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# Mitochondria-targeted antioxidant SkQ1 reverses glaucomatous lesions in rabbits

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## Abstract

Glaucoma is the main cause of irreversible blindness worldwide. This disease is characterized by apoptosis of retinal ganglion cells (RGC) and visual field loss that seems to be related to elevated intraocular pressure (IOP). Several lines of evidences have implicated the crucial role of mitochondrial dysfunction in the pathogenesis of glaucoma. Increased mitochondrial oxidative stress in RGC may underlie or contribute to susceptibility of RGC to apoptosis. In our work we (i) designed a rabbit model of chronic, moderately elevated IOP for studying glaucoma and (ii) demonstrated efficacy of mitochondria-targeted antioxidant SkQ1 as a tool to reverse several traits of experimental glaucoma induced by a series of injections of hydroxypropylmethylcellulose (HPMC) to the anterior chamber of the rabbit eye. It is shown that 6 months instillations of drops of 0.2.5–5 μM solution of SkQ1 normalize IOP and eye hydrodynamics and abolish an increase in lens thickness that accompanies glaucoma.

**KEYWORDS:** Glaucoma, Rabbit Model, Mitochondria-Targeted Antioxidant, Reversal Of Lesions, SkQ1

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## Introduction

Glaucoma is a leading cause of blindness and visual impairment [1]. It is a multifactorial ocular disease characterized by progressive damage to and degeneration of the optic nerve and visual field loss [2]. The progressing and irreversible loss of vision is caused by the apoptotic death of retinal ganglion cells (RGC). Some of the important risk factors of glaucoma development have been identified, the most important being elevated IOP [3].

The exact mechanism of RGC death in glaucoma is not obvious. Several hypotheses concerning the mechanism of RGC death have been suggested. One of them consists in that mechanical compression caused by high IOP hinders normal blood supply to the optic nerve, and irregular blood flow causes a tissue hypoxia, which may be the triggering mechanism of RGC damage and apoptosis [4, 5]. Indeed, RGC have been reported to be particularly sensitive to systemic hypoxic challenge [6, 7]. RGC death has been found to occur in many different models of induced retinal ischemia [8, 9]. The analysis of the expression of a hypoxia-induced transcription factor, HIF-1 , whose synthesis is tightly regulated by cellular oxygen concentration and/or reactive oxygen species (ROS), provided direct evidence that hypoxia occurs in RGC of glaucomatous eyes, and hypoxic signaling is likely one of the pathogenic mechanisms involved in glaucomatous neurodegeneration [4].

A great number of studies focused on the improvement of glaucoma therapy have stimulated a search for animal models of experimental glaucoma [10, 11]. J. Benozzi, M. Moreno and coworkers have reported [12, 13] that injections of a viscoelastic material into the anterior chamber of the rat eye caused persistent IOP increase for up to six months and led to a significant drop in the level of melatonin in the retina, the activation of lipid peroxidation, and a decrease in antioxidant protection reserve in the eye media.

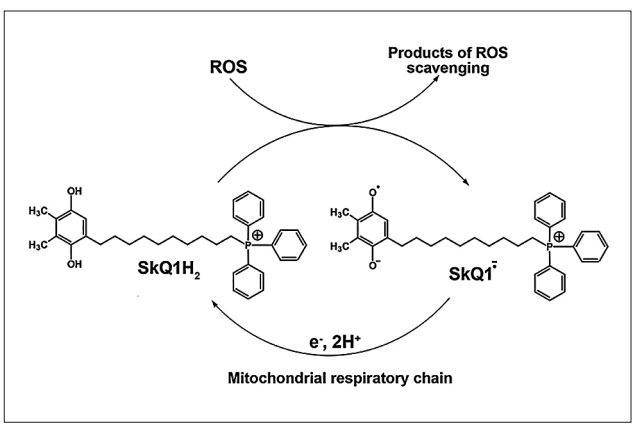


**Fig. 1.** SkQ1H2 (reduced form of SkQ1) scavenges ROS. This results in conversion of SkQ1H2 to its semireduced form (SkQ1•⁻). The latter is reduced by mitochondrial respiratory chain to initial SkQ1H2

However, for ophthalmic pharmacological investigation, the rabbit eye is a more appropriate biological object [14]. The rabbit eye is closer to the human eye than the eye of the rat, in particular, the size of the eyeball, its internal structure, anatomical, optical, as well as biomechanical and biochemical parameters. Further, the conjunctiva cavity volume of the rabbit eye corresponds to this parameter of the human eye. For this reason, we developed a rabbit model of chronic, moderately elevated intraocular pressure for studying glaucoma.

