Determination of Safety of Escalating Doses of Intravitreal Erythropoietin in Rabbit Eyes

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Abstract

***Purpose*:** To determine the maximum non-toxic dose of recombinant human erythropoietin (EPO) in rabbit eyes

***Methods*:** Eight rabbits (sixteen eyes) were scheduled for evaluating side effects of four intravitreal doses of 1000, 2000, 4000 and 5000 IU of EPO, two rabbits for each dose. For each dose (two rabbits), the drug was injected for the right eyes. Balanced salt solution (BSS) was injected into the left eye of one rabbit (placebo eye) and the other left eye remained uninjected (control eye). All eyes were examined in 1, 2, 3, 7, 14 and 28 days after intravitreal injections. Electroretinogarphy (ERG) was performed before and 14 days after intravitreal injection. After four weeks, animals were euthanized and eyes were enucleated and submitted for Hematoxylin & Eosin (H&E) and immunohistochemistry evaluations.

***Results*:** Traumatic cataract developed in one of placebo group eyes. Neither anterior nor posterior segment inflammatory reactions were observed after injections. H&E staining and immunohistochemistry examinations did not revealed any sign of retinal and retinal pigment epithelium toxicity with injected doses. ERG changes were within normal limits in all eyes.

***Conclusion*:** Intravitreal injection of recombinant human EPO in rabbit eyes was not associated with adverse toxic effects up to 5000 IU doses.

***Keywords*:** Erythropoietin, Intravitreal injection, Rabbit, Toxicity

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Introduction

Neuroglial elements of the retina experience injury and death in a wide variety of pathologic insults including inflammatory, ischemic, degenerative and toxic diseases. Therapeutic strategies may aim at the specific pathogenic insult or as a more recently developed concept it may be directed to prevent, decrease or even reverse the common pathways leading to cell injury. The aim of "neuroprotection" is to prevent or decrease of neuronal elements injury or death via mechanisms that lead to apoptosis. This can be achieved by pharmacologic interventions or molecular genetic pathways.[1,2] Brimonidine,[3,4] memantin,[5,6] and erythropoietin (EPO)[7-10] are among pharmacologic agents that have been studied in ophthalmology researches for this purpose.

EPO, a glycoprotein hormone, is originally known for its vital role in red blood cell differentiation by preventing apoptosis of erythroid progenitor cells in bone marrow.[11] Recently, EPO has been shown to have considerable neuroprotective and neuroregenerative effects in the different animal models of neuronal system injuries.[7-10,12] High doses of systemic EPO has been associated with encouraging results for CNS stroke,[13] although further studies have raised systemic (thromboembolic) concerns from its high intravenous doses.[14] In the last decade, intravitreal injections have gained significant popularity in ophthalmology. This method provides large doses of medications in close vicinity to the site of vitreoretinal pathologies with little potential systemic complications. Pharmacokinetics and safety of intravitreal injection of EPO has been studied in some recent animal models, however the experiences were limited to doses up to 1,000 IU.[15,16] Since higher doses might be associated with better outcomes, the safety concerns should be addressed first. The aim of this pilot study was to evaluate the safety of escalating doses of intravitreally injected recombinant human EPO in rabbit eyes.

Methods

Animals

Eight male New Zealand albino rabbits weighing between 2 and 3 kg were treated. The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All of the animals were examined by slit-lamp and dilated funduscopy. In the presence of corneal or lenticular opacity or retinal damage before injection, the animal was excluded from the study. Before ocular examination or intravitreal injection, the rabbits were anesthetized by an intramuscular injection of the mixture of ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg). Topical anesthesia with 0.5% tetracaine eye drops (Sina Darou, Tehran, Iran) was applied to reduce the animals' discomfort. The pupils were fully dilated with 1% tropicamide (Sina Darou, Tehran, Iran) and 2.5% phenyl epherine (Sina Darou, Tehran, Iran) with 5 minutes interval. Then, slit-lamp biomicroscopy and indirect ophthalmoscopy and electroretinogarphy (ERG) were performed. The exclusion criteria were the presence of any corneal or lenticular opacity or retinal damage before injections.

