

The Effect of Erythropoietin on Spatial Memory and Entorhinal Cerebrocortical Level of BDNF in Rat Model of Intrauterine Growth Restriction

Hamideh Rajabpour, Mohammad Amin Edalatmanesh*

Department of Biology, Shiraz Branch, Islamic Azad University, Shiraz, Iran

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***Corresponding author:**

Mohammad Amin Edalatmanesh,
Department of Biology, Shiraz
Branch, Islamic Azad University,
Shiraz, Iran

Phone: +989396335040

Fax: +987136410059

Email: edalatmanesh@iau.ac.ir

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Abstract

Introduction: Intrauterine Growth Restriction (IUGR) causes disruption for the central nervous system of fetus and is followed by cerebral cortex damage of neonates. This study aims to analyze the effects of erythropoietin (EPO) on spatial memory and brain-derived neurotrophic factor (BDNF) in entorhinal cortex of IUGR rat models.

Methods: For IUGR induction, anterior uterine artery occlusion surgery is carried out on rats in embryonic day (ED) 14. From ED12, EPO are injected subcutaneously in 500, 1000 and 2000 IU/kg doses until the birth of their neonates. Spatial memory is analyzed by Morris water maze at postnatal day (PND) 30. Then, BDNF cerebral cortex level is estimated using ELISA. Differences between groups is analyzed with ANOVA and considered statistically significant at $p \leq 0.05$.

Results: A significant decrease is observed in spatial memory and BDNF cortex of untreated IUGR group in comparison with the control group ($p \leq 0.05$). On the other hand, treatment of EPO improves spatial memory by increasing BDNF level in entorhinal cortex ($p \leq 0.05$).

Conclusion: The present study indicates that fetal growth restriction causes cognitive disorder in rat model. Consequently, expression of neurotrophic factors, such as cerebral cortical BDNF, will be decreased. Moreover, neuroprotective effects of EPO could ameliorate cognitive deficits in IUGR model.

Keywords: Entorhinal Cortex, Intrauterine Growth Restriction, BDNF, Erythropoietin

Introduction

Intrauterine Growth Restriction (IUGR) is a fetal disease which restricts or stops growth. IUGR neonates are encountered with long term and short term complications (1). These complications are the latent reasons of neonatal mortality (2). One of the main reasons of IUGR is uterine artery occlusion which causes hypoxia and fetal death (3). In fact, 20% of fetal deaths are caused by intrauterine growth restriction (4). IUGR neonates have growth and cognitive retardations, musculoskeletal disorders, cardiovascular abnormalities, and highest prenatal and neonatal mortality rate (5). In IUGR, changes in glucose level and insulin homeostasis are energy saving processes

which help the fetus to survive in an environment with insufficient nutrients (6). IUGR is mostly caused by utero-placental insufficiency (UPI) which is followed by reduced nutrition and oxygen transmission to fetus (7). Since proliferation and migration of neurons in central nervous system occur in pregnancy and neonatal periods, UPI ventures neuronal migration, and fetal brain development (8). IUGR reduces catecholamine level in fetal brain (9). Therefore, it can possibly damage necessary cognitive functions, such as learning, memory, reward, motivation, and emotion (10). Brain-derived neurotrophic factor (BDNF) and Neurotrophin-3 (NT-3) drop in cerebral cortex of IUGR patients (11). Cerebral cortex

manages many complex functions, such as emotional-perceptual, motion control, speech and thinking, memory, and learning. Damage in cerebral cortex causes many complex cognitive and sensory-movement disorders (12). Entorhinal cortex is the ventral-caudal (VC) part of brain hemispheres which is identified anatomically and histologically with its output axons of layer II to dentate gyrus of the hippocampus (13). In anterior level, entorhinal cortex is connected to piriform cortex, and in lateral level, is connected to pre-amygdala cortex and amygdala posterior nucleus (13). The studies indicate that layer II neurons of entorhinal cortex will be damaged through aging or cognitive disorders, such as Alzheimer's disease (AD) (14). Furthermore, entorhinal cortex is a heterogeneous environment in which cellular adjustment is followed by cognitive disorders (14). BDNF signaling disruption is an attractive mechanism for selective vulnerability analysis of layer II entorhinal neurons in aging and AD (15). Many factors which increase risks of cognitive disorders (such as diabetes or physical inactivity patterns) will reduce BDNF levels in different parts of brain, especially layer II cortex neurons (16). On the other hand, there are some evidences which approve the increase of inflammatory factors in entorhinal cortex of Alzheimer patients (17). In transgenic rat model with AD, a selective increase of microglia activity and pre-inflammatory cytokines, such as TNF- α , were observed (18). Despite the confirmed role of Erythropoietin (EPO) in red blood cell production, EPO and its receptors are expressed in many tissues, such as brain, heart, and retina. Therefore, EPO has a wide range of physiological functions. EPO can reduce oxidative stress and inflammatory responses, and increase BDNF release (19). Furthermore, it increases neuroplasticity and can act as a neuroprotective substance (20). Therefore, the aim of this study is to analyze neuroprotective effects of recombinant human erythropoietin on spatial memory and expression of Brain-

