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Original Article

Effect of erythropoietin on inflammatory response and ischemic brain damage after carotid artery clamp in rat

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Abstract

Introduction: Erythropoietin (EPO) is reported to have a minimizing effect on the ischemic condition, but the detailed association between EPO and the ischemic upshot is still not clearly understood. We aimed to evaluate the effect of EPO on inflammatory response and ischemic brain damage after carotid artery clamp in rats.

Methods: In this experimental, animal trial study, which was conducted at the Animal Facility and laboratory at Tabriz University of Medical Sciences, 50 adult male Wistar rats with (250 to 300 g) were randomly allocated to intervention and control groups. The intervention and control groups were administered intraperitoneally with equal volumes of EPO (5000 U/kg) and normal saline, respectively. Both groups had common carotid arteries clamped for 20 minutes. Using the Nissl staining technique, the slides of brain ischemic areas were observed and the rate of ischemic injury in both groups was determined. The blood level of inflammatory cytokines was also measured.

Results: The levels of inflammatory markers including creatine phosphokinase (CPK), interleukin (IL)-6, IL-1B and tumor necrosis factor alpha (TNF)-α in the intervention group were significantly lower than that of control group. Mean percentage of the ischemic area in the intervention group with an amount of 4.30±2.15%, was significantly lower than that of control group (11.20±2.35%, $P=0.023$).

Conclusion: Findings of this study showed that the injection of EPO before carotid clamping is effective in preventing cerebral ischemic injury in rats.

Introduction

Cerebrovascular diseases are important health issues, which almost always affect the individuals' quality of life by leading to disabling health conditions.[1] Narrowed vessels, thrombosis or hemorrhages occurred in the central nervous system, especially in the brain territory lead to the decreased blood flow and severe lack of oxygen, and subsequently, life-threatening events like stroke.[2-4] Stroke is responsible for 5% of deaths each year.[5] Near 20% of patients experiencing stroke die in a year, indicating that stroke is considered at the top of the list of preventable causes of death.[5-8] Risk factors such as smoking, hypertension, cardiovascular diseases, diabetes and sedentary life, may increase the risk of stroke as they increase the chance of carotid stenosis.[9,10] Carotid artery has a specific vascular area, and any changes in its oxygen-delivering role can cause life-long disabilities by the occurrence of cerebrovascular diseases, mainly stroke. Carotid artery disease causes up to 20% of strokes and its risk of occurrence increases by the age highlighting its clinical importance.[11,12]

Many symptomatic or asymptomatic patients with carotid artery stenosis are candidates for the carotid endarterectomy or stenting to prevent stroke.[13-16] A devastating complication during these procedures is ischemic brain injury. Many techniques have been used to prevent this complication, including carotid artery shunting, electro-encephalographic monitoring, and using protecting devices during carotid stenting.[17-19]

Erythropoietin (EPO) is an anti-inflammatory and antioxidant agent, effective in inducing the formation of red blood cells.[20] In addition, EPO has been shown to have a protective role against ischemic damages in various tissues including liver, extremity, kidney, and neonatal brain.[21,22] Due to the presence of EPO receptors on the most brain's cortical cells, EPO plays a direct role

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in protecting neurons against ischemia by the possible mechanism of increasing the resistance to nitric oxide and glutamate toxicity.[23] However, declaring a precise association between EPO administration and decreased ischemic events is in need of more evidence.[24,25] Therefore, this study was conducted to evaluate the effect of EPO administration on brain inflammatory and ischemic status, following carotid clamp in rats.

Materials and Methods

Study design

In this experiment, 50 mature male Wistar rats (250-300 g) were provided by the Animal Facility of Tabriz University of Medical Sciences (Tabriz, Iran) during 2019 (June) to 2020 (December). All the rats were at the same age and maintained under controlled environmental conditions of sufficient food and water at 21 ± 3°C temperature. Then, rats were randomly allocated to the intervention and control groups using RandList v1.2 software.

