

Locomotion in young rats with induced brain cellular edema – effects of recombinant human erythropoietin

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Submitted: 2018-04-12 *Accepted:* 2018-07-10 *Published online:* 2018-10-20

Key words: **erythropoietin; EPO; recombinant human erythropoietin; rhEPO; cytotoxic edema; water intoxication; open field test; locomotion; young rats**

Neuroendocrinol Lett 2018; **39**(4):310–314 PMID: 30531705 NEL390418A08 © 2018 Neuroendocrinology Letters • www.nel.edu

Abstract

OBJECTIVES: Effect of recombinant human erythropoietin (rhEPO) on spontaneous motor activity was tested in young rats after intraperitoneal (i.p.) administration of rhEPO, followed by induction of cellular brain edema (CE). Induced changes in the spontaneous horizontal locomotor activity was studied by open field test (OFT).

METHODS: CE was induced by water intoxication (WI) using standard method of fractional hyperhydration accompanied with desmopressin administration. Using the accepted method of OFT average time spent in locomotion (s) was determined. 48 young rats at the age of 25, and 35 days were divided into three groups – controls, rats after WI (OFT followed after 44 hours), and rats administered with rhEPO prior to application WI (OFT after 48 hours).

RESULTS: In 35-day-old rats rhEPO administration increased the spontaneous locomotor activity, previously decreased by cellular edema. In 25-day-old rats, rhEPO administration prior to the induced CE, decreased spontaneous locomotor activity.

CONCLUSION: Presented results demonstrate the neuroprotective capacity of rhEPO, manifested by elimination of the suppressive influence of CE on the locomotion in 35-day-old rats. In 25-day-old rats the neuroprotective effect was not present. These results confirmed that the 10 day interval in the development may represent a different stage of brain maturation in the relation to the neuroprotective effect of rhEPO.

Abbreviations:

rhEPO - recombinant human erythropoietin
EPO - erythropoietin
EPOR - erythropoietin receptor
i.p. - intraperitoneal
CE - cellular edema
WI - water intoxication
OFT - Open Field Test
CNS - Central Nervous System

TBI - Traumatic Brain Injury
BBB - Blood-Brain Barrier
AQP - Aquaporine
DW - Distilled water
ADH - antidiuretic hormone
NO - natriumoxid
RNA - ribonucleic acid
DNA - deoxyribonucleic acid

INTRODUCTION

Erythropoietin (EPO) is a cytokine with a precisely defined roles including gene cloning and expression in erythropoiesis. Its therapeutic effect was first used for anemia associated with end-stage renal failure and malignant tumors. Research in the new millennium showed that EPO at the cellular level also affects other systems, especially the immune, cardiovascular, gastrointestinal and nervous (Broxmayer 2013; Brines & Cerami 2006). In the CNS, a neuroprotective effect based on the mechanisms that influence the neurotropic, anti-apoptotic and regeneration promoting properties has been demonstrated (Hand & Brines 2011). The effect of EPO has been studied in two large studies on the patients with stroke (Ehrenreich *et al.* 2009) and with diffuse brain injury (TBI) (Robertson *et al.* 2014) without any convincing results. One important condition enabling an exogenous rhEPO (recombinant human EPO produced by recombinant DNA technology in cultured cells) to achieve its neuroprotective effect in the CNS is its ability to cross the blood-brain barrier (BBB). It is enabled by receptor-mediated transcytosis of an EPO molecule after binding to specific receptors at the endothelial cells, similarly to other proteins with molecular weight exceeding 50 kDa (Brines 2000). From the literary data it can be hypothesized, that the neuroprotective effects of endogenous EPO enhanced by administration of exogenous rhEPO on the development of cellular edema result from its role in the control of expression of aquaporine 4 (AQP4) (Gunnarson *et al.* 2009). Aquaporine 4 is an integral plasma membrane protein that enables selective flow of water along the gradients given by osmolality of the cellular and extracellular compartments. (Agre *et al.* 2004; Wells 1998; Manley *et al.* 2000). AQP4 expression is ontogenetically dependent – in young and immature individuals it is lower (Li *et al.* 2013). Another literary data confirmed that EPO expression is age-dependent; in young individuals being lower (Lourhmati *et al.* 2013). Owing to the ontogenic dependency of both processes – brain edema development and neuroprotective effect of EPO – two age groups of rats were used: 25-day-old rats at the weaning period and 35-day-old rats at the beginning of adolescence (puberty). Different neuroprotective effect of EPO in adult rats from that of young and immature rats has been already described (Schober *et al.* 2010). The aim of our study was therefore to find whether the interval of 10 days between the end of weaning and beginning of puberty, represents the critical period for the onset of neuroprotective effect of EPO.

