CoQ10-Containing Eye Drops Prevent UVB-Induced Cornea Cell Damage and Increase Cornea Wound Healing by Preserving Mitochondrial Function

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Submitted: July 23, 2014
Accepted: September 24, 2014

Citation: Mencucci R, Favuzza E, Boccalini C, et al. CoQ10-containing eye drops prevent UVB-induced cornea cell damage and increase cornea wound healing by preserving mitochondrial function. *Invest Ophthalmol Vis Sci*. 2014;55:7266–7271. DOI:10.1167/iovs.14-15306

Purpose. We evaluated the potential protective effects of Coenzyme Q10 (CoQ10) on human corneal cells and rabbit eyes after ultraviolet B (UVB) exposure and a model of wound healing in rabbit eyes after corneal epithelium removal.

Methods. Human corneal epithelium cells (HCE) were exposed to a source of UVB radiation (312 nM) in the presence of different CoQ10 concentrations or vehicle. The mitochondrial function and cell survival were evaluated by means of 3-(4,5-dimethylthiazole-2-yl)2,5-diphenyl-tetrazolium (MTT) reduction and lactic dehydrogenase (LDH) release. Furthermore, quantitation of oxygen consumption and mitochondrial membrane potential were conducted. In vivo rabbit models were adopted to evaluate the effect of CoQ10 on UVB-induced conjunctival vessel hyperemia and corneal recovery after ethanol induced corneal lesion.

Results. In UVB-exposed HCE cells, CoQ10 addition led to an increased survival rate and mitochondrial function. Furthermore, oxygen consumption was maintained at control levels and adenosine triphosphate (ATP) decline was completely prevented in the CoQ10-treated cells. Interestingly, in an in vivo model, CoQ10 was able dose-dependently to reduce UVB-induced vessel hyperemia. Finally, in a model of corneal epithelium removal, 12 hours from surgery, animals treated with CoQ10 showed a reduction of damaged area in respect to vehicle controls, which lasted until 48 hours.

Conclusions. We demonstrated that CoQ10 reduces corneal damages after UVB exposure in vivo and in vitro by preserving mitochondrial function. Also, for the first time to our knowledge we showed that the administration of CoQ10 after corneal epithelium removal promotes corneal wound healing.

Keywords: CoQ10, corneal wound healing, mitochondrial function

Corneal wound healing is a complex process that involves cells, matrix, and secreted factors to repair the function of the damaged tissue. Renewal of the corneal epithelium, damaged by chemical, physical, or biological insults, is the result of a coordinated process consisting of migration, proliferation, and differentiation of epithelial cells, and involving several growth factors.[1]

Several studies have shown that some therapeutics may modulate and help the corneal wound healing after epithelial damage. Recently, the therapeutic application of several new drugs (like b-glucan, nicergoline, and 17b-estradiol) were demonstrated in laboratory experiments as having positive effects on the wound healing of the cornea.[2]

Adding as another candidate of corneal wound healing drug, coenzyme Q10 (Ubiquinone Q10, CoQ10), a vitamin-like benzoquinone compound, has been evaluated in recent years.[3,4] Coenzyme Q10 is an organic molecule composed of a hydrophobic tail and a redox active quinone ring. It is present in biological membranes, particularly in mitochondria where it serves as an electron transporter in complexes I (NADH–ubiquinone oxidoreductase), II (succinate–ubiquinone oxidoreductase), and III (ubiquinone–cytochrome oxidoreductase).[5] In recent years, the role of CoQ10 in mitochondrial bioenergetics has been extended to regulate mitochondrial apoptosis through modulation of the permeability transition pore (PTP), a mitochondrial inner membrane conductance channel, and, thus, acting as a potential mitochondrial inhibitor of apoptotic signal transduction. Besides, the reduced form of CoQ10 is an effective antioxidant and it can act as a ubiquitous free radical scavenger to protect against oxidative damages to the mitochondrial and lipid membranes.[6]

It has been postulated an essential role of CoQ10 in mitochondrial bioenergetics, regulating the mitochondrial apoptosis and functioning as an ubiquitous free radical scavenger and increasing respiratory rate.[7,8] The CoQ10 has been reported to modulate the PTP, a mitochondrial inner membrane conductance channel, being a potential mitochondrial inhibitor of apoptotic signal transduction.[9,10]

Numerous studies reported the efficacy of CoQ10 implementation in the alleviation of mitochondrial dysfunctions,

such as myopathies, metabolic diseases, aging, and cardiovascular and neurodegenerative diseases.[8,11,12] In ophthalmology, the use of CoQ10 after iatrogenic damage has been supported by its protecting role against harmful free radicals after refractive surgery.[13,14] Brancato et al.[3,4] showed both in vitro and in vivo that CoQ10 inhibited corneal keratocyte apoptosis induced by excimer laser in a way better than other antioxidants. Chen et al.[15] showed that CoQ10 pretreatment reduced cell apoptosis in corneas exposed to ethanol. However, the underlying mechanism of CoQ10 during wound healing process remains to be determined.