Hypoxia causes accumulation of ROS, which have been shown to be cytotoxic to RGC [4]. It was found that a hypoxia-induced mitochondrial dysfunction is associated with RGC death in a mouse model of glaucoma [15–17]. Mitochondrial dysfunction increases ROS production and as a consequence causes cell death due to apoptosis. Mitochondria are major producers of ROS [18]. Superoxide is the primary ROS formed within mitochondria by the reduction of molecular oxygen [19]. The respiratory chain in mitochondria is a powerful source of superoxide and consequently hydrogen peroxide as a product of superoxide dismutation [18–20].

Further evidence for oxidative stress being implicated in the pathogenesis of glaucoma comes from studies demonstrating a benefit from compounds with antioxidant properties: vitamin E, Brimonidine, manganese porphyrin, astaxanthin [16], and timolol [21].

Recently, a group of new antioxidants that are targeted to mitochondria was described [22, 23]. These compounds are selectively accumulated by mitochondria and are regenerated by the respiratory chain after scavenging of ROS. As a result, they can be used as effective antioxidants at micromolar and even nanomolar concentrations. High efficiency of one of these compounds, 10-(6'-plastoquinonyl) decyltriphenylphosphonium (SkQ1) (Figure 1), was confirmed in experiments with artificial lipid membranes, isolated mitochondria, and cells in culture. Pronounced therapeutic effects of SkQ1 were observed in models of ischemic pathology of heart, kidney, and brain and also in models of some eye diseases [23–26]. A related rhodamine-based plastoquinone analogue SkQR1 was shown to alleviate the acute bacterial infections associated with pyelonephritis [27].

In this paper, we report our findings of the effect of SkQ1 on a rabbit model of chronic and moderately-elevated IOP. Our data suggest that SkQ1 reverses experimental glaucoma which was induced by injection of a viscoelastic cellulose-derived polymer HPMC.

## Material and Methods

### Animals

78 male pigmented rabbits weighing 2 to 2.5. kg (RND Company "Manikhino", Moscow region, Russia) were housed in an air-conditioned room. The animals were given food and water ad libitum. All animal care

*Table 1.* **Drops of 5 μM SkQ1 reserved experimental glaucoma in rabbits**

| Group | Number of eyes | IOP ($P_0$, mm Hg) | Total humor outflow (C, $mm^3$/min x mm Hg) | Humor production (F, $mm^3$) | Bekker's coefficient (K) | Lens thickness, mm |
|---|---|---|---|---|---|---|
| Control | 20 | 17,5±0,6 | 0,15±0,07 | 1,19±0,29 | 110±27 | 7,30±0,08 |
| Glaucoma | 10 | 21,3±1,0* | 0,07±0,02* | 0,46±0,14* | 305±66* | 8,30±0,13* |
| Glaucoma+5 μM SkQ1 | 10 | 17,7±1,0* | 0,19±0,04* | 1,81±0,48* | 96,3±19,0* | 7,5±0,2* |

***Note:*** *p<0.0.5 for the eyes with glaucoma vs. control eyes or for eyes with glaucoma + SkQ1 vs. glaucoma eyes (line 2 or 3, respectively).

and experimental procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Chronic, moderately elevated intraocular pressure model

Chronic, moderately elevated intraocular pressure was induced by injections of 0.15 μl 2% of hydroxypropylmethylcellulose (HPMC) in the anterior chamber of the right eye of 24 rabbits. This was done under local anesthesia produced using 0.5% alcain (Alcon). HPMC was injected twice per week for 5 weeks. The left eye remained as the untreated control. This group of rabbits was used for developing the glaucoma model. A total of 54 rabbits were used for the study of the efficacy of mitochondria-addressed antioxidant SkQ1. An injection of 0.15 μl 2% HPMC was made to the anterior chamber of both eyes of these rabbits twice a week for five weeks. SkQ1 eye drops were instilled once a day in the right eye. Instillations were performed during a 6 month period. Treatments with HPMC and SkQ1 were started simultaneously.

### IOP measure

The IOP was measured just before the first HPMC injection and SkQ1 instillation and then every month during half a year, using a Schmitz tonometer. A Glautest 60 tonograph (Russia) was used to study of the hydrodynamic parameters of the aqueous humor. Measurements were done under local anesthesia with 0.5% alcain.

