of the experiment, the rat is slowly placed in the middle or one of the sides of the open environment, and after that, in a certain period of time (usually 5 minutes), some variables are measured in order to calculate the exploratory behavior. One of the most important indicators, the number of times the animal traversing, the number of cleanings, and the number of times it crosses the lines separating the parts of the floor. Habituation to a new environment is one of the most basic forms of learning, in which the reduction of exploration follows repeated exposure to a new environment. The environment is considered as an indicator of memory. The chamber was cleaned with 10% alcohol between each test. All tests were performed at a specific time of the day to avoid the influence of the circadian rhythm on the animal's motor activities.

Statistical analysis

Data Analysis was done using SPSS 21 software. The difference between groups was determined using one-way ANOVA, and then Tukey's test was used to determine the level of significance between groups. The significance levels were $p < 0.01$ and $p < 0.001$. The graphs were drawn using Excel software. The results were presented as Mean ± SEM.

RESULT AND DISCUSSION

There is a significant difference in the relative level of BDNF mRNA expression in different groups. The relative expression level of BDNF mRNA in groups 3, 4 and 5 had a significant decrease compared to the control group ($p < 0.001$). Also, the relative expression level of BDNF mRNA in group 2 ($p < 0.01$) showed a significant decrease compared to the control group (Figure 1).

The number of squares walked in the groups: the results showed a significant difference in the number of squares walked among the different groups. Groups 2 ($p < 0.01$) and 5 ($p < 0.001$) had a significant decrease compared to the control group. Also, no significant difference was observed between the control group and groups 3 and 4 (Figure 2).

The number of standing in groups: the results showed a significant difference in the number of standing among different groups. Results showed that groups 2 ($p < 0.01$), 4 ($p < 0.001$) and 5 ($p < 0.001$) had a significant decrease compared to the control group. Also, no significant difference was observed between the control group and group 3 (Figure 3).

Number of cleaning in groups: The results showed a significant difference in the number of cleaning among different groups. Groups 4 ($p < 0.001$) and 5 ($p < 0.001$) had a significant decrease compared to the control group. Also, no significant difference was observed between the control group and groups 2 and 3 (Figure 4).

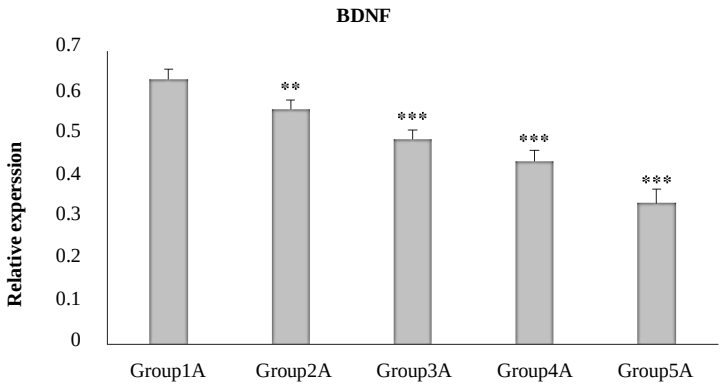

Figure 1. The relative expression level of BDNF in the hippocampus of offspring.
**: $p < 0.01$, ***: $p < 0.001$, significant difference with the control group

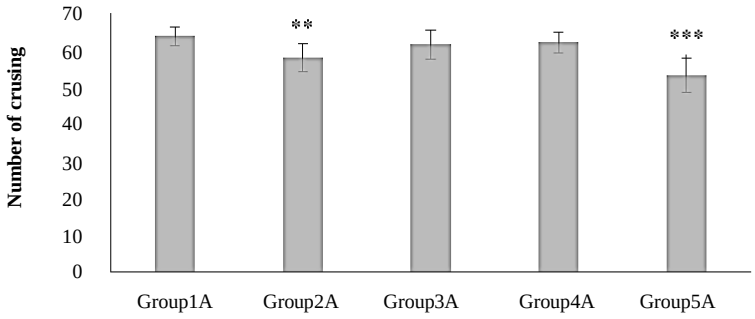

Figure 2. Average number of squares traversed in groups.
**: $p < 0.01$, ***: $p < 0.001$, significant difference with the control group