Erythropoietin preparation

Preloaded syringes containing 4,000 IU/0.4 cc of recombinant human EPO (Eprex 4000, Cliag, Zug, Switzerland) were used in this study. Under sterile conditions and according to the given dose, EPO was drawn into the tuberculin syringe. Since the maximum drug volume of the preloaded syringe was 0.4 cc (4,000 IU), in the case of 5,000 IU injection we drew 0.5 cc of EPO into the tuberculin syringe that was equivalent to 5,000 IU of EPO.

Intravitreal injection

After general anesthesia and under sterile conditions, eyelids were sterilized using 10% povidone iodine and then kept open with sterile speculum. After instilling of 0.5% tetracaine eye drops for more comfort, conjunctiva surface was prepared with 5% povidone iodine. Thirty-gauge needles (Supa Co. Ltd, Tehran, Iran) attached to tuberculin syringe containing desired amount of EPO or sterile balanced salt solution (BSS) were used for intravitreal injections. Intravitreal injections of 1,000 IU/0.1 ml, 2,000 IU/0.2 ml, 4,000 IU/0.4 ml and 5,000 IU/0.5 ml EPO were performed. For each dose, EPO injection was performed for two right eyes and BSS injection was performed for one left eye with the same volume (placebo eye); another left

eye had only a contact with needle (sham injection) and considered as control. The needle was introduced into the vitreous cavity transconjunctivally at superior temporal quadrant, 1.5 mm behind the limbus. For all injections, the same volume of intended intravitreal injections was withdrawn from the vitreous with the same needle. Without removing of the needle and only by changing the syringe, injections were performed. After termination of the injection, needle was withdrawn and sterile cotton-tipped applicator was placed over the injection site to prevent leakage. Central retinal artery perfusion was controlled with indirect ophthalmoscopy and if central retinal artery occlusion was evident, anterior chamber paracenthesis was performed. At the end of the procedure, topical ciprofloxacin eye drops were instilled. The eyes were clinically examined for any sign of lens or retinal injury and intraocular inflammation, at 1, 2, 3, 7, 14 and 28 days after injections.

Electrophysiologic testing

Electrophysiologic examinations were performed after general anesthesia, just before intravitreal injections and 2 weeks after injections. Full field electroretinography responses were recorded using a mini-Ganzfeld bowel and a Roland electroretinography system (Ronald Consult, Wiesbaden, Germany). After anesthesia, pupils were dilated and a ground silver wire electrode was attached to the animal's forehead and a second electrode was placed adjacent to inferior limbus. After 30 minutes of dark adaptation, scotopic responses were recorded. The rabbits were placed in room light for 10 minutes and photopic responses were recorded after a flash light intensity of 2.0 cd.s/m^2. Electroretionographic changes were considered significant if the postinjection differences in a- and b- wave amplitudes were ≥30% from the baseline values.[17]

Histopathologic Examination

Four weeks after injections, rabbits were euthanized with an overdose of intravenous injection of ketamin. After enucleation, rabbit whole globes were fixed in 10% formalin and sent to the ophthalmic pathology laboratory of the Central Eye Bank of Iran. After 24 hours of fixation, the anterior segments of the eyes were removed by a circumferential incision 2 mm posterior to limbus. Each globe was bisected axially and after tissue processing and embedding into the paraffin blocks, 5 µm-sections were prepared and stained with Hematoxylin & Eosin and Periodic acid-Schiff. Immunohistochemical studies for Glial Fibrillary Acidic Protein (GFAP, ready to use, from Dako Diagnostics, Denmark, Copenhagen) as a glial cell marker and bCL2 (ready to use, from Dako Diagnostics, Denmark, Copenhagen) as an apoptotic marker were performed using the Envision kit (from Dako Diagnostics, Denmark, Copenhagen). One section from the posterior part of each section, which was not treated with the antibodies, was considered as the negative control. A section from an optic nerve glioma and a section from an orbital extranodal marginal zone lymphoma were considered as positive control for GFAPCD31 and bCL2, respectively. Then all the stained slides were examined with a light microscopy (Olympus BX43, Tokyo, Japan) by an ophthalmic pathologist (M RK) who was not aware from the details of the cases. Histopathologic data including integrity of the retinal layers, presence of intraretinal hemorrhages and/or exudates, and immunoreactivity for GFAP and bCL2 immunostainings were recorded in a data table. GFAP immunoreactivity was graded as intense and non-intense based on the extension of immunoreactive cells in the retinal layers. When immunoreactivity was observed in all the retinal layers it was graded as intense.