Surgical technique and blood sampling

The intervention group was administered with 5000 units/kg of intraperitoneal (IP) EPO (Roche, Hertfortshire, UK),[26] whilst the comparison group was administered with an equal volume of placebo (normal saline). After ten minutes, both groups received 100 mg/kg IP ketamine (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and 10 mg/kg xylazine (Sigma-Aldrich, Saint-Quentin-Fallavier, France), for anesthesia. Under sterile condition, a 1-cm incision was made on the anterior aspect of the neck, and then both common carotid arteries were exposed. The vagus nerve was denoted and preserved from stimulation and damages. Both of the common carotid arteries were occluded using vascular clamps (Figure 1). The micro clamps were removed after 20 minutes for reperfusion, and 24 hours later blood sampling was done to quantify creatine phosphokinase (CPK), interleukin (IL)-1B, IL-6 and tumor necrosis factor alpha (TNF)-α levels.

Tissue processing technique

Four days after the onset of ischemia, both groups were euthanized and the brain was removed using craniotomy technique and kept in 10% neutral buffered formalin. Then, the fixed brains were excised to 10 µm coronal slices in 2.3 to 5 mm interval by a rotatory microtome and placed on gelatin-coated slides. The slides were colored using the Nissl staining technique and observed under the optical microscope with ×400 magnification. Only pyramidal neurons with clear nucleus and nucleolus were considered intact and alive. Eight photomicrographs were obtained from each sample by the main investigator (MOH), where 3 of them with a minimum of 40 µm interspace were selected randomly and observed. By checking the slides, the percentage of ischemic damage in both groups was determined.

Statistical analysis

All gathered data were reported as mean ± standard deviation and analyzed by SPSS Software v16 (SPSS ltd, Chicago, IL, USA). The normal distribution was assessed using the Kolmogorov-Smirnov test and the quantitative analyses comparing the two groups were done using independent *t* test or the Mann-Whitney U test. Also, the *P* values less than 0.05 were considered statistically significant.

Results

Results of blood sampling

Of 50 Wistar rats involved in this study, consisting intervention and control group with a one-to-one ratio, CPK amount and inflammatory cytokines levels were measured by analyzing blood samples and the ischemic

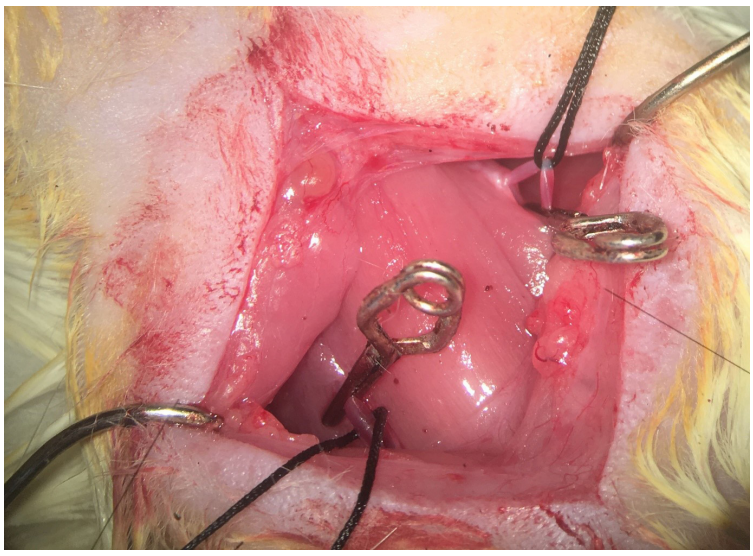

Figure 1. The process of carotid artery clamping in rats in sterile condition in which the anterior aspect of the neck was exposed with one centimeter incision, then both common carotid arteries were clamped.

area was evaluated by microscopic observation of brain slides. The results of blood analysis are shown in Table 1. All inflammatory cytokines had significantly decreased levels in the intervention group.

Results of tissue observation

The ischemic area in the intervention group was 4.30 ± 2.15%, while this value was 11.20 ± 2.35% in the control group. Among the rats with EPO administration, the percentage of the ischemic area was significantly reduced in comparison with the control group ($P = 0.023$).

Discussion

This study shows that the EPO administration has a positive impact on inflammatory responses and brain ischemic injury (4.3% vs. 11.20%, $P = 0.023$), following the carotid clamp in Wistar rats. Carotid clamp simulates the blood flow reduction and just like what happens in stroke, increases ischemic response.[27] An inflammatory response is predictable in either event.[28] The intraperitoneal injection of EPO significantly decreased the levels of CPK, IL-6, IL-1B and TNF-α, which are known as inflammatory cytokines, verifying the anti-inflammatory effect of EPO administration on ischemia induction. Also, the percentage of brain ischemic area was reduced in a similar manner.

