Effectiveness of Erythropoietin on Working Memory, Passive Avoidance Learning and Anxiety-Like Behaviors in Prenatal Food Restriction Model

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Abstract

Introduction: Prenatal Food Restriction (PFR) causes some disorders in prenatal development and neuro-developmental abnormalities. On the other hand, the studies indicated that erythropoietin can act as a neuro-protector. Therefore, the aim of this study is to analyze the effects of erythropoietin on working memory, passive avoidance learning, and anxiety level in rat PFR model.

Methods: In this experimental study, 50 neonatal rats are exposed to PFR. Reduction of standard food portion up to 50% has been started on the embryonic day (ED) 14 in rats until postnatal period. Then, different doses of 500, 1000, and 2000 IU/kg weight erythropoietin were injected to experimental groups, subcutaneously. At post natal day (PND) 30; Y-maze, shuttle box and elevated plus maze (EPM) are used for evaluation of working memory, passive avoidance learning, and anxiety level, respectively. Differences between groups were analyzed by one way ANOVA with Tukey's post hoc ($p < 0.05$).

Results: The results indicate that working memory and avoidance learning have reduced significantly in the control group ($p < 0.05$). Moreover, anxiety level has increased in PFR group in comparison with the control group ($p < 0.001$). On the other hand, working and avoidance memories have increased in those groups which received EPO in comparison with PFR groups and anxiety level have decreased significantly ($p < 0.05$).

Conclusion: Our results indicate that prenatal treatment of erythropoietin can ameliorate behavioral abnormalities in PFR model.

Keywords: Fetal Growth Restriction, Erythropoietin, Memory, Anxiety, Rat

Introduction

Prenatal Food Restriction (PFR) cause many prenatal and neonatal problems (1). Reduction of nutrition, lack of protein, and hypoglycemia in pregnancy result in intrauterine growth restriction (IUGR) (2). Those infants which are affected by IUGR are encountered with many threatening problems, such as prenatal mortality, increased risk of physical or mental disorders, cardiovascular diseases, and diabetes (3). In fact, insufficient function of placenta and uterine abnormalities decrease intrauterine blood flow. These problems have been observed in diabetes mothers and multiple pregnancies (4). Learning, memory, and behavior disorders are other kinds of complications which are caused by IUGR (5). Maternal food restriction results in psychopathy in infants, such as anxiety,

depression, and weak cognitive functions (6). Chu *et al.* indicated that PFR, which causes IUGR in itself, reduces the immunity of the area around placenta, and consequently, increases cell stress and fetal death (7). IUGR complications reduce the volume of gray matter and total weight of brain which cause memory and learning disorders in infants (8). In addition to its main role in the production of erythrocytes, erythropoietin (EPO) as a glycosylated protein has many significant roles in control of different hormones, growth factors, and cytokine signal pathway (9). EPO has physiological effects on the central nervous system and can act as a neuro-protector. Therefore, it can improve nervous functions after a chronic brain traumatic injury (TBI) (10). Meanwhile, human recombinant erythropoietin is known as a new treatment

method for cognitive disorders; it is selected as an efficient medicine in psychotherapy as well (11). EPO increases synaptic plasticity and reduces oxidative stress which can prevent the production of anti-inflammatory cytokines (12). Wang *et al.* claimed that EPO is a potent anti-age medicine for nervous system (13). This combination has been useful in treatment of TBI model by reducing the volume of injury and ameliorating neuro- behavioral symptoms (13). Therefore, the aim of this study is to analyze the effects of recombinant human erythropoietin on memory, learning, and anxiety level of animal models with restricted prenatal growth.