derived neurotrophic factor (BDNF) in anterior cortex of IUGR rat models.

Methods

In this experimental study, 60 male rat neonates were used which were produced from mating of 24 adult virgin female rats (weight: 180 ± 10 g) and 24 adult male Wistar rats. The rats were provided from experimental animal center of Shiraz University of Medical Sciences. They were kept in the laboratory for 8 days in order to adopt with new conditions. In all stages, the animals were kept in standard temperature ($2 \pm 25^{\circ}\text{C}$), humidity ($10 \pm 50^{\circ}\text{C}$), and 12 hour light-dark cycles (6 am to 6 pm). 60 neonate rats in 30 days of birth (PND30) were classified randomly in experimental groups. All the animal trials were approved by the Ethics Committee of Islamic Azad University, Shiraz Branch. Before mating, vaginal smears were taken from female rats. Consequently, those rats which were in their estrous cycle were selected for mating. In fact, the selected female rats had 2 or 3 regular estrous cycles in 12 to 14 consequence days of vaginal smear observations. For vaginal smear, primarily 0.3 ml normal saline was injected to animal's vagina. Then, some drops of this liquid was extracted as smear. The samples were analyzed with using optical microscope (Olympus, Japan) with $\times 400$ magnitude. Rats with estrous cycle were selected for further stages of the studies. Vaginal smear has more stratum cells in estrous cycle in comparison with epithelial cells (It also has no leukocytes) (21). After ensuring about mating, rats were re-examined the next day. In case of observing vaginal plug or spermatozoa cells in vaginal smear, that day was selected as embryonic day zero (E0). In these studies, pregnant rats are classified randomly in 4 groups: healthy control group, IUGR group, and IUGR+EPO500, IUGR+EPO1000, IUGR+EPO2000 groups. After induction of intrauterine growth restriction, 500, 1000, and 2000 UI/kg doses of human recombinant erythropoietin were injected subcutaneously at

ED12 until ED21. For IUGR induction, weighted female pregnant rats were anesthetized after 14 days of pregnancy using a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg). Then, after laparotomy surgery and observance of uterus horns, all the fetuses were driven out of abdomen. After counting live fetuses, right and upper left uterine vessels were totally cauterized using electrocauter, so that no blood flow existed after cauterizing. Afterwards, fetuses were taken back into the abdomen and surgical site was sutured (22). In order to prevent any potential infection, 500 ml/kg penicillin was injected intramuscularly. After recovery, rats were transmitted to recovery cage and supervised for 24 hours. Regarding stillbirths of many female rats which received IUGR surgery, among total 25-30 male neonates, 15 were selected randomly (2 to 3 male neonates from each mother). The same number of male neonates from healthy control group selected. Neonates were weaned at PND24 and analyzed at PND30. Morris water maze was used for spatial memory evaluation in the experimental groups. The maze was a black pool, 136 cm diameter and 60 cm high, which was filled with water up to 25 cm of its depth (temperature: 24 ± 1 degrees centigrade). A platform with 10 cm diameter was placed 1 cm beneath the water and some images were stacked on the wall. Moreover, the location of test taker was fixed all through the test. Each rat was tested 4 times a day for 5 days. The first 4 days (4 blocks) were considered to be learning stages. In the fifth day (probe stage), the platform was removed and the animal was kept in the water for 60 seconds. Escape latency, which is the time it takes to find the hidden platform, and swimming speed were the main variables which were analyzed in learning stage. In probe stage, time spent in each quadrant (target quadrant) was estimated (23). After cognitive tests, the animals were deeply anesthetized by inhaling chloroform gas in desiccator. Then, they were immediately decapitated and their brains are taken out of

