

Safety threshold of intravitreal clonidine in rabbit's eyes

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Abstract

• **AIM:** To determine the safe dose of intravitreal clonidine (IVC), a potential drug for neuroprotection and angiogenesis inhibition in rabbits.

• **METHODS:** A total of 28 rabbits were divided into four groups. Three groups received IVC with concentrations of 15 (Group A), 25 (Group B), and 50 (Group C) µg/0.1 mL and the control group (Group D) received 0.1 mL balanced salt solution (BSS). To investigate IVC safety, electroretinography (ERG) was performed at baseline, then at 1, 4 and 8wk after injection. After last ERG, all rabbits were euthanized, their eyes were enucleated and subjected to routine histopathological evaluation, immunohistochemistry for glial fibrillary acidic protein (GFAP) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) test.

• **RESULTS:** Based on ERG, histopathology, GFAP and TUNEL assay findings, 15 µg IVC was determined as the safe dose in rabbit eyes. While, the results of routine histopathology and TUNEL assay were unremarkable in all groups, toxic effects attributed to 25 and 50 µg IVC were demonstrated by ERG and GFAP tests.

• **CONCLUSION:** Totally 15 µg clonidine is determined as the safe dose for intravitreal injection in rabbits. Contribution of IVC in neuroprotection and inhibition of angiogenesis deserve more studies.

• **KEYWORDS:** clonidine; intravitreal injection; electroretinography; glial fibrillary acidic protein; TUNEL assay

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INTRODUCTION

Clonidine, is an alpha2-adrenoceptors (α2-ADRs) agonist. It exerts its effects on central nervous system via presynaptic α2-ADRs to control blood pressure^[1]. Also, apraclonidine, a derivative of clonidine, has been used to reduce the intraocular pressure spike after anterior segment laser surgery^[2]. Additionally, recent *in vivo* studies have clarified the neuroprotective and anti-vascular endothelium growth factor (anti-VEGF) effects of clonidine^[3-5]. *In vivo* and *in vitro* studies has been demonstrated that clonidine is neuroprotective against retinal neuronal cell apoptosis and death induced by hypoxia and ocular hypertension^[5]. Furthermore, it has been shown that intraperitoneal injection of clonidine increases the level of basic fibroblast growth factor (b-FGF) mRNA in the retina of rats^[3]. b-FGF impede the harmful effects of N-methyl-D-Aspartate (NMDA) in the hypoxemia and protects the neuronal tissue after hypoxemic/ischemic insults^[3,6]. Moreover, it has been detected that brimonidine treatment significantly attenuates retinal and choroidal neovascularization (CNV) and vitreous VEGF concentration in animal models of retinopathy of prematurity (ROP) and CNV^[7].

Neuroprotection is a challenging issue in medicine. In ophthalmology, hypoxemic, ischemic and cytotoxic insults to the optic nerve and retinal tissue leads to permanent and severe visual loss in most cases, emphasizing the importance of neuroprotection. Some drugs and cytokines including erythropoietin^[8-9], brimonidine^[10-11], dexmedetomidine^[12], glutamate antagonists^[13-14], calcium channel blocker^[15], and brain-derived neurotrophic factor (BDNF)^[16-17] were found to exert neuroprotective effects in *in vivo* and *in vitro* models. However, none of them have been used for neuroprotection in ophthalmology. Despite similarities between the brimonidine and clonidine effects, unlike brimonidine, clonidine has an available injecting form and can be used intravitreally. The superiority of intravitreal injection includes achieving high concentration with long term activity after single injection, while the drawbacks of intravitreal route are rare. In this study, we conducted a preclinical study to determine the maximum safe dose of intravitreal clonidine (IVC). It is certain that

the results of this study can be used for investigating the neuroprotective, anti-VEGF as well as anti-glaucoma effects of IVC in future studies.