Methods

In this experimental study, 50 male rat neonates were used produced from mating of 20 adult virgin female and 20 adult male Wistar rats. All the steps are carried out under standard temperature $(25\pm2^{\circ}C)$, humidity $(50\pm10\%)$, and 12 hour light/dark cycle (7 am to 7 pm) conditions and ethical supervision of laboratory animal researches. After determining zero day of pregnancy by vaginal smear, observance of spermatozoa and vaginal plaque, pregnant rats were divided randomly into five groups (n = 4): control, PFR, PFR+EPO500, PFR+EPO1000, and PFR+EPO2000. All groups except control were been put under 50% food restrictions (15) from ED14 until ED21. Recombinant human erythropoietin was injected subcutaneously to treatment groups with different doses (500, 1000, and 2000 IU/kg) from ED14 until ED21 (16). After the birth, the neonate were morphologically evaluated and used randomly (n= 10) in behavioral studies at PND30. Elevated plus maze (EPM) was used for evaluation of anxiety like behavior in rodents. The structure of this maze consists of four arms (two open arms without any walls, and two arms surrounded by walls) with 35 cm length and 15 cm width. These arms were placed 35 cm above ground level. The animal was placed at the center of the device and its behavior was monitored by camera for 300 seconds. In this trial, the mean time of insertion in closed arm was recorded as anxiety index in different groups (17). Y-maze was used for evaluation of working memory. This maze consists of three arms with similar Y shaped condition. At first, the animal was placed in one of the three arms gently and without any stress, and its movement was monitored for 5 minutes. The number of times the animal entered each one of the arms was recorded. To determine of working memory, the percentage of alteration behaviors was calculated from the sum of successive alteration behavior divided by total arm entries minus 2. Successive inputs are meant to be intermittent and serially for all three arms (18). Shuttle box device was used for passive avoidance learning analysis. This device consists of two parts, training box and stimulator. Training box has two light and dark chambers which are separated from each other by a guillotine door. Some shocker steel rods with 2.5 mm diameter are placed with 1 cm distance from each other at the bottom. At first, the animals were placed in the light part of the device, behind the guillotine door, in order to become adoptable. After 30 seconds, the guillotine door was opened gently and the animal was allowed to enter the dark part. Then, the animal was transmitted from the dark part to the light part. This procedure was repeated after 30 minutes and in 3 steps. After 30 minutes of adaptation, learning step was initiated. In this step, the animal was placed in the dark chamber with closed door and a weak shock - 2 mA for 5 seconds with a frequency of 5 Hz – was applied on animal's feet. In the next step, the animal was allowed to come out of the dark part and enter the light one. Then, the animal was removed from the light part after 30 seconds and was returned to the cage. Memory trial was carried out after 24 and 48 hours from the shock. Latency time to the dark room (LTD) was considered as learning index (19). The statistical analysis of different groups was done by SPSS software version 22.

The normal distribution of data was evaluated by Kolmogorov-Smirnov. Then, one way ANOVA and Tukey's post hoc test were used in order to determine the significance of difference between anxiety and behavior indexes ($p < 0.05$).

Results

Following subcutaneous injection of human recombinant erythropoietin with 500, 1000, and 2000 IU/kg doses, the results indicate that mean time in closed arm of EPM, significantly increased in PFR and PFR+EPO500 groups than control ($p < 0.001$ and $p < 0.05$, respectively). Moreover, there was a significant decrease in PFR+EPO2000 compared to PFR group ($p < 0.05$). In fact, anxiety level was decreased significantly in those groups which received maximum dose of EPO in comparison with PFR group (Figure 1). The results of Y-maze test, which was applied in percentage of alteration behaviors in different groups, indicate that although working memory was decreased significantly in PFR group compared with control ($p <$ 0.0001), using treatment of EPO in 1000 and 2000 IU/kg doses could ameliorate working memory in the treatment group. Furthermore, a significant increase had been observed in PFR+EPO1000 and PFR+EPO2000 compared to the PFR group (Figure 1, $p < 0.001$). Moreover, the results were indicative of the role of dose manner recombinant human erythropoietin in amelioration of working memory (Figure 2).