their skulls and preserved in ice. Rats' entorhinal cortex was separated accurately from other parts of brain under stereoscope (Olympus, Japan). The entorhinal cortexes were washed with saline solution using Tris buffer (Sigma, Germany) for 5 minutes and homogenized in homogenizer machine (IKA, Germany) with 5000 rpm. The homogenized solution was centrifuged by refrigerated centrifuge (Hermle, Germany). In order to prevent the destruction of enzymes and proteins, all the stages were carried out in 4°C (refrigerated centrifuge) and 0.5 mM phenyl methyl sulphonyl fluoride (Sigma-Aldrich, Germany) solution was used as protease inhibitors (24). After centrifuge, tissue BDNF amount of the supernatant solution was estimated by ELISA method and Boster kit (Rat BDNF Picokine™, Boster; China). Statistical analysis of different groups was done by SPSS software version 22. In order to determine significant differences among groups, ANOVA test (Probe test and BDNF estimation), repeated measure ANOVA (learning stage of Morris water maze) and Tukey post hoc test were used ($P < 0.05$).

Results

The results of repeated measure ANOVA and Tukey post hoc tests in each 4 blocks of learning stage indicated that escape latency and swimming distance were reduced in all the studies groups (Figure 1). In fact, escape latency in IUGR group has a significant increase in comparison with second block ($p < 0.01$, 32.93 ± 6.1 vs. 22.62 ± 4.5 seconds), third block ($p < 0.01$, 31.17 ± 4.6 vs. 12.63 ± 3.5 seconds), and fourth group ($p < 0.01$, 26.95 ± 4.8 vs. 12.63 ± 3.5 seconds). Moreover, in those groups which received EPO, mean time of finding platform decreased significantly in second, third, and fourth learning blocks in comparison with IUGR group. So that in IUGR + EPO500 group, escape latency decreased significantly in IUGR + EPO500 group in comparison with third block (23.40 ± 4.7 vs. 31.17 ± 6.2 seconds) and fourth group

(18.63 ± 2.7 vs. 26.95 ± 2.9 seconds) ($P < 0.05$). IUGR+EPO1000 indicated a significant difference with IUGR group in escape latency of second block (24.76 ± 3.5 vs. 32.93 ± 5.4 seconds), third block (19.62 ± 4.3 seconds), and fourth block (13.35 ± 2.9 seconds) ($p < 0.01$). Furthermore, IUGR + EPO2000 group indicated a significant decrease of escape latency in comparison with IUGR group in its second block (24.19 ± 4.1), third block (19.49 ± 4.6) and fourth block (15.41 ± 2.2 seconds) ($p < 0.01$). Among those groups who received 500, 1000, and 2000 doses of erythropoietin, a significant difference has been observed in their second block ($p < 0.05$). One way ANOVA revealed no significant difference in swimming speed of different groups based on mean and standard deviation analysis of IUGR and control groups with those groups which received EPO and IUGR ($p > 0.05$). These results indicate that no movement disorder is induced to IUGR rat models (Figure 2). The results of one way ANOVA in target quadrant swimming time indicate a significant decrease in IUGR group (19.52 ± 1.8) in comparison with control group (5.2 ± 5.2 seconds) (Figure 3, $p < 0.0001$). On the other hand, there is a significant difference between IUGR + EPO500 group (25.05 ± 3.4 seconds) and control group. Moreover, there is a significant difference between EPO group (IUGR + EPO500 group: $p < 0.05$, IUGR + EPO1000 group: 29.22 ± 4.2 seconds, $p < 0.01$, and IUGR + EPO2000 group: 34.0 ± 3.8 seconds, $p < 0.0001$) and IUGR group (Figure 3). Moreover, a significant difference exists between IUGR + EPO500 and IUGR + EPO2000 groups ($p < 0.01$). One way ANOVA and Tukey post hoc test showed that IUGR significantly decreases expression level of BDNF in entorhinal cortex in comparison with control group. So that amount of BDNF is lower in cerebral cortex tissues of IUGR group (228.43 ± 54.12 Pg/dl) than the control group (402.14 ± 43.4 Pg/dl) (Figure 4, $p < 0.001$). However, EPO treatment increased BDNF level in treatment groups in comparison

with IUGR group. Therefore, there is a significant difference between IUGR + EPO1000 group (312.78 ± 34.6 Pg/dl) and IUGR + EPO2000 (354.32 ± 37.3 Pg/dl) with IUGR group ($p < 0.05$).

