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The effect of swimming exercise and hesperidin on hippocampal cell damage after pentylenetetrazol induced prenatal seizures in rats

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Abstract

Introduction: Prenatal seizures cause the production of free radicals in fetal nervous system and selective neuronal death of hippocampus. The purpose of this study is to evaluate the effect of swimming exercise (EX) and hesperidin administration (HES) as a potent antioxidant on hippocampal cell damage in rats following pentylenetetrazol (PTZ) induced prenatal seizures.

Materials & method: In this experimental study, infants from 20 females pregnant Wistar rats were randomly put in 5 groups of control, PTZ+NS, PTZ+HES, PTZ+EX, PTZ+HES+EX. From the 14th day of pregnancy, animals were exposed to repeated PTZ injection (50mg/kg, intraperitoneal) for 5 consecutive days. From the first day of pregnancy to the term delivery, swimming exercise training with moderate intensity and 3 times a week and daily hesperidin gavage for 3 weeks was done. Finally, the hippocampal cell density was evaluated in different hippocampus areas of male infants at postnatal day 30.

Results: The increase of cell damage and decrease of cellular density in different regions of hippocampus was seen in PTZ+NS group than the control group ($p<0.05$). Meanwhile, the increase of hippocampal cellular density and decrease of mortality of pyramidal neurons was also observed in PTZ+HES+EX group compared to PTZ+NS group ($p<0.05$).

Conclusions: The interaction of swimming exercise training and hesperidin have neuroprotective effects in rats' prenatal seizure models.

Keywords: Pregnancy, Swimming, Hesperidin, Seizure, Hippocampus.

1. Introduction

Epilepsy seizures causes induction of histopathological changes in brain by making disturbance in releasing and neurotransmitters function (1). Spontaneous and repeated neuronal discharges following seizure caused an extensive cell death in nervous system (2). The first changes which occurred because of long term repetition of this event are followed by the selective death of brain cells and intensification of oxidative stress particularly in hippocampus area (3). Hippocampus as an important part of brain, has a significant role in learning and acquired memory and damage to this area resulted in the life quality decrease, cognitive decline

and memory deficiency (4). Therefore, an effort to control seizure and thus to prevent cognitive disorders can significantly improve the lives of people with this complication. However, different factors such as neuropathology, type of seizure, mental problems and side effects of medication are effective on seizure disorders (5). Among people suffering from epilepsy, more than 75% of them live in developing and poor countries without proper health services (6).

Women with epilepsy have less pregnancy than normal women. Different reasons such as sexual desire reduction, social issues, genetic disorders and side effects of anti-epileptic drugs cause fertility and fetal mortality decrease in epileptic women (7). The use of anti-epileptic drug in such women in pregnancy period has a destructive effect on the developing fetus. The administration of such drugs can lead to the fetal abnormalities, neural tube closure defect, congenital heart defects, cleft lip and face, growth and mental retardations, microcephaly and cognitive disorders (8). Most of the children from epileptic mothers with seizure or using anti-epileptic drugs background during pregnancy have weaker cognitive performance than normal children and suffer from mild to moderate mental retardation (9).

In recent decades, the use of anti-epileptic drugs had various side effects. Using such drugs specially in pregnant women have negative inevitable effects on fetus's brain and deficiency in cognitive performance. Therefore, it seems necessary to find treatment methods to stop seizure and its causes or to find drugs that prevent its cognitive disorders (10). On the other hand, occurrence of seizures intensifies the oxidative stress in hippocampus which is considered as the main reasons of neural degeneration, learning and memory defects in laboratory models (11).

Different evidence confirmed the role of sport and physical activities in physical and mental health. The reinforcement of brain antioxidant defense system and increase of antioxidant enzymes activities arising from the compatibility with exercise after oxidative stress reduces damages to brain (12). Also, sport activities will end at an increase in the phenomenon of long-term plasticity in dentate gyrus of rat hippocampus as well as neurogenesis and improved performance in behavioral tests of learning and memory (13).