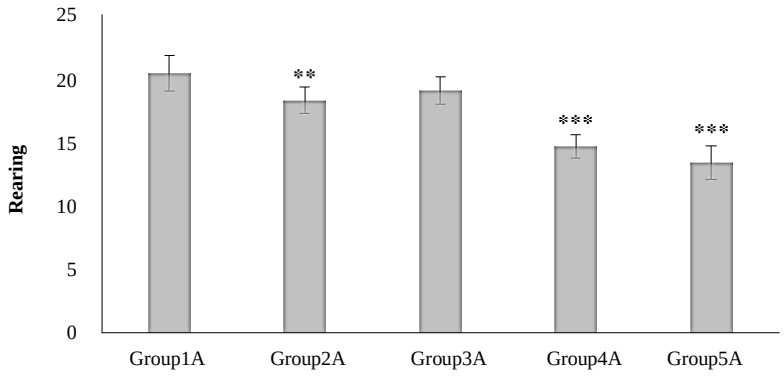

Figure 3. Average number of standing in groups.
**: $p < 0.01$, ***: $p < 0.001$, significant difference with the control group

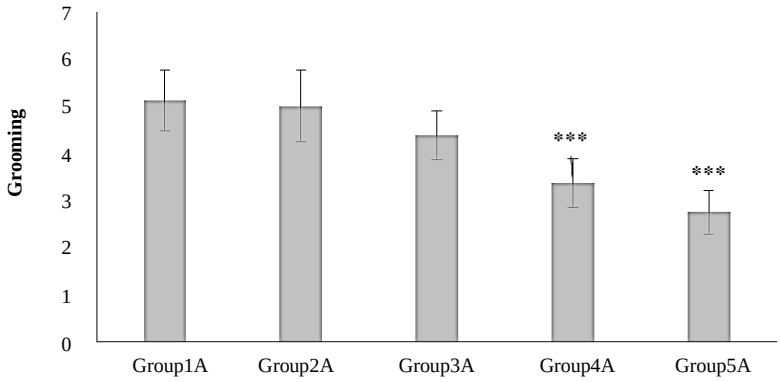

Figure 4. Average number of cleaning in groups.
***: $p < 0.001$, significant difference with the control group

The results of this research showed that the consumption of 4.5 grams/kg of ethanol during pregnancy leads to developmental abnormalities and learning, spatial and cognitive memory disorders in offsprings. Ethanol enters the bloodstream of the fetus through the placenta [17].

The fetus has a limited ability to metabolize alcohol due to the low level of the main alcohol metabolizing enzyme, the hepatic alcohol dehydrogenase enzyme. Therefore, elimination of alcohol from the fetus through passive diffusion from the placenta will follow elimination of alcohol from the mother. In addition, the removal rate of alcohol from amniotic fluid is almost half of the excretion through maternal blood; for this reason, it causes a relatively high concentration of alcohol in amniotic fluid, while alcohol levels are low and excreted from maternal blood. Therefore, the amniotic fluid may act as a reservoir for alcohol and indeed the fetus is exposed to alcohol for a longer period of time than predicted based on the mother's blood alcohol concentration [18].

Understanding the mechanisms that play a role in cognitive disorders resulting from alcohol consumption during fetal development will help to develop therapeutic strategies for these disorders. On the other hand, the results of the molecular section showed a decrease in the expression and activity of BDNF. These molecules, in addition to the role they play in the development and differentiation and survival of neurons, play an important role in synaptic flexibility, which is the basis of learning and memory in the hippocampus. Therefore, the developmental abnormalities and learning and memory disorders observed in this study can be attributed to the reduction of BDNF system activity. The results of present study using the RT-PCR technique showed that ethanol causes a significant decrease in the amount of BDNF mRNA in the hippocampus of newborn children compared to the control group (groups 3, 4 and 5, significant decrease compared to the control group ($p < 0.001$). Also, the relative expression level of BDNF mRNA in group 2 ($p < 0.01$) showed a significant decrease compared to the control group).