Discussion

In this study, effects of EPO prescription on cognitive disorder and expression level of BDNF in entorhinal cortex followed by uterine artery occlusion in rat neonates are analyzed. IUGR neonates are encountered with growth and cognitive retardations (25). This model can be used for evaluation of neuronal damages in different parts of brain, such as cortex, and their effects on cognitive disorders. In the present study, blood flow of anterior uterine artery to uterine and placenta is totally de-vascularized which causes fetal hypoxia and neuronal-cognitive disorders in neonates. Some events, such as hypoxia, stress, toxicity, inflammation, and decreases blood flow to placenta can affect fetus growth in pregnancy. Chronic UPI and discontinuous consumption of oxygen and nutrients can cause abnormal growth of the fetus. Determining the significant effective factors on changes of brain growth in IUGR neonates is a key for prevention and treatment of brain damages (26).

Recent human studies indicate that nervous disabilities, learning disorders, paramnesia, and mood disorder are prevalent in those children who had experienced IUGR (27) In this case, the highest brain damages is caused by UPI. BDNF decrease in entorhinal cortex of IUGR rat models in comparison with control group indicates that a change in expression pattern of neurotrophins in cortex of IUGR neonates can create cognitive disorders (28). Cerebral cortex has multiple functions in memory processes, including planning, decision making, learning and attention, knowledge, thinking, and understanding (12). Cortex damage can significantly damage spatial memory (29).

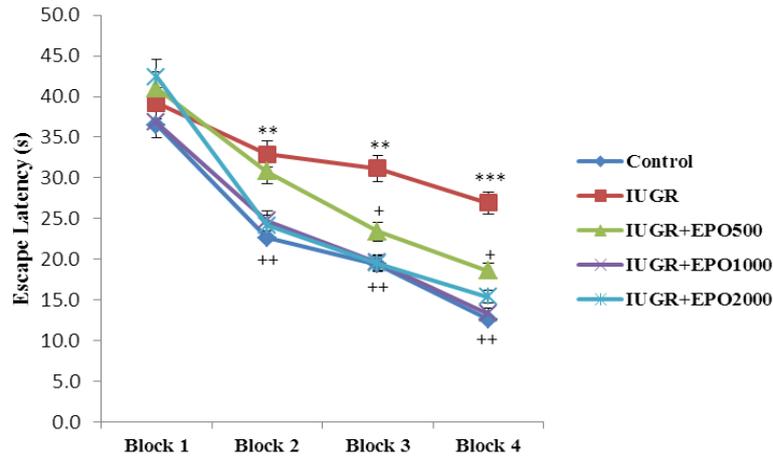


Figure 1. Mean \pm standard deviation of escape latency in four trial blocks of Morris water maze groups. The results indicate that there is significant different between IU GR group and control group ($**p < 0.01$) and treatment groups of EPO and IU GR ($^{++}p < 0.01$, $^{+}p < 0.05$).

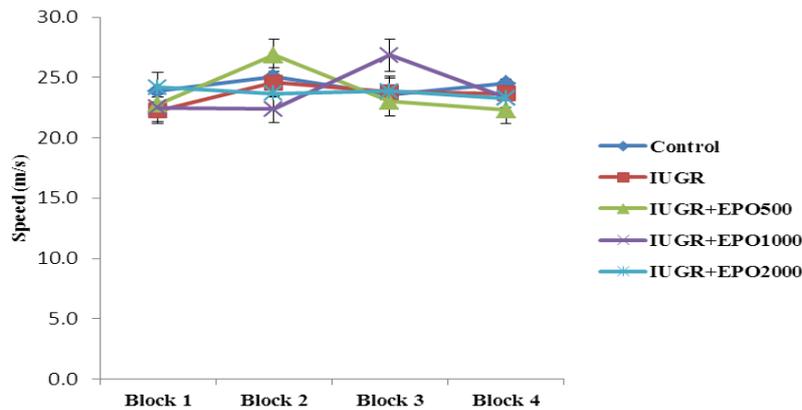


Figure 2. Mean \pm standard deviation of swimming speed in four trial blocks (Learning) of Morris water maze groups. The results indicate no significant difference between these groups.

