ORIGINAL ARTICLE

Evaluation of erythropoietin effects on cerebral ischemia in rats

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Abstract

OBJECTIVE: Majority of severe disabilities in adults are caused by stroke. The aim of our study is to learn the effects of erythropoietin (EPO), on infarct size in cerebral ischemia and to determine neurological behavioral scores and histopathological evaluation.

MATERIAL & METHODS: In this study 30 adult Sprague-Dawney rats were used. Cerebral ischemia was constituted by intraluminal filament method with a 4-0-nylon suture. Reperfusion was started after two hours of middle cerebral artery occlusion. The rats were randomly divided into two groups as follow: control and EPO groups. Saline 0.9% (0.5 ml/kg) and EPO (5 000 U/kg) was administered intraperitoneally in the groups. Three coronal slices in two millimeters thickness were obtained from cerebrum, cerebellum and brain stem, and were stained with a 2% solution of triphenyltetrazolium chloride. Transparent sheets were placed over each section and the areas of the brain and infarct were measured. The neurological scores were determined at 24th, 48th and 72nd hours after reperfusion.

RESULTS: Percent of ischemic area (%) in cerebrum, cerebellum and brain stem level in EPO groups were less than those of control group ($p<0.0001$). In addition, we determined that EPO group was better than controls of neurologic score and histopatologically after cerebral ischemia.

CONCLUSIONS: We concluded that EPO may decrease ischemic area in experimental cerebral ischemia in rats and it seems that EPO may be beneficial.

INTRODUCTION

The so-called "stroke", meaning the sudden occlusion of one or more brain vessels resulting in an insufficient perfusion of the associated brain area, presents, together with cardiovascular diseases and cancer [13]. The treatment of stroke is still limited with the optimal supportive measures in spite of recombinant tissue plasminogen activator (r-TPA). Any therapeutic approach in stroke promising to favorably influence the course of this disease therefore merits to be followed up emphatically. This has been done in recent years with a number of substances hope to positively influence the course of recovery after a stroke due to their neuroprotective properties. All of these studies have failed so far [13].

Erythropoietin (EPO) is a hematopoietic growth factor and cytokine and is most notably recognized for its central role in erythropoiesis [15,18]. It possesses neuroprotective effects on hypoxic/ischemic cerebral damages and experimental subarachnoid hemorrhage [1,22,34]. The neuroprotective properties are extending from anti-apoptotic, anti-oxidative, anti-inflammatory, glutamate inhibitory, and stem cell modulating to neurotrophic and angiogenic effects. Thus, EPO may protect neurons by a combination of these mechanisms [13,14,15,33]. But there is limited data about effect of EPO on the cerebral ischemia.

In this study we aimed to effectiveness of EPO on percent ischemic area in experimental ischemic brain injury and on neurological outcome after temporary middle cerebral artery occlusion (MCAO) and reperfusion in rats.

MATERIAL AND METHODS

All the experimental procedures were performed in accordance the guidelines of the Experimental Research Institute of Dicle University (DUSAM), after approval of Dicle University Ethic Committee (#02-224). Thirty adult male Sprague-Dawney rats, weighing 300 to 350 g, obtained from the DUSAM were used in this study. The rats were kept in a room having interior temperature 21–23 °C. Fans and illuminated 12 hours ventilated the room continuously in a day. The rats were randomly divided into two groups; control (C) (n=15), and erythropoietin group (EPO) (n=15) (Recombinant human erythropoietin, Neorecormen® 5 000 IU, Roche, Switzerland). The rats were fed with *ad libitum* standard pellet chow and daily fresh tap water during the experimental procedure.