In passive avoidance memory test, there was no significant LTD difference between groups in acquisition period. 24 and 48 hours after shock, a significant LTD difference had been observed between PFR+EPO1000, PFR+EPO500, and PFR groups with control group ($p < 0.001$). In addition, a significant difference had been observed between PFR and PFR+EPO2000 groups after this time ($p < 0.001$). In sum, LTD increased significantly in high dose of EPO treated groups in comparison with PFR which indicates the amelioration of fear-based memory in PFR+EPO2000 group (Figure 3).
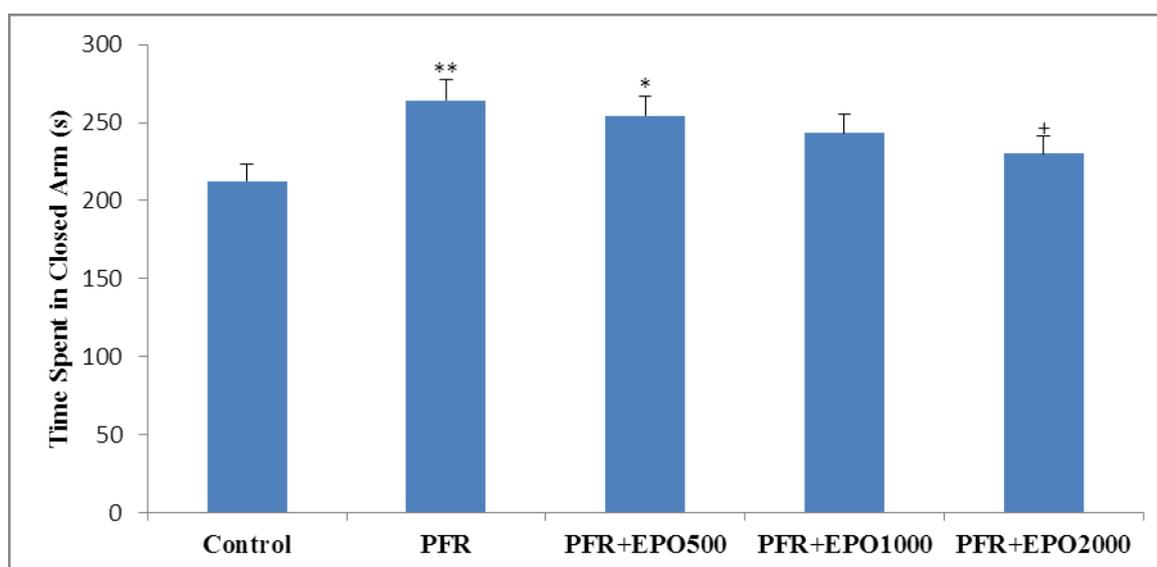
Figure 1. Mean ± standard deviation of average time spent in closed arm of EPM. There is a significant difference between control with PFR and PFR+EPO500 (**$p < 0.001$, *$p < 0.05$). Also, a significant difference between PFR and PFR+EPO2000 was observed (+$p < 0.05$).
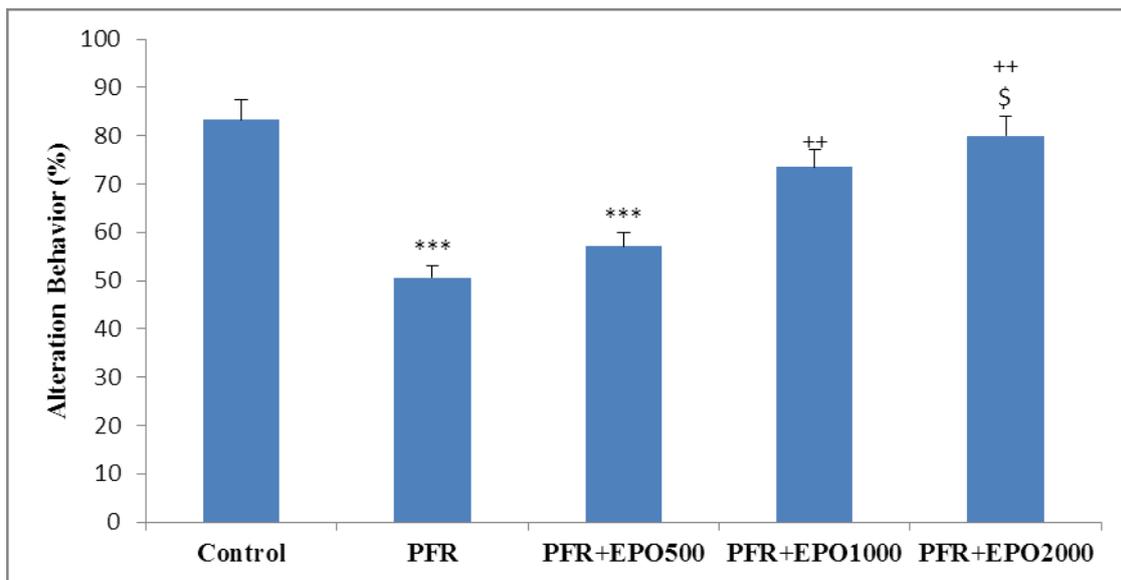
Figure 2. Mean ± standard deviation of alteration behaviors in Y-maze. A significant difference between control with PFR and PFR+EPO500 (***$p < 0.0001$), and between PFR with PFR+EPO1000 and PFR+EPO2000 (++$p < 0.001$) were observed. Also, there is a significant difference between PFR+EPO500 and PFR+EPO2000 (+$p < 0.05$).
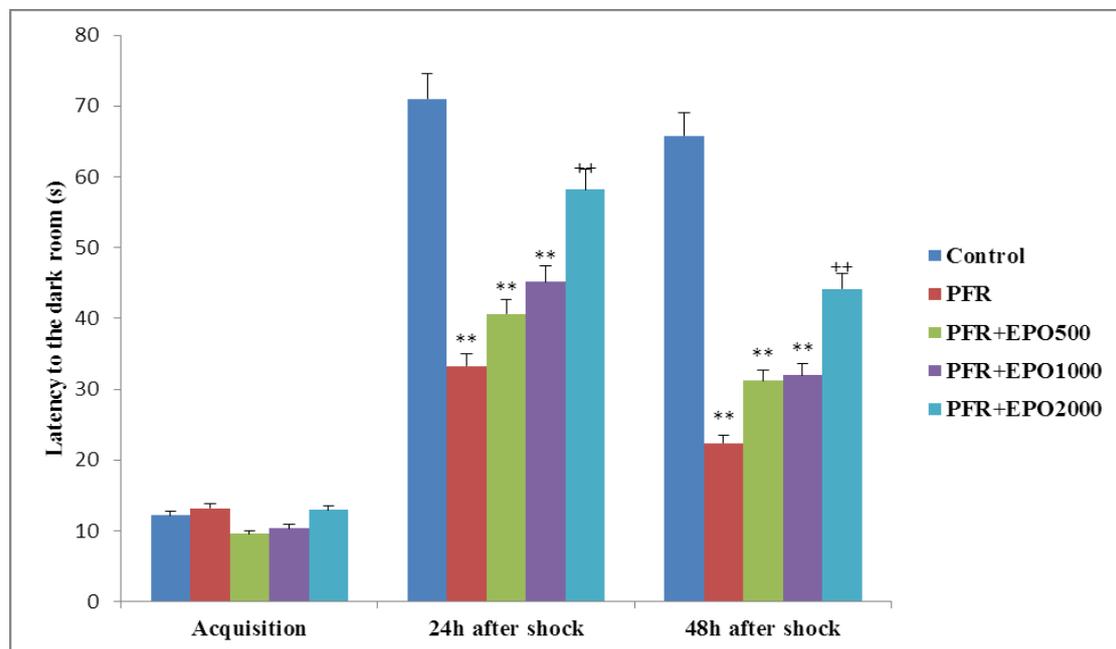
Figure 3. Mean ± standard deviation of LTD in shuttle box. In acquisition there is no significant difference between groups. 24 and 48 h after shock a significant decrease was observed in PFR, PFR+EPO500 and PFR+EPO 1000 in comparison with control group (**$p < 0.001$), and PFR+EPO 2000 had a significant increase of LTD with PFR group (++$p < 0.001$).