Alcohol treatment during brain development causes a decrease in the amount of BDNF and changes in its receptor, tyrosine-kinase B (TrkB). This action is performed through disruption of the intracellular signaling pathways involved in cell survival, i.e. MAPK/ERK and PI3K/AKT pathways. These effects and events lead to an increase in cell death due to apoptosis and necrosis in the cerebellar cortex [19].

Habituation to a new environment is one of the most basic forms of learning, where the reduction of exploration following repeated exposure to an environment is considered as an indicator of memory. Groups 4 and 5 showed a significant decrease compared to the control group and it indicates a memory disorder. Therefore, it can be concluded that the effect of ethanol consumption during pregnancy in reducing the amount of mRNA in the hippocampus of children, which was observed in this research, can also play a role in learning and memory disorders.

Alcohol consumption during pregnancy in rats (from the 5th day of pregnancy to the end of pregnancy) decreased the level of BDNF expression and protein in the cortex and hippocampus of 8-day-old offspring and also inhibited TrkB receptor phosphorylation in hippocampus, but it did not change the total amount of TrkB protein. They suggested that changes in BDNF expression and function could play a role in alcohol-related deficits in offspring [20].

BDNF and other neurotrophins are necessary and vital for neurogenesis in the hippocampus. In fact, BDNF is a powerful synaptic facilitator, and animals that are deficient in this protein have impaired long-term reinforcement and impaired learning and memory [21].

There are some contradictions in the results of hippocampal BDNF levels of mice exposed to ethanol [22, 23], which can probably be related to the tested animal, the time of exposure of animals to ethanol, age and dose of ethanol [24].

Oxidative stress induced by ethanol can reduce the level of BDNF by the following mechanisms: 1- reducing the activity of activator protein I (which is a protein that binds to DNA), and the transcription factor CREB, which is reduced by the expression BDNF gene is related. 2- Energy depletion through the induction of oxidative stress and as a result the dysfunction of the NMDA channel, which is related to the reduction of BDNF gene expression. 3- Oxidative stress with oxidative damage to

macromolecules, including oxidation of nucleic acids, can cause mistakes in transcription and translation. As a result, DNA damage and disruption of the DNA repair system can lead to a decrease in BDNF mRNA and its downstream factors [25].

Traumatic brain injury through oxidative stress can cause a decrease in BDNF and its effectors such as synapsin I and CREB in the hippocampus of rats, which can play a role in cognitive disorders [26].

In the present study, ethanol consumption led to a decrease in the expression of important molecules involved in the underlying mechanisms of memory and learning such as BDNF in the hippocampus, which reduced and improved learning and memory disorders in children.

CONCLUSIONS

According to the present data, it can be concluded that alcohol consumption (v/v 20%, 4.5 g kg^{-1}) during the second decade of pregnancy can lead to the induction of fetal growth and development disorders and disorders related to learning and spatial memory. And to make children aware that these indicate the role of oxidative stress in inducing developmental and behavioral abnormalities in FASD patients. On the other hand, according to the decrease in BDNF mRNA level, it can be concluded that the oxidative stress resulting from ethanol consumption in the second decade of pregnancy causes a change and decrease in mRNA levels and thus participates in learning and memory disorders caused by ethanol. As a result, this study can show the relationship between oxidative stress and BDNF in learning and memory disorders induced in FASD patients. In the present study, new evidence was presented regarding the relationship between oxidative stress and BDNF for the effect on synaptic plasticity and cognitive functions such as learning and memory.

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ETHICAL CONSIDERATION

All experiments were carried out in compliance with the ethical principles with the code of ethics IR.IAU.DAMGHAN.REC.1401.016.

Conflicts of interests

The authors declare no conflict of interest.

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