To confirm our hypothesis, brain cellular edema was induced in both age groups by water administration along with rhEPO administration. Behavioural changes were identified by analysing spontaneous motor activity in the open-field test (Hall, 1934; Meng *et al.* 2015; Kozler *et al.* 2017a).

MATERIAL AND METHODS

All experiments were approved by the Ethical Committee of the First Faculty of Medicine (Charles University in Prague) and were in agreement with the Guidelines of the Animal Protection Law of the Czech Republic and Guidelines for the treatment of laboratory animals EU Guidelines 86/609/EEC.

For experiments, male rats of the Wistar strain aged 25 and 35-day of our own breed were used.

Water intoxication

WI was achieved by the fractionated hyperhydration accompanied with the antidiuretic effect of desmopressin. Distilled water (DW) in a total amount corresponding to 20% of the body weight, divided into three doses, was applied intraperitoneally (i.p.) within the period of 24 hours. Each sub-doses of water was completed with one third of the total dose of desmopressin. Desmopressin (1-desamino-8-D-arginine vasopressin) is a synthetic form of the human hormone arginine vasopressin (the antidiuretic hormone, or ADH). Desmopressin (OCTOSTIM®, Ferring) was given in a dose of 0.032 mg/kg (www.rxmed.com/b.main/b2.pharmaceutical/OCTOSTIM.html). Hyperhydration procedure corresponds with the literature data (Manley *et al.* 2000; Yamaguchi *et al.* 1997; Silver *et al.* 1999).

Open field test

To test the motor activity of rats, we used the system Laboras (Metris, B.V., Netherland) for continuous registration and analysis of physical activity. It consists of triangular shaped sensing platform (carbon fiber plate 700 mm × 700 mm × 1000 mm × 30 mm), positioned on two orthogonally placed sensor-transducers and third fixed point attached to bottom plate. Makrolon cage (type III, 840 cm²) is placed on this platform. Any mechanical vibrations caused by the movement of the animal are converted into electrical signals, which are then evaluated using software Laboras. Animals were tested in a darkened room at a constant room temperature 22 to 23 deg C, always in the same time, between 9:00 and 12:00. Horizontal locomotor activity – average time spent in locomotion (s), during one hour at time intervals of ten minutes were recorded and analyzed.

Groups of animals

In both age categories (25 and 35-day-old), the control group (C) consisted of 8 animals, 8 animals had the induced cellular edema (HH group), and 8 had the induced cellular edema preceded with EPO (EHH group). In the HH group (fractional administration of three doses of DW and desmopressin within 24 hours), locomotion analysis on the open field test began 20 hours after the completion of induction of cellular edema. For the EHH group, the procedure was the same,

but 4 hours before the induction of cellular edema, animals were treated with rhEPO at 5 000 µg/kg i.p.

Statistical evaluation

Results of all measurements were statistically evaluated using the tests of the GraphPad Prism program (parametric ANOVA and nonparametric Kruskal-Wallis test, the statistical significance was set at 5%).

RESULTS

Figure 1 and Figure 2 illustrate the average time spent in locomotion in individual groups of rats. Spontaneous locomotor activity, expressed as the average time spent in locomotion (s) was tested for 60 minutes at 10 minute intervals.

In the group of 25-day-old rats, no significant difference in motor activity was found between the control group (C) and the group with induced cellular edema (HH). In animals with rhEPO applied before the induction of CE, the locomotion was significantly suppressed. In the group of 35-day-old animals, a locomotion decline was observed in animals with the induced cellular edema (HH). In animals with rhEPO applied before the induction of CE the locomotor activity did not differ from that in controls. Results have shown the neuroprotective effect of rhEPO manifested in 35-day-old rats by the increase of the spontaneous locomotor activity, which was suppressed by the induced CE. In 25-day-old rats rhEPO administration to rats with induced CE decreased spontaneous locomotor activity.