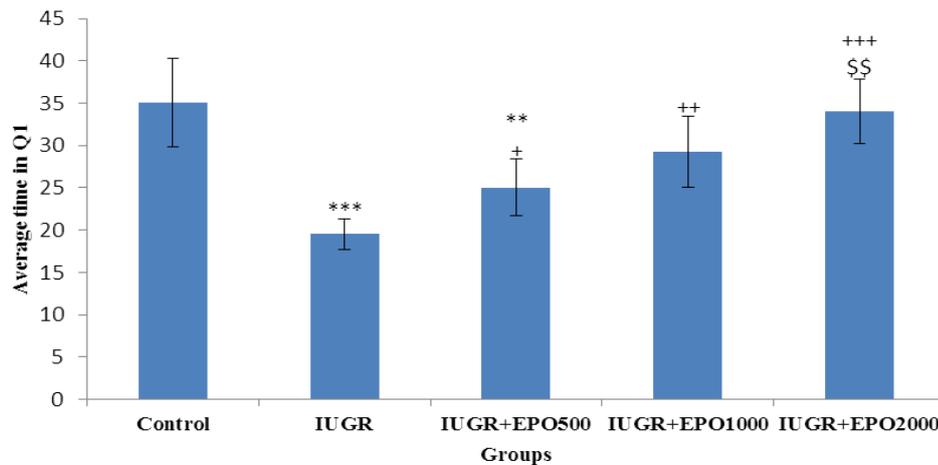


Figure 3. Mean \pm standard deviation of average time the subject spends in in target quadrant in trial stage (probe). There is a significant difference between IU GR and treatment groups ($^{***}p < 0.0001$, $^{**}p < 0.01$). There is a significant difference between EPO and IU GR groups ($^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.01$). There is a significant difference between IU GR + EPO500 and IU GR + EPO2000 groups ($^{ss}p < 0.01$).

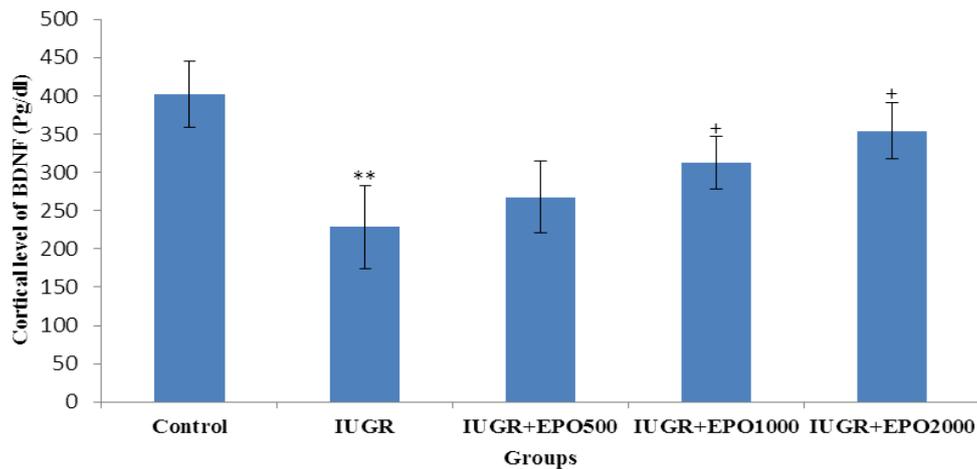


Figure 4. Mean \pm standard deviation of cortex BDNF level in different groups. There is a significant difference between IUGR and control groups (** $p < 0.001$). Moreover, there is significant difference between IUGR + EPO1000 group and IUGR + EPO2000 group with IUGR group († $p < 0.05$).

In a normal brain, neurotropic expression is higher in cortex and hippocampus in comparison with other parts (30). Waterhouse and Xu indicated that BDNF level decreases in those patients with brain damage and premature fetuses (31). Moreover, decreased connectivity of BDNF to NTRP75 causes apoptosis by means of JNK in cortex (32). Recent studies indicate that increased level of BDNF in cortex creates erythropoietin which prevents cognitive disorders and improves spatial memory of IUGR rat models. EPO treatment improves spatial memory and fear memory in old rats (33). EPO can decrease oxidative stress and inflammatory responses, and also, adjust BDNF production (33). Previous findings indicate the effects of EPO on synapses plasticity of brain. It can act as a neuroprotective substance and improve neurological functions after a chronic injury (34). Furthermore, EPO acts as a neuroprotective for cortex and adjust neurotropic factors, and consequently, decreases neuronal mortality (34).

Conclusion

Erythropoietin improves spatial memory by increasing BDNF expression of entorhinal cortex in UPI model. Since EPO has

neuroprotective properties, it can probably prevent and improve brain symptoms caused by UPI or IUGR. Because many parts of the brain have reached their final development during prenatal period, it should be noted that treatment and prevention proceedings of potential brain damages in IUGR neonates should start from prenatal period.

Ethical issues

Not applicable.

Authors' contributions

All authors equally contributed to the writing and revision of this paper.

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