Due to the importance of the issue, many studies have been conducted previously on the prevention of ischemic complications and especially, on the effect of EPO and based on the results, many models have been suggested. In a study conducted on male rats, EPO had made an impact of 50% decrease in IL-6 and TNF-α levels in ischemic brain injury.[29] The researchers suggested that EPO administration has a decreasing effect on the inflammatory cytokines by increasing the resistance to inflammatory injury and cessation of the neural apoptosis pathways, unlike other anti-inflammatory agents which inhibit the TNF-α production directly.

In another study, conducted on Sprague-Dawley rats, the delayed administration of EPO improved the revascularization process in the ischemic brain hemisphere and reduced the affected area.[30] Also, the study demonstrated that EPO increases the neurogenesis in the subventricular zone and progenitor migration to the ischemic cortex. The positive effect on reducing ischemic damage was significant.

In a review done on the neuroprotective measures against ischemic events, the role of EPO was defined as a mediator to reduce the volume of infarction and neurobehavioral dearth.[31] Another study which discussed the effect of EPO on ovarian ischemic damage highlighted that EPO administration has a remarkable effect on tissue damage caused by ischemic/reperfusion events.[32] Also, long-term improvement in neurological complications has been reported previously.[33]

Other studies suggest a variety of mechanisms for the discussed results, such as inhibiting the cell death process, stimulating angiogenesis, reducing the influx of inflammation agents into the CNS during injury episodes, immunomodulation, activating neural stem cells and inducing several metabolic actions, like activating the cAMP response element-binding pathway.[34-37] Apoptosis inhibition is carried out by activating Jak2 kinase, which induces signaling cascades leading to BCL-2 and BCL-xL antiapoptotic genes activation.[36,38]

The current study benefited from a proper sample size and reported the EPO effect on both inflammatory cytokines and ischemic area of the brain. Unlike the previous studies, more inflammatory agents were measured in the blood and by highlighting the margins between different agents helps define more underlying mechanisms of EPO effect. However, more studies with higher sample sizes are needed to obtain more detailed results. Also, the lack of clinical trials prevents the application of these results in clinical practices. More studies on the effects of other factors, such as the type of EPO administration, dosage, administration intervals and the race effect should be conducted.

Table 1. Comparison of Inflammatory cytokines levels in both groups

Inflammatory marker	Groups		*P*
	Intervention group (n = 25)	Control group (n = 25)	
Creatine Phosphokinase (mU/mL)	50.12 ± 11.12	73.61 ± 14.51	0.001
Interleukin-6 (pg/mL)	241.68 ± 40.52	290.02 ± 49.78	0.001
Interleukin-1B (pg/mL)	34.71 ± 9.49	47.20 ± 11.08	0.038
Tumor necrosis factor alpha (pg/mL)	112.51 ± 22.86	135.46 ± 25.32	0.026

Data was shown as mean ± standard devation.

Conclusion

The administration of EPO has a protective effect on ischemic brain injury which is associated by the decreasedinflammatory effect due to the reduced CPK, IL-6, IL-1B and TNF-α blood levels. Therefore, it may be used as a prophylactic measure in carotid stenosis procedures. Further studies and clinical trials are suggested to assess neuroprotective effects of EPO in patients with cerebrovascular events.

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Author Contributions

Study design and conceptualization: MBAF, SH, MF, MOH. Methodology and investigation: JM, MH, MSH, MOH. Statistical Analysis: MSH, MOH. Original draft preparation: All of co-authors. Approving final version: All of co-authors.

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Study Highlights

What is current knowledge?

- Many techniques have been used to prevent this complication, including carotid artery shunting, electro-encephalographic monitoring, and using protecting devices during carotid stenting.

What is new here?

- The administration of EPO has a protective effect on ischemic brain injury, it may be used as a prophylactic measure in carotid stenosis procedures

Ethical Approval

The study was conducted based on the Experimental Animal Laboratory Guidelines and the protocol was approved by the Ethics Committee of Tabriz University of Medical Sciences (TBZMED54/651). Despite keeping statistical accuracy, the minimum number of rats was involved and the harm and loss were decreased to the least possible.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper

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