Experimental protocol and groups

The all groups were anesthetized with ketamine hydrochloride (90 mg/kg) intraperitoneally, and by using intraluminal filament method cerebral ischemia was constituted [3,23]. The method consists of introducing a 4-0-nylon intraluminal suture into the cervical internal carotid artery (ICA) and advancing it intracranially to block blood flow into the MCA; collateral blood flow was reduced by interrupting all branches of the external carotid artery (ECA) and all extracranial branches of the ICA. The intraluminal filament was withdrawn after two hours of MCAO, and reperfusion started again and passed to therapeutic stages for all the groups. After, Saline 0.9% (0.5 ml/kg) to the C group, and EPO (5 000 U/kg) were administered intraperitoneally in EPO group. All animals were sacrificed under pentobarbital by decapitating 72 hours after MCAO. Afterwards the whole brains were immediately removed, briefly cooled in ice-cold saline, and three coronal slices in two millimeters thickness were obtained from cerebrum, cerebellum and brain stem respectively.

Neurological evaluation:

The neurological scores were determined at 24th, 48th and 72nd hours after reperfusion by using the modification described by Bederson *et al.* [2]. In neurological evaluation, the worst score was determined as "12" and the best score as "0". The rats were evaluated in 24th, 48th and 72nd hours after MCAO.

Histopathological procedure and examination

The sections were stained with a 2% solution of triphenyltetrazolium chloride (TTC) in a warm water bath 37 °C for 30 minutes [2]. The stained sections were immersed in 10% phosphate-buffered formalin and the infarct size examined after 1 week. Transparent sheets were placed over each section and the areas of the brain and of the infarct (as outlined by TTC staining) were traced on the overlay. The tracings were digitalized and total pixel counts of the ischemic area and the whole brain in all three surfaces were determined. The sums of the three surfaces were calculated and the ischemic area was expressed as a percentage of the whole brain area. The histopatological evaluation is made according to the Kirino and *et al* who described the ischemic neuronal changes [20].

Statistical analysis

Percent of ischemic area of three groups were presented as mean ± standard deviation (SD). ANOVA test was used to compare the measurements on three groups. Post hoc Tukey analyses were used. Values $p<0.05$ was accepted as statistically significant.

RESULTS

All results of two groups are shown in Table 1. Percent of ischemic area (%) in cerebral level of EPO group was lower than control group (14.4±2.23%, 19.6±2.67% respectively, $p<0.0001$). Percent of ischemic area (%) in cerebellar level of EPO group was also lower than control rats (11.6±2.73%, 24.2±4.75% respectively, $p<0.0001$). Again percent of ischemic area (%) in brain stem level of EPO group was also lower than control rats (5.7±1.42%, 18.1±2.29% respectively, $p<0.0001$). After two hours of

Table 1. Comparison of infarction areas in the coronal sections in the control and EPO groups.

	Ischemic area of Cerebrum (%)	Ischemic area of Cerebellum (%)	Ischemic area of Brain stem (%)	p-value
Control	19.6±2.67	24.2±4.75	18.1±2.29	
EPO	14.4±2.23	11.6± 2.73**	5.7±1.42**	p<0.0001*

*p<0.0001 EPO group vs control.

middle cerebral artery occlusion (MCAO) we determined a improvement neurologic score at 24th, 48th and 72nd hours in the rats that have been given EPO (Table 2 and 3). The EPO-group showed significantly better recovery than the control group.

In histopatological evaluation, the EPO group had a microvacuolisation phase in pericardium (Stage I) and the control group had a ischemic cell changes included the cell had narrowed and its nucleus was dyed darkly and pushed to edge of cell (Stage II–III), (Figures 1 and 2). Figure 3a and 3b demonstrate sample of whole brain and ischemic area.

DISCUSSION

In this study we have shown that EPO have positive effects on experimental cerebral ischemia in rats, because the mean infarct size treated with EPO was significantly lower than that of control group.