### Ultrasonic biometry and biomicroscopy

The same local anesthesia was used for ultrasound biometry using an Angiodin ultrasound computer ophthalmoscope (Russia). Slit lamp biomicroscopy was used for monitoring of the eye media. Photographs of the eye fundus were made after two consecutive instillations (with 15 min interval) of mydriatic (1% Mydriacil, Alcon), using a Canon fundus camera (Japan).

### Morphology

After the six month period, the rabbits were anesthetized and sacrificed with a lethal intravenous injection of 10 mg/kg ketamine. Each eye was enucleated and post-fixed by immersion in 10% paraformaldehyde in PBS. Subsequently, they were dehydrated in ethyl alcohol and embedded as a cross section in paraffin for sectioning. Cross sections (thickness, 5 μm) were cut on an ultra-microtome, mounted on glass slides, and stained with hematoxylin-eosin [28].

## Results

### Rabbit glaucoma model

The mean baseline IOP value for 24 healthy rabbit eyes ($P_0$) was 15.3±1.7 mm Hg (here and below, M±SE is indicated). This coincides with previously published values for healthy rabbits [29]. HPMC resulted in IOP elevation during first two weeks to approximately 20.5±1.4 mm Hg. This IOP proved to be stable at this level over 6 months. Eyeball biometry demonstrated a change in some anatomical and optical parameters during the development of experimental glaucoma. In particular, the development of induced glaucoma is accompanied by thickening (swelling) of the lens and an increase in anterior–posterior length of the eyeball by an average of 1±0.3 mm. It is well established that the drainage system of the eye is responsible for controlling IOP. The analysis of flow values in experimental eyes showed changes in aqueous humor dynamics *(Table 1)*.

After 10 injections of HPMC, the estimated total outflow facility (C) significantly decreased. The Bekker's coefficient ($K=P_0/C$) strongly increased. These results reveal a violation of hydrodynamics of intraocular fluid in the eyes. Morphological study of the drainage area revealed significantly enhanced intrascleral channels and watery veins that confirm the development of a glaucomatous damage. Fundus pattern analysis showed that in all eyes with experimental glaucoma optic nerve excavation was observed. In the optic disk

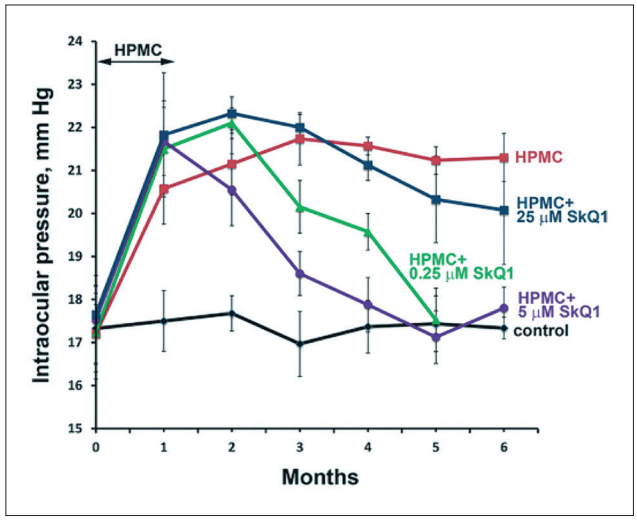


**Fig. 2.** SkQ1 reverses effect of viscoelastic HPMC on IOP in rabbit eyes.

of the eye with normal IOP, the vessels appear to originate from the center of the optic disk and have straight directions. In experimental glaucoma eyes with elevated IOP, the inferior vessels do not appear to originate at the center of the optic disk and the directions of the vessels are more tortuous. To assess the potential effect of moderately elevated IOP on the posterior eye segments, histological studies were performed. Evidence of glaucomatous damage was obtained in the HPMC-treated eyes: i.e. deep excavation and vacuolar degeneration of the optic nerve, RGS loss, and the detachment of pigment epithelium and the layer of rods and cones. Significant changes in Elschnig's inner limiting membrane were noted. The membrane was thinner in comparison to healthy eyes, especially in the central area of the optic disk where there was a sharp thinning of the membrane and even its complete disappearance. Changes similar to those typical for glaucomatous process were observed in the optic nerve prelaminar compartment. Especially pronounced vacuolization processes were revealed on the border of the prelaminar area of the optic nerve.