MATERIALS AND METHODS

Study Design and Animals Twenty-eight female New Zealand white rabbits weighting about 1.5 kg were used. Animals were housed in plastic cages under a 12/12-h dark-light cycle, with access to the food and water ad-libitum. The rabbits were divided into 4 groups (A to D), seven rabbits in each. The groups A to C received 0.1 mL IVC (American Regent, Shirley, NY, USA) corresponding to doses of 15 µg, 25 µg, and 50 µg, respectively and Group D received 0.1 mL of balanced salt solution (BSS) and considered as control group. All intravitreal injections were performed in the right eyes of rabbits. Clonidine hydrochloride with concentration of 5000 µg/10 mL was used. It was diluted as necessary with BSS in sterile condition and controlled pH to produce the required concentration. The rabbits were sedated with intramuscular ketamine (10 mg/kg) (Ketamine-Rotex, Rotexmedica, Trittau, Germany)^[18]. Then injections were performed under sterile conditions and direct visualization using a surgical microscope, by an expert ophthalmologist who was blind to the study. After aspiration of 0.1 mL aqueous humor, intravitreal injection of clonidine or BSS was performed from 2 mm posterior to the limbus at the superotemporal sclera, using a 30-gauge needle. Immediately and three days after the intravitreal injection, all eyes were examined by slit lamp and indirect ophthalmoscope for developing intraocular inflammation, cataract formation, and retinochoroidal damage.

All experiments were designed and conducted in accordance with the ARVO statement for use of animals in ophthalmic and vision research and the guidelines for animal research at the Ophthalmic Research Center, Shahid Beheshti Medical University, Tehran, Iran.

Electroretinography Electroretinography (ERG) examinations were carried out at the baseline, 1, 4 and 8wk after injections. Animals were dark adapted for 12h, and all the procedures were carried out under dim red illumination. The pupils were dilated with one drop of tropicamide 1% (Mydrax, Sina Darou, Tehran, Iran) and anesthesia was achieved with intramuscular injection of ketamine hydrochloride (Ketamine-Rotex, Rotexmedica, Trittau, Germany) (50 mg/kg) and xylazine hydrochloride (Xylazin, Animedica, Germany) (5 mg/kg). The animals were placed in the recording chamber on a warm platform (38°C) to keep their body temperature constant. The ERG signals were recorded using the RETI-port/scan 21 electrophysiological diagnostic systems (Roland Consult, Brandenburg, Germany). *Via* a contact lens, a gold wire electrode (Roland Consult, Brandenburg, Germany) covered with one drop of 2% methylcellulose gel (EyeGel, Eyeol, UK) was placed to touch the central cornea. The ground electrode was placed on pinna and the negative electrode was positioned

in forehead subcutaneous tissue. The rabbits were dark adapted again for 10min and scotopic recordings were made by using scotopic flash ERG at light intensity of 3 cd·s/m². After 10min of light adaptation, photopic cone responses was documented by use of a photopic flash ERG at light intensity of 3 cd·s/m². For both scotopic and photopic flash ERGs, the analogue filters of the instrument were set to the frequency ranges of 0.5 to 200 Hz. ERG analysis was made on amplitude of the b-wave. It was measured from the trough of the a-wave to the peak of the b-wave. The b-wave ratio (experimental eye b-wave amplitude/control eye b-wave amplitude) was calculated for each rabbit in each ERG recording session to decrease the effect of daily and individual variability and considering the control eyes in the ERG analysis^[19-20]. The mean of b-wave ratio in both photopic and scotopic conditions was calculated for any dose of IVC in every session of ERG and used as an index of retinal function in the experimental eyes^[21].

Histopathological and Immunohistochemical Studies

Eight weeks after intravitreal injections, the rabbits were euthanized by an intravenous injection of a lethal dose of sodium pentobarbital (0.36 mg/kg)^[22]. The eyes were enucleated carefully, fixed in 10% formalin and subjected to histopathological examinations. After bisecting the eyes axially into 2 calottes, tissue processing, and embedding into paraffin blocks, 5 µm tissue sections at three different tissue planes (200 µm apart) were prepared and stained with hematoxylin & eosin. Blank sections were then subjected to immunohistochemical staining for Glial Fibrillary Acidic Protein (GFAP) (Z 0334; Dako, Glostrup, Denmark) to investigate alterations of retinal Müller cells immune reactivity for GFAP. Müller cells show increased GFAP expression in retinal damaging^[23]. All the stained sections were examined under light microscopy (BX41; Olympus, Tokyo, Japan) by a masked ocular pathologist (MRK) for the presence of hemorrhage, inflammation, necrosis, atrophic changes, and GFAP immunoreactivity in the retina. The GFAP results, as described previously, were scored from 0 to 5^[24]. Zero score was when there was no staining. Score 1 was when staining was limited to internal limiting membrane and nerve fiber layer. Focal and diffuse staining of Müller cells involving partial length of the cells were regarded as scores 2 and 3, respectively. Score 4 was defined as focal staining of Müller cells involving full length of the cells. When there was a diffuse staining of Müller cells involving full length of the cells, it was regarded as score 5. Mean score of 2.5 or more in each group was considered significant.