EPO has a well-known erythropoietic effect, and it has also been shown to be neuroprotective in various animal models [10,12,13,33]. Although the mechanisms of EPO in protecting brain were likely to be complex, several different pathways have been studied. EPO and its receptor (EPO-R), present in brain tissue after ischemic injury, were involved in preventing the glutamate toxicity that augments neurons against hypoxia-ischemia in vitro and in vivo [4,17,25,26,30]. It was claimed that EPO acted at EPO-R to activate Jak2 (kinase-2), which initiates phosphorylation of IKB (inhibitor of nuclear factor-kappaB (NFκB)), to activate NFκB and induce NFκB neuroprotective genes [7]. Jak2 also activates PI3K to phosphorylate Akt, which leads to phosphorylation and deactivation of the pro-apoptotic bad protein [28]. In contrast to EPO, carbamylated EPO (CEPO) does not bind to the EPO-R on UT7 cells or have any haematopoietic/proliferative activity on these cells. In vivo studies in mice and rats showed that even high doses of CEPO for long periods are not erythropoietic. However, in common with EPO, CEPO does inhibit the apoptosis associated with glutamate toxicity in hippocampal cells. Like EPO, CEPO is neuroprotective in a wide range of animal models of neurotoxicity: middle cerebral artery occlusion model of ischemic stroke, sciatic nerve compression, spinal cord depression, experimental autoimmune encephalomyelitis and peripheral diabetic neuropathy [19]. They are found in the human cerebral cortex and hippocampus and in vitro, the cytokine is synthesized by astrocytes and neurons, has neuroprotective activity, and is upregulated after hypoxic stimuli [19]. The transcription factor hypoxia-inducible factor 1 (HIF-1) appears to be a universal molecular master switch, controlling cellular survival, glucose metabolism and transport, and metabolic adaptation. One of the most relevant target genes of HIF-1 is the EPO. EPO gene expression in the

Table 2. Neurologic scores of control group.

Number	24 hours	48 hours	72 hours	Mean
1	9	9	9	9
2	8	8	8	8
3	9	9	9	9
4	7	8	8	8
5	7	7	7	7
6	6	7	7	7
7	7	8	8	8
8	8	9	9	9
9	9	8	8	8
10	8	8	8	8
11	9	9	9	9
12	10	9	9	9
13	8	8	8	8
14	9	8	8	8
15	9	9	9	9
Mean of control group	**8**	**8**	**8**	**8**

Table 3. Neurologic scores of EPO group.

Number	24 hours	48 hours	72 hours	Mean
1	6	5	5	5
2	5	5	4	5
3	5	5	5	5
4	5	5	4	5
5	4	4	4	4
6	6	5	5	5
7	5	4	4	4
8	7	6	5	6
9	6	5	4	5
10	6	4	4	5
11	6	5	5	5
12	6	5	4	5
13	5	4	4	4
14	6	5	5	5
15	5	5	4	5
Mean of EPO group	**6**	**5**	**4**	**5**

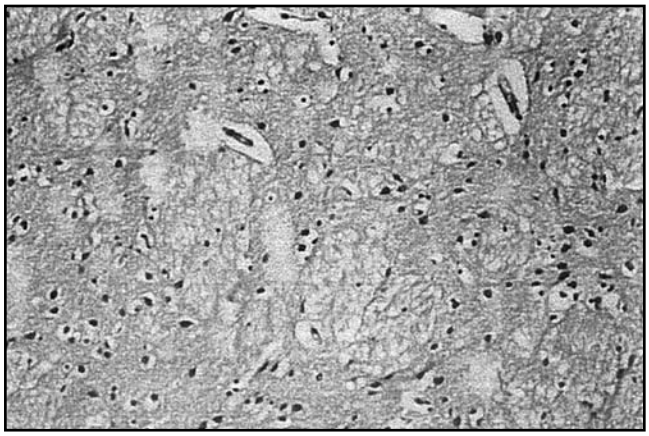

Figure 1. In control group "Stage II-III ischemic neuronal changes" were observed frequently.