Hesperidin is a flavanone from the flavonoid family that is extracted from citrus peels. Flavonoid are aromatic polyphenolic compounds that have strong antioxidant properties due to the presence of hydroxyl groups and play a protective role against free radicals caused by oxidative stress (14). The ability to remove hydroxyl and superoxide anions by strong antioxidant system of this compound protects cells (15).

The purpose of this study is the investigation of the effect of swimming exercise training and hesperidin administration on cellular damage and density in different areas of hippocampus in rats which were exposed to the induced seizure by pentylenetetrazol in prenatal period.

2. Materials and methods

Animals and experimental protocol

In this experimental research, 40 infants with 1-month old (n=8 in each group) exposed to PTZ during embryonic period were used. Infants were the mating result of 20 virgin female rats with average weight of 180 ± 10 grams and 20 mature male Wistar rats. After preparing animals from the Laboratory Animal Breeding Center of Shiraz University of Medical Sciences, they were transferred to the specialized laboratory of animal sciences of the Islamic Azad University, Shiraz Branch. Animals were kept in the animal room for 1 week to adapt to the new conditions and then treated. In all stages, animals were put in standard temperature condition of 25 ± 2°C, relative humidity of 50 ± 10% and lightness/darkness cycle of 12 hours (from 6:00 a.m. to 6 p.m.). Tap water and standard food for laboratory rats (purchased from Fars 110 Company) were freely available throughout the study. Ethical principles were observed in accordance with the international law and the recommendation of the Ethics Committee of Laboratory Animals of Islamic Azad University of Shiraz (Ethics Code: IR. IAUSHIRAZ. 1398. 14.23).

Before mating, vaginal smear was prepared from female rats to determine the estrus cycle and mating preparation. Therefore, 0.3 ml normal saline was injected to the rat's vagina and then a drop was taken from vaginal fluid and the smear was prepared. Smear samples then

investigated after dying with Giemsa paint under the light microscope (40X magnification). After the identification of cornified cells without nuclei in the vaginal smear, which indicates the estrous cycle, they were mated with male rats in the early hours of sunset. Female animals were examined in the early hours of the morning to ensure mating. Zero day of pregnancy was determined by observing vaginal plaque or the presence of spermatozoa in a vaginal smear (16). Pregnant rats were randomly divided into 5 groups of 4. Healthy control groups, PTZ+NS (pentylenetetrazol and normal saline receiver group), PTZ+HES (pentylenetetrazol and hesperidin with 100mg/kg dosage receiver group), PTZ+EX (pentylenetetrazol receiver and swimming exercise group), PTZ+HES+EX (pentylenetetrazol and hesperidin with 100mg/kg dosage receiver and swimming exercise group). Injection of PTZ with repeated dosage of 50 mg/kg of the animal's body weight from the 14^{th} day of pregnancy for 5 consecutive days was done intraperitoneally. Rats were investigated half an hour after PTZ injection for seizure behavior (17).

Score 0: no response. Score 1: hyperactivity, shake, tension. Score 2: head shaking, head muscle seizure and myoclonic jump. Score 3: unilateral anterior muscle seizure. Score 4: expansion of seizures with bilateral anterior muscle seizure. Score 5: generalized tonic-clonic seizure with elongation reflex (18).

Swimming exercise and hesperidin (Sigma, Germany) gavage of 100 mg/kg dosage for 3 weeks and from the first day of pregnancy was performed in pregnant mothers. After the term delivery, 8 infants (2 male infants from each mother) were selected randomly among all offspring of 4 pregnant rats in each group and then were studied. Mothers' mortality during seizure or after that, abortion, stillbirth, weight loss, and infants' genetic disorders cause limitation for the study and criteria for the exclusion of animals from the study. Infants were kept with their mothers up to postnatal day 30.

Swimming exercise protocol

This protocol includes 3 weeks of swimming (from the first day of pregnancy to the end of pregnancy or term delivery) in a circular pool specified for rodents with 1m diameter, 50cm water height, water temperature of 25±2° C for 20 minutes each session (with average

intensity) and 3 sessions in a week. Animals were entered to the pool before exercise to get acquainted with the environment and warm up. In the main phase of exercise, they left a fixed part of the pool and swam to the hidden platform. The platform which had 10 cm diameter and made of transparent Plexiglas was 1 cm under water. As soon as they were on the platform, the platform position was changed for the animal to continue swimming (19).