Apoptosis Assay The apoptosis assay was done using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit according to the manufacturer's instructions (*In Situ* Cell Death Detection Kit, Fluorescein; 11684795910, Roche) to detect the presence of DNA fragments of apoptotic cells in retinal sections. The paraffin sections were de-waxed

Table 1 B-wave amplitude ratio in different treatment groups, photopic response mean±SD

Time	15 µg/ 0.1 mL (Group A)	25 µg/ 0.1 mL (Group B)	50 µg/ 0.1 mL (Group C)	¹ P
Pre-injection	0.92±0.31	1.19±0.44	0.97±0.24	
1wk	0.9±0.16	0.9±0.26	1.09±0.33	0.91
² P	0.866	0.249	0.401	
4wk	1.1±0.26	1.03±0.18	0.93±0.23	0.154
² P	0.237	0.686	0.779	
8wk	1.2±0.34	0.93±0.19	0.85±0.76	0.259
² P	0.063	0.225	0.612	

¹Adjusted for the pre-injection, based on multiple linear regression. These *P* values show the effect of dosage increments on the b-wave amplitude decrement; ²Based on Linear mixed model, adjusted for multiple comparisons by Bonferroni method.

Table 2 B-wave amplitude ratio in different treatment groups, scotopic response mean±SD

Time	15 µg/ 0.1 mL (Group A)	25 µg/ 0.1 mL (Group B)	50 µg/ 0.1 mL (Group C)	¹ P
Pre-injection	1.03±0.46	0.95±0.27	0.99±0.21	
1wk	0.99±0.36	0.87±0.15	1.05±0.13	0.473
² P	0.499	0.345	0.674	
4wk	0.83±0.42	1.09±0.28	1.03±0.2	0.334
² P	0.398	0.345	0.484	
8wk	1.02±0.13	0.85±0.12	0.33±0.21	<0.001
² P	0.735	0.500	0.018	

¹Adjusted for the baseline, based on multiple linear regression. These *P* values show the effect of dosage increments on the b-wave amplitude decrement; ²Based on Linear mixed model, adjusted for multiple comparisons by Bonferroni method.

by immersing in xylene for 10min, absolute alcohol for 10min, and double distilled water. Then the sections were permeabilized in the permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) for 8min. After washing with phosphate-buffered saline (PBS) for 5min twice, the slides were placed in citrate buffer pH 6 and microwaved (high irradiation or 750 W) for 1min. Following rapid cooling in double distilled water (20°C-25°C) and PBS (20°C-25°C), the slides were immersed in Tris-HCL 0.1 mol/L pH 7.5, 3% BSA, and 20% FBS for 45min. Subsequent to washing with PBS for 5min twice, the positive control slide from a retinoblastoma case was incubated in DNase I, Tris-HCL 50 mmol/L pH 7.5, 1 mg/mL BSA for 10min (20°C-25°C). Then 50 µL TUNEL reaction mixture (50 µL enzyme solution in 450 µL label solution) was added to each slide except negative control that received 50 µL label solution. After incubation at 37°C for 60min in the dark, the samples were washed with PBS for 5min three times. The sections were observed and images were captured using an inverted fluorescence microscope (Olympus IX71, 515-565 nm filter). The presence of apoptotic cells in the clonidine-treated cases was assessed as compared to the control group.

Statistical Analysis All statistical analyses were performed using SPSS software (IBM Corp. Released 2014. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp., USA). All values were expressed as the mean±standard deviation. To evaluate difference of the ratio from 1 we used one sample *t*-test. To compare the results within each group

with baseline ratio during the follow ups, we used Linear Mixed Models (LMM) option in the SPSS. The Bonferroni correction method was considered for multiple comparisons. The Mann-Whitney *U* test was used to compare staining intensities values between groups. To evaluate the trend of b-wave ratio values with the increment in clonidine dosage, we used multiple linear regression. *P* values less than 0.05 were considered statistically significant.

RESULTS

All animals tolerated the injections well. Post-IVC eye examination demonstrated no intraocular inflammation, cataract formation, retinal detachment, optic atrophy or endophthalmitis. Two rabbits (one in Group B and one in Group C) developed mild vitreous hemorrhage in their right eyes due to injection technique and were included in the analysis. One rabbit in Group B died during the study period and was excluded from the final analysis.