Discussion

The present study analyzed the effects of EPO on learning, memory, and anxiety-like behavior resulted from PFR. The result indicated that those animals which were exposed to PFR are in the risk of increased anxiety level and decreased long and short term memories and learning capacities. Some events, such as hypoxia, stress, toxicity, inflammation, decreased blood flow of placenta, and maternal protein restriction can affect the fetus development. Chronic utero-placental insufficiency, low oxygen and nutrition result in abnormal growth of the fetus (20). Human studies indicate that nervous disabilities, learning and memory disorders, and mood disorders are prevalent in children with IUGR (21). However, paying attention to nutritional status of the mother during pregnancy has a significant role in reducing cognitive disorders. Therefore, using some interventions for better nutrition of the mother in prenatal and postnatal phases can prevent weight loss of the infant and improve his/her health (22). Growth restriction, latency in neonatal reflexes, permanent movement disorders such as muscle weakness during puberty, mood disorders, and hyperactivity during puberty have been observed in prenatal protein restriction model (23). Illa *et al*. claimed that anxiety level have increased significantly in IUGR rabbit model (24). Increased corticosterone level in blood serum of IUGR rats is considered to be one of the reasons for anxiety in female rats (25). On the other hand, Naik *et al*. believe that hyperactivity of IUGR rats in Open Field test is indicative of decreased anxiety level (26). However, it should be studied more. Neuro-protective role of EPO in this study caused improvement of working memory, fear-based memory, and reduction of anxiety level in those groups which received maximum dose of EPO in comparison with PFR group. The studies indicate that EPO has a significant role in improvement, protection, and recovery of nervous system. In prenatal and postnatal periods, EPO causes a significant reduction in apoptic cells in preventricular area of brain (27). Treatment by EPO increases the expression of anti-inflammatory cytokine (*i.e.* IL-10), while it decreases the expression of anti-inflammatory cytokinein (*i.e.* IL-1β and TNF-α) in damaged brain tissue (28). Therefore, EPO can ameliorate neuro-cognitive function and regulate immune-inflammatory reactions in brain impairments (29). Thalamus and basal ganglia volume reduction (30), abnormal secretion of adrenal hormones (31), and reduced expression of neurotrophins, especially BDNF (32) are considered to be underlies of mood disorders in IUGR model. However, EPO could increase neurotrophins in animal models (33).

Conclusion

PFR and induction of maternal hypoglycemia are underlies of stable and permanent cognitive and mood disorders in infants. In the present study, amelioration of working memory, passive avoidance memory, and decreased of anxiety level have been observed in EPO-treated rats in comparison with PFR groups. It is indicative of neuro-protective effects of EPO.

Ethical issues

Not applicable.

Authors' contributions

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

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