Histopathology

Separation and stabilization of brain tissue in animals was performed under deep anesthesia with chloroform trans-cardiac perfusion method. Following washing blood from the whole body by injection of 100 ml normal saline into the left ventricle and cutting right atrium, 200 ml of 10% v/v formalin was replaced. After the end of perfusion and sacrifice, brain was separated from skull and weighed by a sensitive digital scale. Brain sample was kept in fixator (4% paraformaldehyde) for 72 hours.

In order for microscopic investigations of the target tissue, it was dehydrated by ethylic alcohol solution after tissue fixation by fixator. After keeping the tissue for 6 hours in alcohol, the alcohol was removed from tissue by xylene three time of 25-30. Then, paraffin impregnation and sample blocking were performed. Finally, paraffin small blocks were fixed on rotary microtome and thin cuts of 10-micron thickness were made. Then, samples dying was done by hematoxylin-eosin. Slides were studied and compared by light microscope (Olympus BH_2, Japan).

To determine cellular density in different regions of hippocampus, first 4 hippocampi areas (CA1, CA2, CA3, DG) were identified by Atlas of Paxinos and Watson and then cells were counted in place by Dissector method. Neural density was calculated by using density formula of $NV=\Sigma Q/\Sigma P\times AH$ in which the total number of counted cells in a sample was ΣP: the number of sampling in a sample, A: space of sampling frame, H: thickness of each cut (20).

Statistical analysis

The statistical analysis was done by SPSS software version 20 between different groups. Data were reported as mean $\pm$ standard deviation. To investigate the normal distribution of data, Shapiro-Wilk test was used.

Also, a two-way analysis of variance and Tukey post-hoc test were applied to determine whether there is a significant difference between the groups or not. The values of p <0.05 were considered statistically significant.

3. Results

The investigation of histopathological effect on hippocampus

The results of cellular density were compared in 4 different areas of hippocampus in control and treatment groups (Table 1). As you can see in table 1, cellular damage, cellular density decrease in all areas of CA1, CA2, CA3 and dentate gyrus were observed in 4 areas of hippocampus in groups receiving PTZ (igure 1). Cell damage decrease and increase of neural density were shown in PTZ+HES+EX group which received hesperidin in embryonic period and mothers had swimming exercise in comparison to PTZ+NS group.

Cellular density and degeneration effects of PTZ in groups receiving this substance than control group and treatment groups were assessed. There was a significant difference between PTZ+NS group and control group in CA1, CA2, CA3 areas and dentate gyrus (table 1. $p<0.001$). In CA1 area, there was a significant difference between PTZ+NS and all other treatment groups (table 1. $p<0.001$). In fact, there was a significant increase between PTZ+HES, PTZ+EX, PTZ+HES+EX compared to PTZ+NS group (table 1. $p<0.001$). In this area, there was also a significant difference between PTZ+HES group and PTZ+EX and PTZ+HES+EX groups ($p<0.05$). Cellular density had a significant increase in CA2 area of PTZ+HES+EX group compared to PTZ+NS group ($p<0.01$). There was a significant difference between PTZ+HES+EX group with PTZ+HES and PTZ+EX groups ($p<0.01$). In CA3 area, there was a significant difference between PTZ+HES, PTZ+EX, and PTZ+HES+EX groups in comparison to PTZ+NS group ($p<0.01$). In this area, there wasn't a significant difference between treatment groups ($p>0.05$). Cellular density in DG area had a significant increase in groups receiving hesperidin and swimming exercise compared to PTZ+NS group ($p<0.0001$) that this difference is the indicator of the neuroprotective effect of hesperidin and swimming exercise training with

cellular damage decrease in infants' hippocampus by convulsive substance of PTZ (figure 1). Comparison between treatment groups showed a significant increase of neural density in PTZ+HES+EX group with PTZ+HES and PTZ+HES+EX ($p<0.01$).