Electroretinography There was no difference in the ERG patterns, recorded in treated compared to the control eyes. As it was mentioned in the methods, we defined the b-wave amplitude ratio to evaluate the functional damage to the retina quantitatively. Tables 1 and 2 showed the mean of b-wave amplitude ratio in different time points (baseline, 1, 4 and 8wk after IVC) in the study groups. There were not any toxic effects related to different dosages of IVC, based on photopic ERG responses (*P*>0.05 for all) (Table 1). Considering the scotopic responses, while 15 µg IVC was safe, 50 µg was

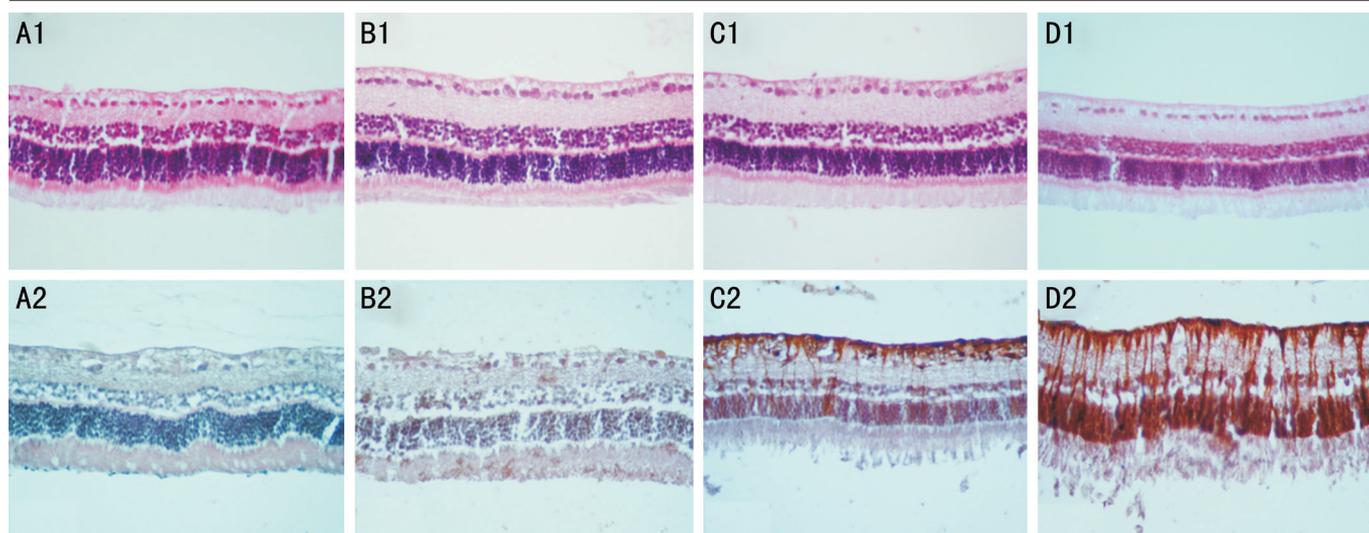


Figure 1 Representative unremarkable neurosensory retina with intravitreal injection of 15 µg (B1), 25 µg (C1), and 50 µg (D1) clonidine as compared to the BSS-injected (A1) eyes (hematoxylin & eosin stain, magnification $\times 400$). Note non-significant GFAP immunoreactivity of the retina in a representative eye injected with 15 µg (B2) clonidine and BSS (A2) and significant retinal GFAP immunoreactivity in a representative eye injected with 25 µg (C2) and 50 µg (D2) clonidine (magnification $\times 400$).

obviously toxic (Table 2). The mean of b-wave amplitude ratio reduced significantly eight weeks after 50 µg IVC compared to the baseline value ($P=0.018$). In Group B, mean of b-wave amplitude ratio decreased eight weeks after clonidine injection compared to the pre-injection value, but the decrement was not statistically significant ($P=0.50$). However, as an additional analysis, the mean of b-wave amplitude in the 25 and 50 µg groups were significantly less than the same value in the control group at eight weeks follow up ($P=0.049$ and $P<0.001$, respectively). Also, the P value for the trend of b-wave ratio rates with the increment in clonidine dosage was significant in scotopic responses, when comparing mean of the b-wave ratio between study groups eight weeks after clonidine injection ($P<0.001$) (Table 2). It shows that increment of the IVC dosage significantly reduced the mean of b-wave amplitude ratio.