Results

Clinical findings

Postinjection slit-lamp and funduscopic examinations of anterior and posterior segments revealed no sign of inflammation or infection. Traumatic posterior subcapsular cataract developed in the 0.4 ml BSS injected eye. It was detected on the first postoperative day in the superior temporal portion of the lens that was consistent with the site of needle entrance. Histopathologic examination of this eye revealed the disruption of the posterior lens capsule.

Electroretinography findings

Tables 1 to 3 show the amplitude of a- and b-waves in the term of cone response, rod response and maximal combined response

and their differences before and after EPO injections, placebo injections and sham injections. There was no significant decrease in the postinjection ERG amplitude (a- and b-waves) compared to the preinjection ERG amplitude in all of the cases indicating that EPO did not lead to gross retinal functional changes.

Histopathologic findings

On histopathologic examinations, the integrity and the appearance of all retinal layers were well preserved in the injected and control eyes. No intraretinal hemorrhage or exudate was observed. The GFAP immunoreactivity was intense in all the EPO injected and placebo retinas compared to mild immunoreactivity in the controls. Although this was considered as a sign of stress on müller cells and not as a sign of toxicity, but this finding must be interpreted cautiously. Both the case and control retinal ganglion cells showed similar immunoreactivity for bCL2 immunostaining (Figure 1).

Table 1. Changes in the amplitude of a- and b-waves (Rod response)

Eye	Rod response							
	a- wave (micro volt)				b- wave (micro volt)			
	Preinjection	Postinjection	Difference	Difference (%)	Preinjection	Postinjection	Difference	Difference (%)
1000R1	27.30	47.00	19.70 ↑	72.16 ↑	52.90	107.00	54.10 ↑	102.26 ↑
1000C	20.30	31.70	11.40 ↑	56. 15 ↑	73.90	71.60	2.30 ↓	3.11 ↓
1000R2	80.60	65.80	14.80 ↓	18.36 ↓	170.00	145.0	25.00 ↓	14.70 ↓
1000P	23.00	30.50	7.50 ↑	32.60 ↑	98.30	157.00	58.70 ↑	59.71 ↑
2000R1	21.80	39.70	17.90 ↑	82.11 ↑	50.40	79.10	28.70 ↑	56.94 ↑
2000C	45.30	31.90	13.40 ↓	29.58 ↓	86.90	85.40	1.50 ↓	1.72 ↓
2000R2	33.30	43.60	10.30 ↑	30.93 ↑	73.10	126.00	52.90 ↑	72.36 ↑
2000P	36.60	26.70	9.90 ↓	28.10 ↓	113.00	110.00	3.00 ↓	2.65 ↓
4000R1	15.90	11.46	4.44 ↓	27.92 ↓	123.00	95.50	27.50 ↓	22.35 ↓
4000C	87.80	62.20	25.60 ↓	29.15 ↓	86.90	70.20	16.70 ↓	19.21 ↓
4000R2	18.30	48.60	30.30 ↑	165.57 ↑	170.00	215.00	45.00 ↑	26.47 ↑
4000P	19.70	93.00	73.30 ↑	372.08 ↑	77.10	171.00	93.90 ↑	121.78 ↑
5000R1	22.40	16.40	6.00 ↓	26.70 ↓	98.90	141.00	42.10 ↑	42.56 ↑
5000C	5.13	27.80	22.67 ↑	441.91 ↑	109.00	82.50	26.50 ↓	24.31 ↓
5000R2	8.54	23.40	14.86 ↑	174 ↑	91.30	81.40	9.90 ↓	10.84 ↓
5000P	30.91	40.10	9.20 ↑	29.71 ↑	86.50	64.80	21.70 ↓	25.08 ↓