DISCUSSION

EPO is a cytokine with a significant neuroprotective effect that influences the neurotropic, antiapoptotic and angiogenic properties of cell populations and pro-

motes regeneration in the CNS (Hand & Brines 2011; Yatsiv *et al.* 2005; Xiong *et al.* 2011). In experimental models of brain homeostasis disorders (e.g., ischemia), increased expression of EPO in endothelial cells, pericytes and neurons and increased expression of EPOR receptors in astrocytes was demonstrated. EPO increases blood perfusion in the brain by stimulating endothelial NO production, it also improves microcirculation by inhibiting endothelial cell apoptosis, improves tissue oxygenation by inducing neoangiogenesis, and it increases neuronal tolerance to ischemia. EPO also significantly prevents development of brain edema. It can dampen the entry of water into cells due to the existing co-localization of EPOR and AQP4 at astrocytic cell membranes. Increased AQP4 activity in the initial stages of cellular edema is mediated by glutamate, and EPO reduces this mediation (Hand & Brines 2011; Brines & Cerami 2006; Gunnarson *et al.* 2009).

As stated in Introduction, EPO expression is age-dependent, being lower in younger individuals. Lower EPO expression was proved in the model of experimental cerebral hypoxia in young rats (Lourhmati *et al.* 2013). In another study, EPO expression was measured (by determining mRNA levels) in hippocampus in the experimental model of diffuse brain injury and results were compared between adult rats and those 17-day-old. In young rats, the increased EPO expression occurred in up to 7 days after the injury, while in adults as early as in 12 hours (Schober *et al.* 2010).

Expression of AQP-4 is also ontogenetically related – in young or immature individuals it is low. Li and co-workers in their study in mice proved low expression of AQP-4 during the first postnatal week, the significant increase in the second week and attaining the adult levels in the fourth week of life (Li *et al.* 2013). Wen and co-workers found very low postnatal expres-

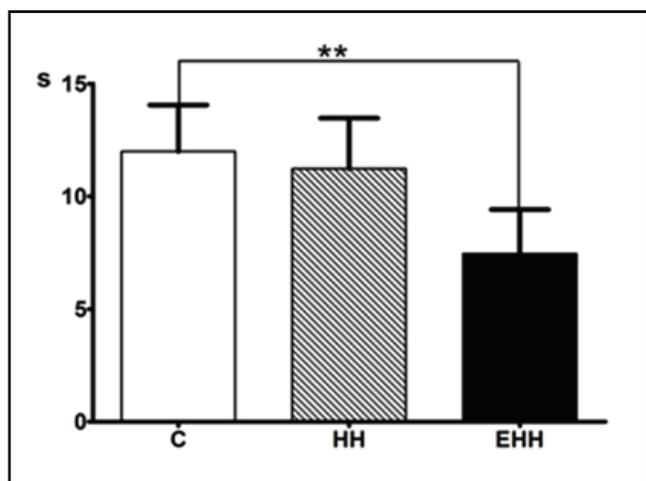


Fig. 1. Average time spent in locomotion - 25-day-old rats. X axis: C – control group, HH – induced cellular edema, EHH – rhEPO was given before water administration, ** = $p < 0.1$, Y axis: average time spent in locomotion (s), s=seconds.

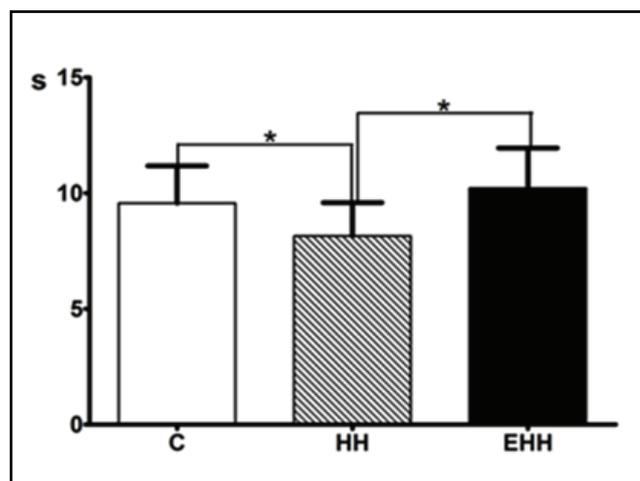


Fig. 2. Average time spent in locomotion - 35-day-old rats. X axis: C – control group, HH – induced cellular edema, EHH – rhEPO was given before water administration, * = $p < 0.5$, Y axis: average time spent in locomotion (s), s=seconds.

sion of AQP4 in rats with only 2% of the adult values at the first week and 25% in the age of two weeks (Wen *et al.* 1999).

In the present study, a model of impaired cerebral homeostasis based on the induced brain cellular edema by water intoxication was used (repeated water and desmopressin administration—see methods). It is a model of cytotoxic, cellular and diffuse brain edema which, regardless of its origin (osmotic, ischemic, traumatic), duration and effects, inhibits brain activity (Liang *et al.* 2007).