### IOP lowering by means of SkQ1

In next series of experiments, a dose/response study was performed to determine the effective dose of SkQ1. Solutions of 50 nM, 250 nM, 5 $\mu$M, and 25 $\mu$M SkQ1 were tested. The data for three SkQ1 concentrations are shown in Figure 2. It is seen that SkQ1 instillation does not prevent IOP increase caused by HPMC injections. However, SkQ1 was quite effective in reversing the HPMC-induced pathology in the post-HPMC period, i.e. when the viscoelastic treatment ceased. Several months were needed to completely normalize the IOP level by 0.25 or 5 $\mu$M SkQ1, the higher the SkQ1 concentration the lower the time required. Further increase in the SkQ1 concentration (up to 25 $\mu$M) strongly decrease the favorable effect of the antioxidant *(Figure 2)*. Lowering SkQ1 to 0.05 $\mu$M was also unfavorable for the treatment of glaucoma (not shown).

The results of Figure 2 demonstrate that eye drops with 5 $\mu$M of SkQ1 most effectively reverse IOP elevation. Therefore, 5 $\mu$M SkQ1 was used in further experiments. Studies of SkQ1 efficacy in reversal of the symptoms of experimental glaucoma in rabbits included the measurement of IOP and hydrodynamic parameters of all examined rabbit eyes before and after six months of instillation of SkQ1 *(Table 1)*. After six months, the $P_0$ value in glaucoma eyes without SkQ1 instillations was 21.3±1.0 mm Hg, i.e. 3–4 mm Hg above baseline values (17.5±0.6); the total humor outflow facility coefficient (0.07±0.02) was reduced compared with the initial level (0.15±0.07) ($p<0.05$). In contrast, in glaucomatous eyes treated with 5 $\mu$M SkQ1, the IOP (17.7±1.0) and total outflow coefficient (0.19±0.04) were similar to the baseline values for healthy eyes *(Table 1)*. Humor production was dramatically decreased in glaucomatous eyes without SkQ1 and increased with SkQ1 *(Table 1)*. Another typical trait of glaucoma, increase in lens thickness, disappeared after SkQ1 treatment *(Table 1)*. As to Bekker's coefficient, it was 110±27 in control eyes and 305±66 in glaucoma eyes. This huge effect was completely abolished by SkQ (96±19). A similar tendency seemed to be inherent in changes in the anterior chamber depth and anterior posterior axis, but the effect of HPMC was too small to be statistically valid. To assess the potential effect of SkQ1 on the posterior segments, glaucomatous eyes were analyzed histologically. In rabbit eyes treated with SkQ1, the area of the optic nerve was well expressed; the Elschnig's membrane had the normal thickness with well-defined structure in the septum of the prelaminar area. Significantly greater preservation of axons in the prelaminar area and fewer vacuoles compared with the SkQ1-nontreated control were noted (not shown).

## Discussion

Elevated IOP is the important risk factor of glaucoma development and degeneration of the optic nerve [2]. Here we describe a suitable experimental rabbit model of glaucoma for pharmacological screening of potential neuroprotective agents. We demonstrated that a series of 10 injections of HPMC into the anterior chamber of rabbit eyes during 5 weeks leads to a moderate elevation of IOP, which remains increased for at least next 5 months. In our model, instillations of eye drops containing the mitochondria-targeted antioxidant SkQ1 reversed the increase in IOP as well as some other symptoms of glaucoma.

Like most neurodegenerative diseases, the mechanisms underlying glaucoma are difficult to study in humans. Clinically, most cases of glaucoma in humans are characterized by chronic elevated IOP, structural changes in the optic disc, and a progressive loss of

vision [2]. Thus, considerable effort has been directed toward the development and use of animal models of experimental glaucoma [10]. These animal models have been widely used in various studies that aimed to find IOP-decreasing drugs. However, only a few models of glaucoma are relevant to long-existing and chronic glaucomatous destruction factors. Laser-or HPMC-induced glaucoma with 6 months elevated IOP and optic nerve degeneration can be produced in monkeys, but the cost of pharmacological screening using these animals is very high [10]. Rat and mouse models with up to 30 weeks of elevated IOP, such as the "microbeads occlusion model" or inbred animal strains, may be useful [10]. However, rabbits, rather than mice and rats, are more appropriate animals for this purpose. Indeed, rabbits are now a common animal used by pharmaceutical companies to test such drugs [14].