R: Erythropoietin injected eye, P: Placebo (BSS) injected eye, C: Control (sham) eye

Table 2. Changes in the amplitude of a- and b-waves (Cone response)

Eye	Cone response							
	a- wave (micro volt)				b- wave (micro volt)			
	Preinjection	Postinjection	Difference	Difference (%)	Preinjection	Postinjection	Difference	Difference (%)
1000R1	13.10	9.53	3.57 ↓	27.50 ↓	65.70	53.00	12.70 ↓	19.33 ↓
1000C	12.50	10.86	1.64 ↓	13.12 ↓	73.70	59.30	14.40 ↓	19.53 ↓
1000R2	10.30	9.91	0.36 ↓	3.79 ↓	81.90	69.50	12.40 ↓	15.14 ↓
1000P	16.40	14.60	1.80 ↓	10.97 ↓	73.20	64.60	8.60 ↓	11.74 ↓
2000R1	9.24	7.66	1.58 ↓	17.09 ↓	71.50	60.50	11.00 ↓	15.38 ↓
2000C	2.73	2.01	0.72 ↓	26.30 ↓	71.30	66.30	5.00 ↓	7.01 ↓
2000R2	10.00	26.00	16.00 ↑	160.00 ↑	44.70	70.20	25.50 ↑	57.04 ↑
2000P	6.19	15.70	9.51 ↑	153.63 ↑	61.10	77.40	16.30 ↑	26.67 ↑
4000R1	4.64	3.49	1.15 ↓	24.78 ↓	73.40	48.80	24.60 ↓	33.51 ↓
4000C	9.64	20.60	10.96 ↑	113.69 ↑	63.90	67.90	4.00 ↑	6.25 ↑
4000R2	3.50	8.54	5.04 ↑	144.00 ↑	96.10	71.50	24.60 ↓	25.59 ↓
4000P	2.83	6.95	4.12 ↑	145.58 ↑	75.00	76.40	1.40 ↑	1.86 ↑
5000R1	9.77	19.90	10.13 ↑	103.68 ↑	58.10	64.70	66.00 ↑	11.39 ↑
5000C	22.20	18.02	4.18 ↓	18.82 ↓	64.20	56.20	8.00 ↓	12.46 ↓
5000R2	20.57	23.00	2.43 ↑	11.81 ↑	26.60	38.20	11.60 ↑	43.66 ↑
5000P	11.89	17.80	5.91 ↑	49.70 ↑	46.80	33.70	13.10 ↓	27.99 ↓

R: Erythropoietin injected eye, P: Placebo (BSS) injected eye, C: Control (sham) eye

Table 3. Changes in the amplitude of a- and b-waves (Maximal combined response)

Eye	Maximal combined response							
	a- wave (micro volt)				b- wave (micro volt)			
	Preinjection	Postinjection	Difference	Difference (%)	Preinjection	Postinjection	Difference	Difference (%)
1000R1	24	40.80	16.80 ↑	70.00 ↑	73.00	110.00	37.00 ↑	50.68 ↑
1000C	70.50	53.40	17.10 ↓	24.25 ↓	16.00	39.90	23.90 ↑	149.37 ↑
1000R2	36.60	26.70	9.90 ↓	28.10 ↓	133.00	106.00	27.00 ↓	20.30 ↓
1000P	113.00	126.00	13.00 ↑	11.50 ↑	15.00	54.70	39.7 ↑	264.66 ↑
2000R1	18.30	16.90	1.40 ↓	7.65 ↓	40.50	88.50	48.00 ↑	118.50 ↑
2000C	69.30	80.40	11.10 ↑	16.01 ↑	25.60	28.80	3.20 ↑	12.50 ↑
2000R2	19.10	17.80	1.30 ↓	6.81 ↓	55.30	114.00	58.70 ↑	106.14 ↑
2000P	110	115.00	5.00 ↑	4.54 ↑	72.70	56.30	16.40 ↓	22.55 ↓
4000R1	8.43	30.00	21.57 ↓	255.87 ↑	100.00	94.00	6.00 ↓	6.00 ↓
4000C	76.80	67.20	9.60 ↓	12.50 ↓	39.10	28.40	10.7 ↓	27.36 ↓
4000R2	86.40	65.90	21.00 ↓	24.30 ↓	163.00	22.30	66.00 ↑	36.80 ↑
4000P	79.70	174.00	94.30 ↑	118.31 ↑	89.80	90.40	0.60 ↑	0.67 ↑
5000R1	19.10	17.80	1.30 ↓	6.81 ↓	104.00	140.00	36.00 ↑	34.61 ↑
5000C	121.00	121.00	0.00	0.00	16.40	48.00	31.60 ↑	192.68 ↑
5000R2	13.40	28.30	14.90 ↑	111.19 ↑	102.00	76.20	25.80 ↓	25.29 ↓
5000P	80.70	89.30	8.60 ↑	10.65 ↑	9.27	22.50	13.23 ↑	142.71 ↑