To test the effect of rhEPO on the spontaneous motor activity, horizontal motor activity was studied – the pattern of locomotion where the rat moves on all four limbs. For the vertical motor activities (rearing and grooming), the rat uses the forelimbs for other purposes than locomotion (Kozler *et al.* 2017b). For the spontaneous horizontal locomotion the dominant limbs are forelimbs. Rat can be characterized as “front-wheel drive” (Schallert *et al.* 2003). From the point of anatomy, for the normal function of the forelimbs, the intact corticospinal pathway and the support from extrapyramidal system are necessary (Whishaw *et al.* 1993). Induced cellular brain edema reduces locomotor activity without affecting the motor centers or motor pathways that are related to those movements functionally or anatomically (Inoue *et al.* 1993).

Results of our study has shown (see Figure 1 and 2) that in 35-day-old rats the locomotion was suppressed by the induced cellular edema in comparison with the group with induced cellular edema and rhEPO administration. This result can be considered as a neuroprotective effect of EPO on the development of cellular brain edema, when rhEPO prevents water to enter the cell by the above described mechanisms.

In 25-day-old rats rhEPO administration to animals with induced cellular edema decreased spontaneous locomotor activity, when compared to control animals. It is possible to conclude, that in this age, neuroprotective effect of EPO has not been manifested. It can be due to low AQP4 expression along with low expression of EPOR at the cell membranes of astrocytes (Li *et al.* 2013; Lourhmati *et al.* 2013; Brines *et al.* 2000). The developmental period between the weaning and the beginning of puberty is assumed to be a critical period for the formation of neuronal circuits (Křeček 1971) and also for the stabilization of processes that maintain the microenvironment of the brain (Boison & Masino 2016). The coincidence with the period of manifestation of the neuroprotective effect of EPO indicates, that one of the factors that participates on the ability to maintain the stability of the brain microenvironment is the expression of AQP4 and the mechanism that can control it (expression of EPOR). The weaning and the post-weaning period may represent a critical period also for the manifestation of the neuroprotective effect of EPO. We can conclude that the neuroprotective capacity of rhEPO, manifested by elimination of the

suppressive influence of brain edema on the locomotion in 35-day-old rats was not present in 25-day-old rats. These results confirmed that the 10-day interval in the development may represent a different stage of brain maturation in the relation to the ability to maintain the stability of the brain microenvironment.

ACKNOWLEDGEMENTS

Supported with grant Q35/LF 1.

REFERENCES

- Agre P, Nielsen S, Ottersen OP eds (2004). Brain Water Homeostasis. *Neuroscience*. **129**: 849–1054.
- Boison D, Masino SA (2016). Homeostatic control of brain function. Oxford University Press. Oxford, p.192–195.
- Brines M, Cerami A (2006). Discovering erythropoietin's extrahematopoietic functions: biology and clinical promise. *Kidney Int.* **70**: 246–250.
- Brines ML, Ghezzi P, Keenan S, Agnello D, de Lanerolle NC, Cerami C, et al (2000). Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. *Proc Natl AcadSci U S A.* **97**: 10526–10531.
- Broxmeyer HE (2013). Erythropoietin: multiple targets, actions, and modifying influences for biological and clinical consideration. *J Exp Med.* **210**: 205–208.
- Ehrenreich H et al (EPO Stroke Trial Group) (2009). Recombinant human erythropoietin in the treatment of acute ischemic stroke. *Stroke.* **40**: 647–656.
- Gunnarson E, Song Y, Kowalewski JM, Brismar H, Brines M, Cerami A et al (2009). Erythropoietin modulation of astrocyte water permeability as a component of neuroprotection. *Proc Natl AcadSci U S A.* **106**: 1602–1607.
- Hall, CS (1934). Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. *Journal of Comparative Psychology.* **18**: 385–403
- Hand CC, Brines M (2011). Promises and pitfalls in erythropoietin-mediated tissue protection: are nonerythropoietic derivatives a way forward? *J Investig Med.* **59**: 1073–1082.
- Inoue T, Lin A, Ma X, McKenna SL, Creasey GH, Manley GT et al (2013). Combined SCI and TBI: recovery of forelimb function after unilateral cervical spinal cord injury (SCI) is retarded by contralateral traumatic brain injury (TBI), and ipsilateral TBI balances the effects of SCI on paw placement. *Exp Neurol.* **248**: 136–147.
- Kozler P, Maresova D, Pokorny J (2017a). An experimental model of the “dual diagnosis”: Effect of cytotoxic brain edema plus peripheral neuropathy on the spontaneous locomotor activity of rats. *Neuro Endocrinol Lett.* **38**: 408–414.
- Kozler P, Maresova D, Pokorny J (2017b). Study of locomotion, rearing and grooming activity after single and/or concomitant lesions of central and peripheral nervous system in rats. *Neuro Endocrinol Lett* **38**: 495–501.
- Křeček J (1971). The theory of critical developmental periods and postnatal development of endocrine functions. In: Tobach E, Aronson LR, Shaw E, editors. *The Biology of Development*. New York: Acad. Press.
- Li X, Gao J, Ding J, Hu G, Xiao M (2013). Aquaporin-4 expression contributes to decreases in brain water content during mouse postnatal development. *Brain Res Bull.* **94**: 49–55.
- Liang D, Bhatta S, Gerzanich V, Simard JM (2007). Cytotoxic edema: mechanisms of pathological cell swelling. *Neurosurg Focus.* **15**: E2
- Lourhmati A, Buniatian GH, Paul C, Verleysdonk S, Buecheler R, Buadze M et al (2013). Age-dependent astroglial vulnerability to hypoxia and glutamate: the role for erythropoietin. *PLoS One.* **8**: e77182.