Polymeric substances, viscoelastics, were originally introduced as protective agents to prevent endothelial cell loss during intraocular lens implantation. Barron et al. showed that viscoelastics can significantly increase postoperative IOP in patients [30]. Liesegang et al. found that the maximum IOP of 70 patients who were given 2% HPMC during extracapsular cataract extraction increased by up to 30 mm Hg [31]. It has been demonstrated that viscous solutions mechanically restrict outflow of aqueous humor, causing viscosity-dependent ocular hypertension [30]. These properties of viscoelastics were used for creation of an experimental glaucoma model in rabbits [32]. It was demonstrated what even a single injection of viscoelastics into anterior chamber of animals led to IOP elevation [30, 32]. After injection of some viscoelastics, maximum IOP (up to 50–60 mm Hg) increase was observed in the first few hours, after which it gradually decreased to the pre-injection level during the next 2–15 days [32].

In our study, we described a rabbit glaucoma model that is based on 10 injections of 2% HPMC during five weeks. It was shown that this procedure leads to a long-lasting IOP elevation of up to 20–25 mm Hg. Characteristic changes occurred in the optic nerve, including destruction of the optic nerve axons and appearance of numerous vacuoles. The Elschnig's membrane of the optic disc become dramatically thinner, up to complete disappearance of this membrane.

While the exact mechanism of the glaucoma pathogenesis remains unknown, numerous studies suggest it is somehow linked to mitochondrial dysfunction, oxidative stress, and a mitochondria-mediated apoptotic cell death [15–17]. Recent evidence shows that elevated IOP may have a direct impact on mitochondria. Studies in mice subjected to ocular hypertension have shown fission of long mitochondrial profiles and depletion of cristae [33]. In addition, increase in IOP correlated with changes OPA1 expression and induction of OPA1 release from mitochondria. These findings further substantiate the possible role of mitochondrial dysfunction in pressure-related glaucomatous optic nerve degeneration.

We addressed the question of whether mitochondrial oxidative stress is involved in development of glaucoma. To this end, mitochondria-targeted antioxidant SkQ1 was used. In contrast to antioxidants traditionally used to treat glaucoma, SkQ1 specifically accumulates in mitochondria [16]. As shown by our and some other groups, the antioxidant properties of SkQ1 are due to its direct ROS scavenging activity [23, 24, 26, 34]. SkQ1 prevents oxidative damage to mitochondria and exhibits neuroprotective properties in retinal cells under condition of oxidative stress [23–26, 28].

In the present work, we demonstrated that long-term instillations of SkQ1 showed complete reversal of such glaucomatous symptoms as increased IOP, decreased humor outflow and humor production, elevation of lens thickness, and morphological changes of RGS. Perhaps, the favorable influence of the SkQ1 was associated with improved blood supply to the area damaged by elevated intraocular pressure. Moreover, SkQ1 may improve the situation directly in RGS. As shown by Patten et al. [34], SkQ1 interrupts a signal transmission chain required to activate HIF-1 by mitochondrial ROS. It was already mentioned in the Introduction that HIF-1α activation is a symptom of a glaucomatous state in RGS [4]. Another effect of SkQ1 on neurons is related to amyloid-β. It is known that amyloid-β is strongly increased in those RGS of glaucomatous animals that are dying by means of apoptosis. Targeting the amyloid-β formation and aggregation pathway can effectively reduce glaucoma-induced apoptosis of RGC [35, 36]. It was found in our group that in vivo SkQ1 treatment lowers concentration of amyloid-β in the brain cortex and hippocampus of OXYS rat suffering from permanent oxidative stress [37]. We also found that SkQ1 prevents a toxic effect (inhibition of long-term potentiation) of amyloid-β added to hippocampus slices [38].

Results of our preliminary pilot experiments on HPMC-treated rabbits were shortly reviewed in [25, 28].

## Conclusions and Outlook

Our results indicate that mitochondria-targeted antioxidant SkQ1 shows a strong therapeutic effect on the rabbit model of chronic, moderately elevated intraocular pressure, suggesting that the mitochondrial oxidative stress plays an important role in the glaucomatous RGS damage, so the SkQ1-type antioxidants may be a promising drug candidate for the treatment of glaucoma. Clinical trials for the SkQ1 treatment of glaucoma are currently in progress in two of the Moscow hospitals.

***Abbreviations:*** intraocular pressure (IOP); retinal ganglion cells (RGC); 2-hydroxypropyl methylcellulose (HPMC); 10-(6'-plastoquinonyl) decyltriphenylphosphonium (SkQ1); reactive oxygen species (ROS)

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