R: Erythropoietin injected eye, P: Placebo (BSS) injected eye, C: Control (sham) eye

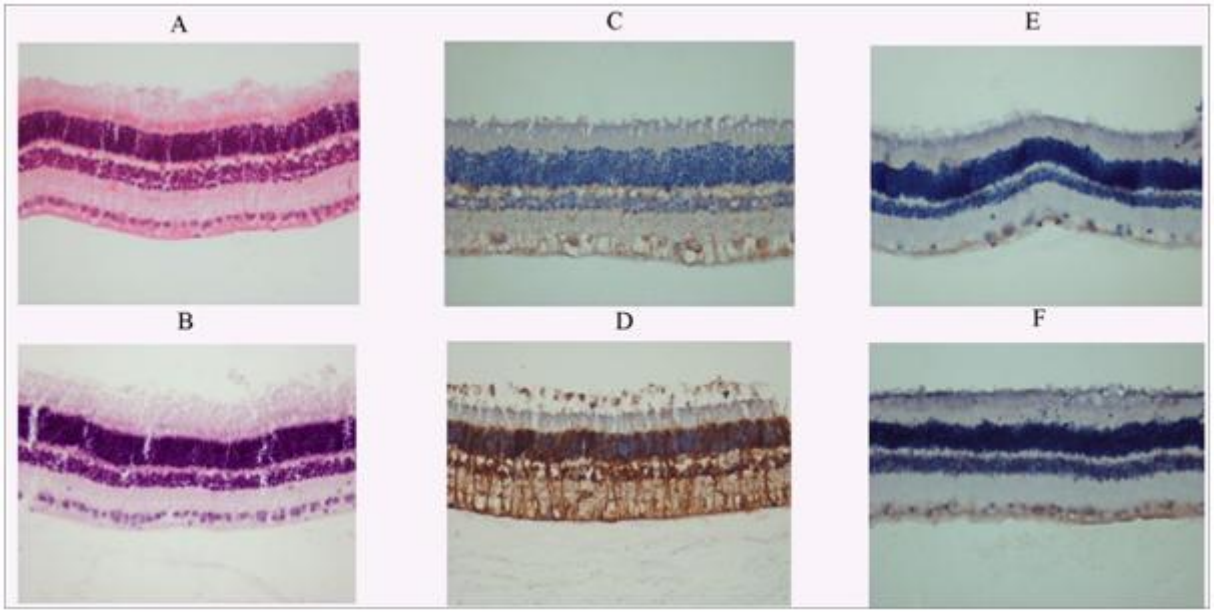

Figure 1. Note the normal-looking retinal layers in both control (A) and EPO injected (B) eyes (Hematoxylin & Eosin, magnification × 200); mild immunoreactivity for GFAP in the control (C) compared to the high immunoreactivity for GFAP in the EPO injected (D) eye (magnification × 200); and immunoreactivity of the ganglion cells in both the control (E) and EPO injected (F) eyes (magnification × 200)