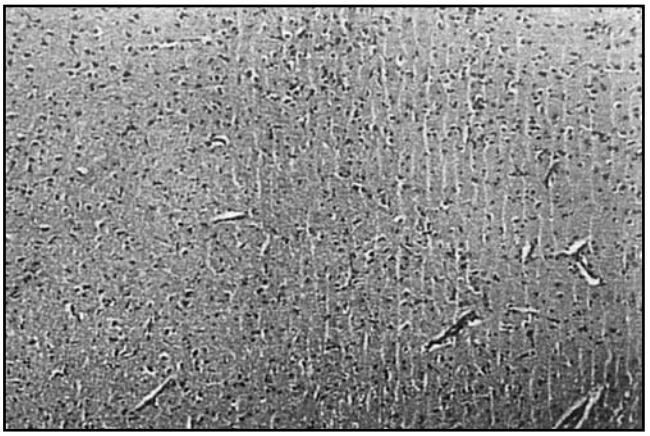

Figure 2. In EPO group "Stage I ischemic neuronal changes" were observed.

brain is regulated by hypoxia-inducible factor-1 that is activated by a variety of stressors, including hypoxia [31]. Another study has demonstrated that EPO infused into the cerebral ventricles of stroke-prone spontaneously hypertensive rats with permanent MCA occlusion improved cognitive tests, reduced cortical infarction, and increased numbers of surviving thalamic neurons and also in situ hybridization revealed that EPO-R mRNA was up regulated at 24 hours in the ischemic penumbra after MCA occlusion [29]. In addition, infusion of EPO into the lateral ventricles prevented ischemia-induced learning disability and rescued hippocampal CA1 neurons from global cerebral ischemic injury in gerbils [30]. A human study suggested that early administration of EPO following stroke improved outcome in the patient population [11]. We could not evaluate these probable mechanisms of EPO in protecting brain due to technical difficulties. Therefore, these results need further investigation for determining of exact mechanism of EPO on cerebral ischemia.

CONCLUSION

In this study shown that EPO reduced infarct volume, and improved neurologic score and histopatologically after cerebral ischemia. We concluded that EPO may decrease ischemic area in experimental cerebral ischemia.

REFERENCES

1 Alafaci C, Salpietro F, Grasso G, Sfacteria A, Passalacqua M, Morabito A, Tripodo E, Calapai G, Buemi M, Tomasello F. Effect of recombinant human erythropoietin on cerebral ischemia following experimental subarachnoid hemorrhage. Eur J Pharmacol. 2000; **406** (2): 219–25.

2 Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke. 1986; **17** (3): 472–6.

3 Belayev L, Alonso OF, Busto R, Zhao W, Ginsberg MD. Middle cerebral artery occlusion in the rat by intraluminal suture. Neurological and pathological evaluation of an improved model. Stroke. 1996; **27** (9): 1616–22;

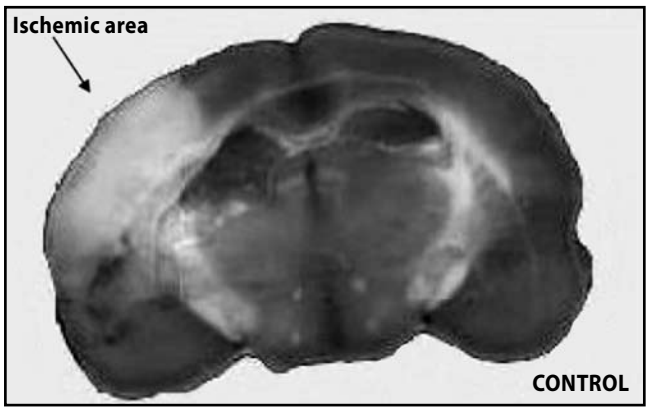

Figure 3a. Infarction samples in brains of control group.