- 17 Manley GT, Fujimura M, Ma T, Noshita N, Filiz F, Bollen AW et al (2000). Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. *Nat Med* **6**: 159–163.
- 18 Meng FT, Zhao J, Ni RJ, Fang H, Zhang LF, Zhang Z et al (2015). Beneficial effects of enriched environment on behaviors were correlated with decreased estrogen and increased BDNF in the hippocampus of male mice. *Neuro Endocrinol Lett*. **36**: 490–497.
- 19 Robertson CS et al (Epo Severe TBI Trial) (2014). Effect of erythropoietin and transfusion threshold on neurological recovery after traumatic brain injury: a randomized clinical trial. *JAMA*. **312**: 36–47.
- 20 Schallert T, Woodlee MT (2003). Brain-dependent movements and cerebral-spinal connections: key targets of cellular and behavioral enrichment in CNS injury models. *J Rehabil Res Dev*. **40**: 9–17.
- 21 Schober ME, Block B, Beachy JC, Statler KD, Giza CC, Lane RH (2010). Early and sustained increase in the expression of hippocampal IGF-1, but not EPO, in a developmental rodent model of traumatic brain injury. *J Neurotrauma*. **27**: 2011–2020.
- 22 Silver SM, Schroeder BM, Bernstein P, Sterns RH (1999). Brain adaptation to acute hyponatremia in young rats. *Am J Physiol*. **276**: 1595–1599.
- 23 Wells T (1998). Vesicular osmometers, vasopressin secretion and aquaporin-4: a new mechanism for osmoreception? *Mol Cell Endocrinol*. **15**: 103–107.
- 24 Wen H, Nagelhus EA, Amiry-Moghaddam M, Agre P, Ottersen OP, Nielsen S (1999). Ontogeny of water transport in rat brain: postnatal expression of the aquaporin-4 water channel. *Eur J Neurosci*. **11**: 935–945.
- 25 www.rxmed.com/b.main/b2.pharmaceutical/OCTOSTIM.html.
- 26 Whishaw IQ, Pellis SM, Gorny B, Kolb B, Tetzlaff W (1993). Proximal and distal impairments in rat forelimb use in reaching follow unilateral pyramidal tract lesions. *Behav Brain Res*. **56**: 59–76.
- 27 Xiong Y, Zhang Y, Mahmood A, Meng Y, Qu C, Chopp M (2011). Erythropoietin mediates neurobehavioral recovery and neurovascular remodeling following traumatic brain injury in rats by increasing expression of vascular endothelial growth factor. *Transl Stroke Res*. **2**: 619–632.
- 28 Yamaguchi M, Yamada T, Kinoshita I, Wu S, Nagashima T, Tamaki N (1997). Impaired learning of active avoidance in water-intoxicated rats. *Acta Neurochir Suppl*. **70**: 152–154.
- 29 Yatsiv I, Grigoriadis N, Simeonidou C, Stahel PF, Schmidt OI, Alexandrovitch AG et al (2005). Erythropoietin is neuroprotective, improves functional recovery, and reduces neuronal apoptosis and inflammation in a rodent model of experimental closed head injury. *FASEB J*. **19**: 1701–1703.