Discussion

Neuroprotection is a newly defined term which refers to "halt or possibly reverse the common pathway leading to neuronal cell injury or death" instead of classical treatment modalities directed toward cure or alleviation of specific disease etiologies.[1] This concept, entails that in different neurologic diseases including ischemic, traumatic or degenerative insults, destruction of neuronal tissue is mediated by a number of common pathophysiologic mechanisms leading to programmed cell death or apoptosis.[18-20] EPO, a well known hematopoietic drug, has recently shown to have potent neuroprotective and neuroregenerative effects.[7,10,12]

Zhong et al showed that intraperitoneal injection of EPO in mice model of glaucoma could promote retinal ganglion cell survival.[7] Zhang and colleagues studied pharmacokinenics and toxicity of intravitreal EPO in rabbits.[15] They showed that doses up to 1,000 IU are well tolerated and safe in rabbit eyes. Zhang and associates showed that EPO receptors up-regulate in retinal tissues of experimental diabetic rats to protect them from diabetic stress. They showed that intravitreal injection of EPO may prevent retinal tissue death and protect the retinal-blood barrier function.[21] Song and colleagues have recently published their safety study on intravitreal injection of EPO in rabbits. They also injected up to 1,000 IU of EPO and showed that this dose does not appear to cause adverse effects in retinal vasculature, retinal anatomy, or ERG function of albino rabbits.[22]

While its clinical usage for different brain and spinal cord injuries is being investigated, there is little clinical experience with its intraocular use in human.[2,23-25] In a small case series, Lagreze et al reported that intravitreal injection of 2,000 IU EPO in human eye is safe.[2] They did not observe positive results in terms of visual acuity (VA) nor visual field. Li and associates injected EPO intravitreally to five patients with chronic and progressive macular edema.[23] They performed three injections 6 weeks apart. VA improved by 3 or more lines in 3 eyes and 1 line in 2 eyes. Improvement in vision occurred within 1 week after the first injection and was maintained until 18 weeks (end point of this study). Only minor improvement in leakage on fluorescein angiography was observed. The largest series of patients underwent intravitreal EPO injection was reported by our team.[24] We evaluated the effects of intravitreal injection of EPO for the treatment of 31 eyes with non-arteritic anterior ischemic optic neuropathy (NAION). The results showed significant improvement of VA.

It is suggested that many neuroprotective drugs exhibit U-shaped curves in which, concentrations higher or lower than optimum are toxic or ineffective. So it is necessary to explore a wider range of doses to find the exact effective and toxic dose.[1] In this study, we showed that intravitreal doses up to 5,000 IU are safe in rabbit eyes. Considering that the rabbit vitreous volume is about one third of emmetropic human vitreous volume, it could be extrapolated that intravitreal injection of 15,000 IU EPO into human eye might be safe. Although vascular proliferation has been observed in cyclosporine-induced nephrotoxicity model of rats treated by systemic EPO,[26] we did not observe any abnormal angiogenesis in clinical and histopathologic examinations.

Small number of animals in each dose group is one of the limitations of our pilot study. According to withdrawing of high vitreous volume before EPO injection, sudden vitreous traction may be accompanied by interfering effects on interpretation of the results; however, retinal detachment and other complications were not seen in the ophthalmoscopic and histopathologic examinations. While ERG is used frequently to assess retinal toxicity of intravitreal injection of different medications, subtle toxicities might be overlooked with this method.[27] Although electroretionographic changes were considered significant if the postinjection decrease in a- and b- wave amplitudes were ≥30% from the baseline values, but there were cases with increasing of the postinjection a- and b- wave amplitudes; so because of this variability, interpretation of the results must be done cautiously. We performed single postinjection ERG, and the ERG examination in different postinjection times may show signs of transient toxic effects. This study addressed safety of single intravitreal injection. Further studies are needed to show whether repeated intravitreal injections of EPO are safe.

Conclusion

Intravitreal injection of recombinant human EPO in rabbit eyes was not associated with adverse toxic effects up to 5000 IU doses.

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