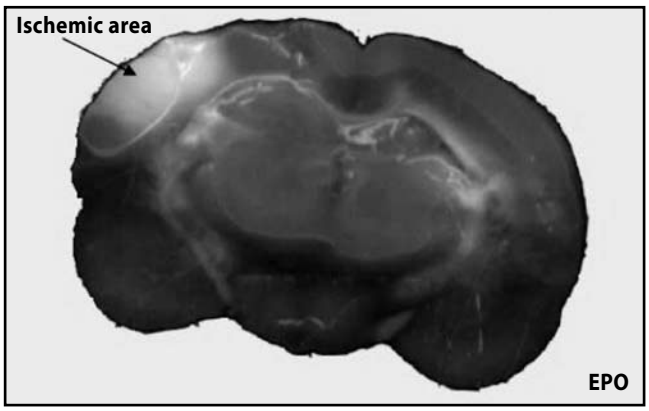

Figure 3b. Infarction samples in brains of EPO group.

4 Bernaudin M, Marti HH, Roussel S, Divoux D, Nouvelot A, MacKenzie ET, Petit E. A potential role for erythropoietin in focal permanent cerebral ischemia in mice. J Cereb Blood Flow Metab. 1999; **19** (6): 643–51.
5 Bormann J. Memantine is a potent blocker of N-methyl-D-aspartate (NMDA) receptor channels. Eur J Pharmacol. 1989; **166** (3): 591–2.
6 Dawson TM. Preconditioning-mediated neuroprotection through erythropoietin? Lancet. 2002; **359** (9301): 96–7. Erratum in: Lancet 2002; **359** (9319): 1782.
7 Digicaylioglu M, Lipton SA. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. Nature. 2001; **412** (6847): 641–7. Comment in: Nature. 2001; **412** (6847): 601–2.
8 Dogan A, Eras MA, Rao VL, Dempsey RJ. Protective effects of memantine against ischemia-reperfusion injury in spontaneously hypertensive rats. Acta Neurochir (Wien). 1999; **141** (10): 1107–13.
9 Doggrell SA. A neuroprotective derivative of erythropoietin that is not erythropoietic. Expert Opin Investig Drugs. 2004; **13** (11): 1517–9.
10 Ehrenreich H, Degner D, Meller J, Brines M, Behe M, Hasselblatt M, Woldt H, Falkai P, Knerlich F, Jacob S, von Ahsen N, Maier W, Bruck W, Ruther E, Cerami A, Becker W, Siren AL. Erythropoietin: a candidate compound for neuroprotection in schizophrenia. Mol Psychiatry. 2004; **9** (1): 42–54.
11 Ehrenreich H, Hasselblatt M, Dembowski C, Cepek L, Lewczuk P, Stiefel M, Rustenbeck HH, Breiter N, Jacob S, Knerlich F, Bohn M, Poser W, Ruther E, Kochen M, Gefeller O, Gleiter C, Wessel TC, De Ryck M, Itri L, Prange H, Cerami A, Brines M, Siren AL. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med. 2002; **8** (8): 495–505.
12 Ehrenreich H, Hasselblatt M, Knerlich F, von Ahsen N, Jacob S, Sperling S, Woldt H, Vehmeyer K, Nave KA, Siren AL.A hematopoietic growth factor, thrombopoietin, has a proapoptotic role in the brain.Proc Natl Acad Sci U S A. 2005; **102** (3): 862–7.
13 Ehrenreich H, Timner W, Siren AL. A novel role for an established player: anemia drug erythropoietin for the treatment of cerebral hypoxia/ischemia. Transfus Apher Sci. 2004; **31** (1): 39–44.
14 Genc S, Akhisaroglu M, Kuralay F, Genc K. Erythropoietin restores glutathione peroxidase activity in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine-induced neurotoxicity in C57BL mice and stimulates murine astroglial glutathione peroxidase production in vitro. Neurosci Lett. 2002; **321** (1–2): 73–6.
15 Genc S, Koroglu TF, Genc K. Erythropoietin as a novel neuroprotectant. Restor Neurol Neurosci. 2004; **22** (2): 105–19.
16 Gorgulu A, Kiris T, Unal F, Turkoglu U, Kucuk M, Cobanoglu S. Protective effect of the N-methyl-D-aspartate receptor antagonists, MK-801 and CPP on cold-induced brain oedema. Acta Neurochir (Wien). 1999; **141** (1): 93–8.
17 Jelkmann W. Biology of erythropoietin. Clin Investig. 1994; **72** (6): 3–10.
18 Jelkmann W. Erythropoietin: structure, control of production, and function. Physiol Rev. 1992; **72** (2): 449–89.