Light and Fluorescence Microscopy Light microscopic examinations of the eyes in the study groups disclosed no evidence of intraretinal hemorrhage, inflammation, necrosis or atrophic changes. Retinal layers integrity was not violated in any of the cases. The GFAP immunoreactivity was significantly increased in the Groups B (25 µg) and C (50 µg) with the mean scores of 2.67 ± 1.15 and 3.33 ± 1.53 , respectively. No significant GFAP immunoreactivity was observed in the retinas of Groups A (15 µg) and D (BSS-injected eyes) with the mean scores of 1.67 ± 0.58 and 1.33 ± 0.58 , respectively (Figure 1). The GFAP results were well correlated with the ERG results, demonstrating retinal toxicity attributable to 25 and 50 µg IVC.

TUNEL assay to characterize the retinal cells death disclosed no significant positivity in the rabbit retinas of Groups A (15 µg), B (25 µg), and C (50 µg) as compared to the control (Figure 2). Overall, given the ERG, histopathology,

immunohistochemical and TUNEL assay results, 15 µg/0.1 mL IVC was determined as the maximum safe dose.

DISCUSSION

In the present study, various doses of clonidine were injected intravitreally into the rabbit eyes to determine its maximum safe dose. It was found that 15 µg IVC was the safe dose, based on, functional, histological and immunohistochemical studies. Furthermore, 15, 25 and 50 µg IVC did not cause retinal cells apoptosis using TUNEL assay. Although the TUNEL assay was unremarkable, retinal toxicity was demonstrated with the doses of 25 µg and 50 µg IVC, based on scotopic ERG and GFAP results, corresponding to the dysfunction of retinal rods and Müller cells, respectively.

Clonidine as an α_2 -ADRs agonist seems to have neuroprotective and anti-VEGF effects^[3-4]. Yoles *et al*^[25] found that single dose of intraperitoneal clonidine, administered immediately after partial crush injury of the rat's optic nerve induced neuroprotective effects compared to the controls, demonstrated by electrophysiological measurements. Weigert *et al*^[26] used intravenous clonidine with dosage of 0.2 µg/kg/min over 10min in healthy volunteers to evaluate its effects on ocular blood flow. They found that intravenous clonidine increased the choroidal and optic nerve head blood flow, displayed by laser Doppler flowmetry. They supposed that it was due to vasodilatory effect of clonidine and concluded that it may contribute to the retinal ganglion cells (RGC) and neuronal protection. Peng *et al*^[27] found that intraperitoneal clonidine contributed to photoreceptor protection in rat retina. They discovered that clonidine induced extracellular-signal regulatory kinases (ERKs) phosphorylation, mainly in Müller cells. Naziri *et al*^[5] also demonstrated that intraperitoneal clonidine decreased RGCs death in an experimental-glaucoma