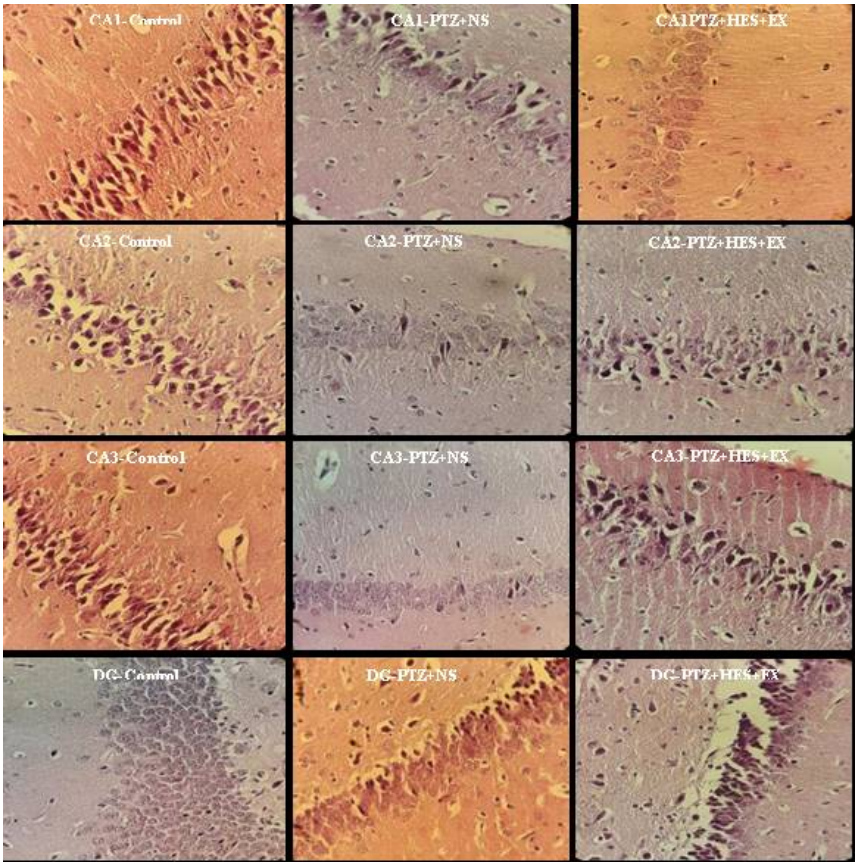

Figure 1. Micrographs prepared from different areas of the hippocampus in the groups. Neuronal density is higher in PTZ+HES+EX group than PTZ+NS group. Hematoxylin-eosin staining (H&E). Magnify 40X.

Table 1. Neural density mean in hippocampus areas

Group	CA1	CA2	CA3	DG
Control	55.10±1.5	23.3±6.1	18.6±5.1	76.10±5.4
PTZ+NS	22.4±6.1	10.2±2.5	8.3±1.5	32.9±6.7
PTZ+HES	38.7±2.8	11.2±2.8	16.2±2.8	50.8±8.3
PTZ+EX	42.6±6.4	15.4±3.6	15.2±4.9	52.11±7.1
PTZ+HES+EX	48.5±2.1	20.3±4.3	16.4±2.8	62.13±5.9

Results are reported in form of mean ± standard deviation. Groups with common letters don't have significant difference with each other. Significant level in one-way analysis of variance is considered $p<0.05$.

4. Discussion

The results of the study showed that repeated injection of PTZ in pregnancy period causes cell damage to different areas of fetal hippocampus by making myoclonic seizures. Infant who were exposed to PTZ in their embryonic period, suffer from cellular damage in hippocampus and cell density decrease that the damage is permanent and lasts until 1 month old. Injection of PTZ is one of the appropriate methods to make an epileptic model in animals (21). The previous studies have shown that maternal seizure which was induced by PTZ reduces learning and memory significantly in infants (22). Also, the 14^{th} day of pregnancy is better for PTZ injection and kindling start. Because in this phase of pregnancy brain structure is forming and maternal seizures have negative effects on neurogenesis, neural migration to hippocampus and finally on infant's mind (23).