19 Jumbe NL. Erythropoietic agents as neurotherapeutic agents: what barriers exist? Oncology (Williston Park). 2002 ; **16** (9): 91–107.
20 Kirino T, Tamura A, Sano K. Selective vulnerability of the hippocampus to ischemia-reversible and irreversible types of ischemic cell damage. Prog Brain Res. 1985; **63**: 39–58.
21 Kornhuber J, Bormann J, Retz W, Hubers M, Riederer P. Memantine displaces [3H]MK-801 at therapeutic concentrations in postmortem human frontal cortex. Eur J Pharmacol. 1989; **166** (3): 589–90.
22 Kumral A, Ozer E, Yilmaz O, Akhisaroglu M, Gokmen N, Duman N, Ulukus C, Genc S, Ozkan H. Neuroprotective effect of erythropoietin on hypoxic-ischemic brain injury in neonatal rats. Biol Neonate. 2003; **83** (3): 224–8.
23 Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke. 1989; **20** (1): 84–91
24 Marvanova M, Lakso M, Pirhonen J, Nawa H, Wong G, Castren E. The neuroprotective agent memantine induces brain-derived neurotrophic factor and trkB receptor expression in rat brain. Mol Cell Neurosci. 2001; **18** (3): 247–58.
25 Masuda S, Okano M, Yamagishi K, Nagao M, Ueda M, Sasaki R. A novel site of erythropoietin production. Oxygen-dependent production in cultured rat astrocytes. J Biol Chem. 1994; **269** (30): 19488–93.
26 Morishita E, Masuda S, Nagao M, Yasuda Y, Sasaki R. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. Neuroscience. 1997; **76** (1): 105–16.
27 Nasr MS, Peruche B, Rossberg C, Mennel HD, Krieglstein J. Neuroprotective effect of memantine demonstrated in vivo and in vitro. Eur J Pharmacol. 1990; **185** (1): 19–24.
28 Ruscher K, Freyer D, Karsch M, Isaev N, Megow D, Sawitzki B, Priller J, Dirnagl U, Meisel A. Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model. J Neurosci. 2002; **22** (23): 10291–301.
29 Sadamoto Y, Igase K, Sakanaka M, Sato K, Otsuka H, Sakaki S, Masuda S, Sasaki R. Erythropoietin prevents place navigation disability and cortical infarction in rats with permanent occlusion of the middle cerebral artery. Biochem Biophys Res Commun. 1998; **253** (1): 26–32.
30 Sakanaka M, Wen TC, Matsuda S, Masuda S, Morishita E, Nagao M, Sasaki R. In vivo evidence that erythropoietin protects neurons from ischemic damage. Proc Natl Acad Sci U S A. 1998; **95** (8): 4635–40.
31 Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol. 2000; **88** (4): 1474–80.
32 Spandou E, Papadopoulou Z, Soubasi V, Karkavelas G, Simeonidou C, Pazaiti A, Guiba-Tziampiri O. Erythropoietin prevents long-term sensorimotor deficits and brain injury following neonatal hypoxia-ischemia in rats. Brain Res. 2005; **1045** (1–2): 22–30.
33 Villa P, Bigini P, Mennini T, Agnello D, Laragione T, Cagnotto A, Viviani B, Marinovich M, Cerami A, Coleman TR, Brines M, Ghezzi P. Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis. J Exp Med. 2003; **198** (6): 971–5.
34 Yu YP, Xu QQ, Zhang Q, Zhang WP, Zhang LH, Wei EQ. Intranasal recombinant human erythropoietin protects rats against focal cerebral ischemia. Neurosci Lett. 2005; **387** (1): 5–10.