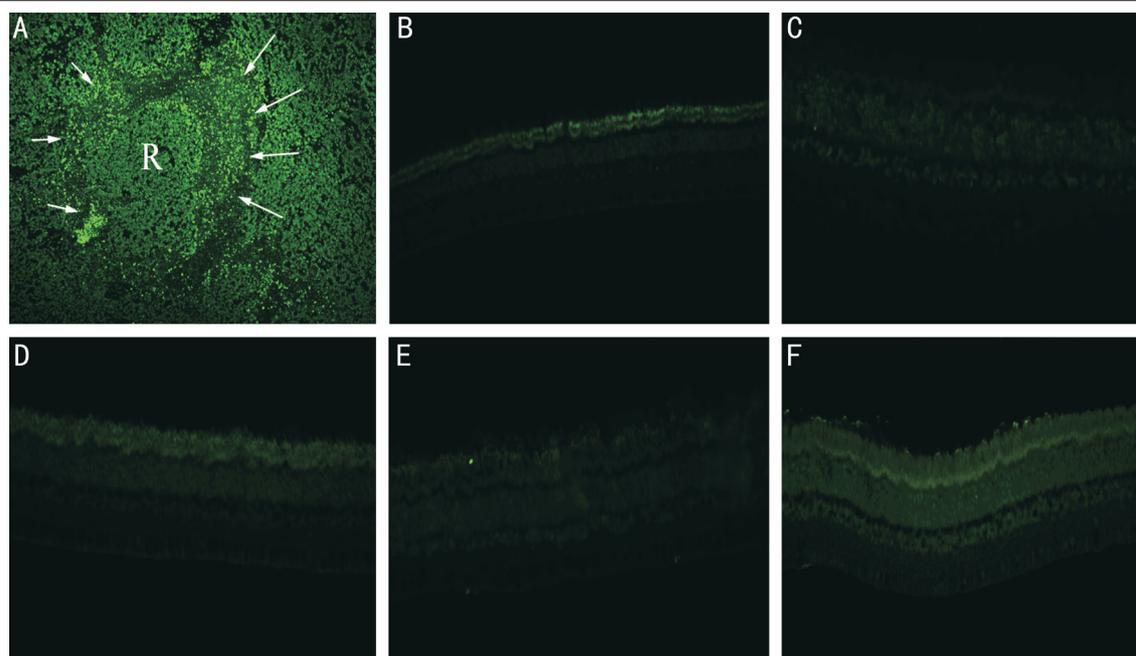


Figure 2 Determination of the apoptotic effects of 15, 25, and 50 µg of intravitreal clonidine injections on rabbit retinas by using TUNEL assay. Positive control is a section of retinoblastoma with viable tumor cells (see R in A) surrounded by apoptotic cells and negative control was not treated by TUNEL reaction mixture (B). Results indicated that none of examined clonidine concentrations 15 µg (C), 25 µg (D), and 50 µg (E) imposed apoptotic effects on the treated groups when compared to the control (F) (all images magnification ×400).

model. It was found that clonidine reduced amyloid beta (Aβ) and amyloid protein precursor (APP) in the RGC layer and protected them from a neurodegenerative process. The anti-VEGF effects of α₂-ADRs agonists have been confirmed in different *in vivo* and *in vitro* studies. Watanabe *et al*^[4] demonstrated that clonidine had an inhibitory effect on VEGF mRNA and protein expression in the RPE cells stimulated by IL1β. Kusari *et al*^[7] clarified that subcutaneous brimonidine significantly reduced laser induced CNV in brown Norway rats. Additionally, they found that retinal neovascularization diminished significantly in an animal model of ROP following systemic brimonidine administration. In most of these studies it was disclosed that the neuroprotective effects of α₂-ADRs agonists such as clonidine, can be blocked by the selective α₂-ADRs antagonist, indicating that the therapeutic effects of these agents were exerted through adrenoreceptors such as α₂-receptors^[25,27]. Bylund *et al*^[28] demonstrated that these receptors (α_{2A} subtype) are also present in the human neurosensory retina. Furthermore, Woldemussie *et al*^[29] reported that all three subtypes of α₂-ADRs (α_{2A}, α_{2B} and α_{2C}) were present in human retina, differentially localized in various cellular layers.

ERG analysis in our study disclosed a significant decrease in b-wave amplitude and b-wave amplitude ratio in dark adapted state, detected 8wk after 25 and 50 µg IVC, respectively. More reduction of b-wave amplitude ratio was observed in those rabbits who had received 50 µg IVC compared to those who had received either 25 or 15 µg IVC. Furthermore, unlike the ERG and GFAP results, routine histopathology and TUNEL

assay did not show any abnormal changes related to different dosages of IVC in the rabbit retinas. This disparity may be indicating that the 25 and 50 µg doses of IVC were sufficient for induction of retinal rods and Müller cells dysfunction but insufficient for inducing cellular apoptosis. In other words, as demonstrated by ERG and GFAP results of the current study, higher doses of IVC disturbed retinal function and induced Müller cells dysfunction but their toxic effects were not too severe to cause retinal ganglion cells apoptosis in TUNEL assay or retinal atrophic changes on light microscopy.

To the best of our knowledge, current study is the first survey in which that the maximum safe dose of intravitreal clonidine was investigated through an experimental preclinical study. The obvious advantage of intravitreal administration is that the concentration achieved with this route is many-fold greater than the one achieved with either topical or systemic administration, while inducing the least systemic side effects. In conclusion, as described before clonidine have four summarized effects including neuroprotection, anti-VEGF, increasing choroidal blood flow and anti-glaucoma. Our data suggests that 15 µg IVC is safe for intravitreal injection, however, it is still uncertain whether this dosage may also contribute to neuroprotection and/or anti-VEGF effects in animal models or in human retina, which deserves further investigations.

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Conflicts of Interest: Nikkhah H, None; Garfami KH, None; Kanavi MR, None; Nashtaei EM, None; Karimi S, None; Soheilian M, None.

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