Studies have shown that induced seizures by PTZ have direct effects on expression level of protein kinase A (PKA). PKA is a main molecule in synapsis transfers which interfere in molecular and synapsis events of epilepsy (24). In maternal epilepsy, the reduction of GABA receptor expression in fetus lead to the intracellular changes in PKA expression level (24). Also, reduction of mRNA expression of GABA in CA1 and CA2 areas of hippocampal pyramidal cells following seizures has been observed in rats (25). Since seizure causes the increase of acidosis and hypoxia that results in irreversible damages in central nervous system (CNS) and other fetus's organs. Maternal epilepsy causes a change in the form of fetus's hippocampus and this causes their cognitive disorders (26). In fact, neurons in hippocampus areas have damages because of epilepsy pathologic changes and will suffer neural death (26).

It is seen in the present study that swimming exercise training in mother's during their pregnancy (3-week exercise) reduces the cellular damages of fetus hippocampus in a way that cellular density has had a significant increase in different areas of hippocampus of PTZ+EX group than PTZ+NS group in CA1, CA3, and DG areas. Studies have shown

that maternal seizures produce free radicals, oxidative stress induction in CNS in embryonic period and after that (27). Oxidative stress has an important role in neural destruction. Brain is the most sensitive organ to oxidative stress because it needs a lot of oxygen. High oxygen consumption causes lack of electron in cell respiratory chain and produces radicals and results in oxidative stress (28). The previous studies have shown that following physical activities, increased oxygen consumption increases the production of reactive oxygen species (ROS) to the point that it optimizes oxidative signals and regulates signaling pathways and gene expression in various tissues, including the brain and heart. This improves the function of mitochondrial respiration, reduces lipid peroxidation and reinforces the antioxidant defense system in these tissues (29). Exercise also improves the function of nervous cells by replicating cells in the hippocampus and inhibiting apoptosis in the dentate gyrus (DG) of the hippocampus and increasing synaptic space in various parts of the brain (30).

Reduction of cell damage following treatment by hesperidin increases cellular density in CA1, CA3 and DG areas. There is also a significant increase in neural density in all hippocampus areas in swimming exercise and hesperidin groups (PTZ+HES+EX) which assess the interaction of swimming activities and hesperidin. As oxidative stress is the main factor of degeneration and induction of oxidative damage in hippocampus and pyramidal cell damage, antioxidants will be able to prevent hippocampus cell damage (31). Hesperidin is a flavonoid found abundantly in citrus fruits. It is proved that this combination has the quality of reducing oxidative stress in the experimental model of Alzheimer's disease (AD) induced by amyloid-beta (32). It also reduces brain damage and improves destruction of the blood-brain barrier following hypoxia (33) and prevents neural damage in experimental model of Parkinson's disease through reducing the expression of Irrk2 and GSK-3β kinases with caspases 3 and 9 (34). Hesperidin in citrus can reduce Bax expression and antioxidant enzymes, and improve cognitive disorders and reduce apoptosis in AD (35).

The results of the present study showed that 3-week swimming exercise training with hesperidin consumption have more effect on the improvement of cell damage and increase of cellular density in

hippocampus than swimming exercise and hesperidin separately in PTZ induced prenatal seizures. It is concluded by the review of previous studies that swimming exercise and hesperidin consumption by same effect may improve the cellular damage in hippocampus and therefore, the present study is also proved by the synergic of these two treatments. Lack of oxidative stress parameters, measurement of antioxidant enzyme activity, total antioxidant capacity and ROS level in hippocampus are some of the limitations in the present study that can be effective in elucidating the mechanism of physical activity and hesperidin treatment on hippocampal function in prenatal seizure model.

5. Conclusion

Overall, the findings of this study showed that 3 weeks of swimming in rats' pregnancy period and hesperidin consumption reduce the cellular damage in fetal hippocampus of prenatal seizure modal. Given that compared to other neuroprotective drugs, antioxidants have much fewer side effects, and the need for moderate physical activity in at all stages of life has been emphasized. If clinical trials are performed to confirm these effects, it is recommended to use swimming and hesperidin supplementation to reduce cognitive disorders and prevent fetal hippocampal damage in epileptic mothers.

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