In light of the well appreciated protective effects of CoQ10 on different pathological conditions and considering the possible boosting effect of this molecule on mitochondrial bioenergetics, the aim of our study was to evaluate the potential healing efficacy of CoQ10 ophthalmic solution on UVB-exposed human corneal epithelial cells in vitro, and on UVB- and ethanol-exposed rabbit eyes in vivo through its effects on mitochondrial function.

Materials and Methods

CoQ10-Containig Eye Drops

The sterile ophthalmic solution was prepared freshly for every experiments to avoid use of any preservative. The CoQ10 was used at the different concentrations, as indicated in every experiment, ranging from 0.1% to 1%. Where not differently stated, the CoQ10 was 0.1%. The solution contained 0.5% of vitamin E, 0.5% of D-α-tocopheryl polyethylene glycol succinate (TPGS) and 0.2% of Hipromelose.

Cell Culture

Human corneal epithelium cells (HCE) were cultured under standard conditions. Briefly, the cells were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO_2 to 95% air and passaged at a 1:3 ratio with trypsin every 5 to 7 days. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 10% fetal bovine serum, and antibiotics. Cultures were brought to 50% to 70% confluence and used for the experiments. In indicated experiments, cells were exposed to 312 nM ultraviolet B (UVB) radiation (30 seconds, 120 Joules, Bio-link BLX; Vilber Lourmat, Cedex, France). Where indicated, the cells were treated with CoQ10 ophthalmic solution or Hipromelose (vehicle) alone. When not differently stated, measurements of cell viability or mitochondrial functions were performed 24 hours after the insult.

Oxygen Consumption Analysis

Quantitation of oxygen consumption was conducted by means of the Oxygraph system (Hansatech Instruments, Norfolk, UK). Cells (250,000) were loaded in the chamber containing 400 μL of respiration buffer (70 mM sucrose, 220 mM mannitol, 2 mM HEPES, pH 7.4, 5 mM MgCl2, 5 mM K2HPO4, 1 mM EDTA, and 0.1% BSA), and oxygen consumption was monitored for 10 minutes at 37°C.

Evaluation of Mitochondrial Membrane Potential

Mitochondrial membrane potential was evaluated by means of flow cytometry.[16] Briefly, cells were washed with PBS, incubated with trypsin (50 μL/0.25%/2 minutes) and then diluted with 350 μL complete DMEM. After gentle pipetting, 200 μL of the cell suspension were further diluted with 400 μL of PBS and analyzed by the flow cytometer Coulter EPICS XL (Beckman Coulter, Inc., Pasadena, CA, USA) equipped with the EXPO32 Flow Cytometry ADC software (Beckman Coulter, Inc.). Tetramethylrhodamine ethyl ester (TMRE) 2.5 nM was present in all the solutions used for cell preparation and measurement.

Cell Viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay and phase-contrast microscopy.

ATP Measurement

The cellular adenosine triphosphate (ATP) contents were measured by means of an ATPlite kit from PerkinElmer Life and Analytical Sciences (Zaventem, Belgium) according to the manufacture's instruction.

LDH Measurement

Cell damage in HCE cells was quantitatively evaluated by measuring the amount of the soluble cytosolic enzyme lactic dehydrogenase (LDH) released from injured cells into the extracellular fluid at indicated time points after UVB irradiation.

All experiments were carried out in triplicate. The activity of LDH was quantified using a Cytotoxicity Detection Kit (LDH) from Roche Diagnostics (Basel, Switzerland).

UVB Exposure

The UVB source was a Vilber Lourmat Lamp with a wavelength set at 280 to 350 nm, centered at 312 nm and with an intensity of 2.2 mW/cm^2. After exposure for 30 seconds the total UVB irradiation to the eye was 120 mJ/cm^2. Five animals were used for every treatment group.

Vessel Hyperemia

Five New Zealand male rabbits (12 weeks; Harlan, Milan, Italy) were used for every treatment group. Rabbit eyes were treated with two different treatment paradigms. As for the "during and after UVB-exposure" treatment, CoQ10 ophthalmic solution was instilled in rabbit eye (2 drops per eye) immediately before UVB-exposure and for the following 3 days every 8 hours starting 1 hour after exposure. As for "after UVB-exposure" treatment paradigm, CoQ10 ophthalmic solution was administered 1 hour after the exposure and for the following three days every 8 hours (2 drops per eye). All procedures of UVB exposure were performed on the right eyes of experimental animals. Vessel reactivity as reflected by the hyperemia of iris, sclera, and limbal vessels, was assessed in each eye at 24, 48, and 72 hours after UVB irradiation. Parameters were measured by two independent operators as previously described.[17] The score was expressed as: 1, absent; 2, very mild; 3, mild; 4, medium; 5, strong.

Removal of the Corneal Epithelium

All animal manipulations were performed according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and approved by the Committee for Animal Care and Experimental Use of the University of Florence. All procedures were performed with animals under general anesthesia, induced by 0.2 mg/kg Zoletil intramuscularly (250 mg Tiletamine and 250 mg Zolazepam; Vibrac, Carros, France). A wire lid speculum

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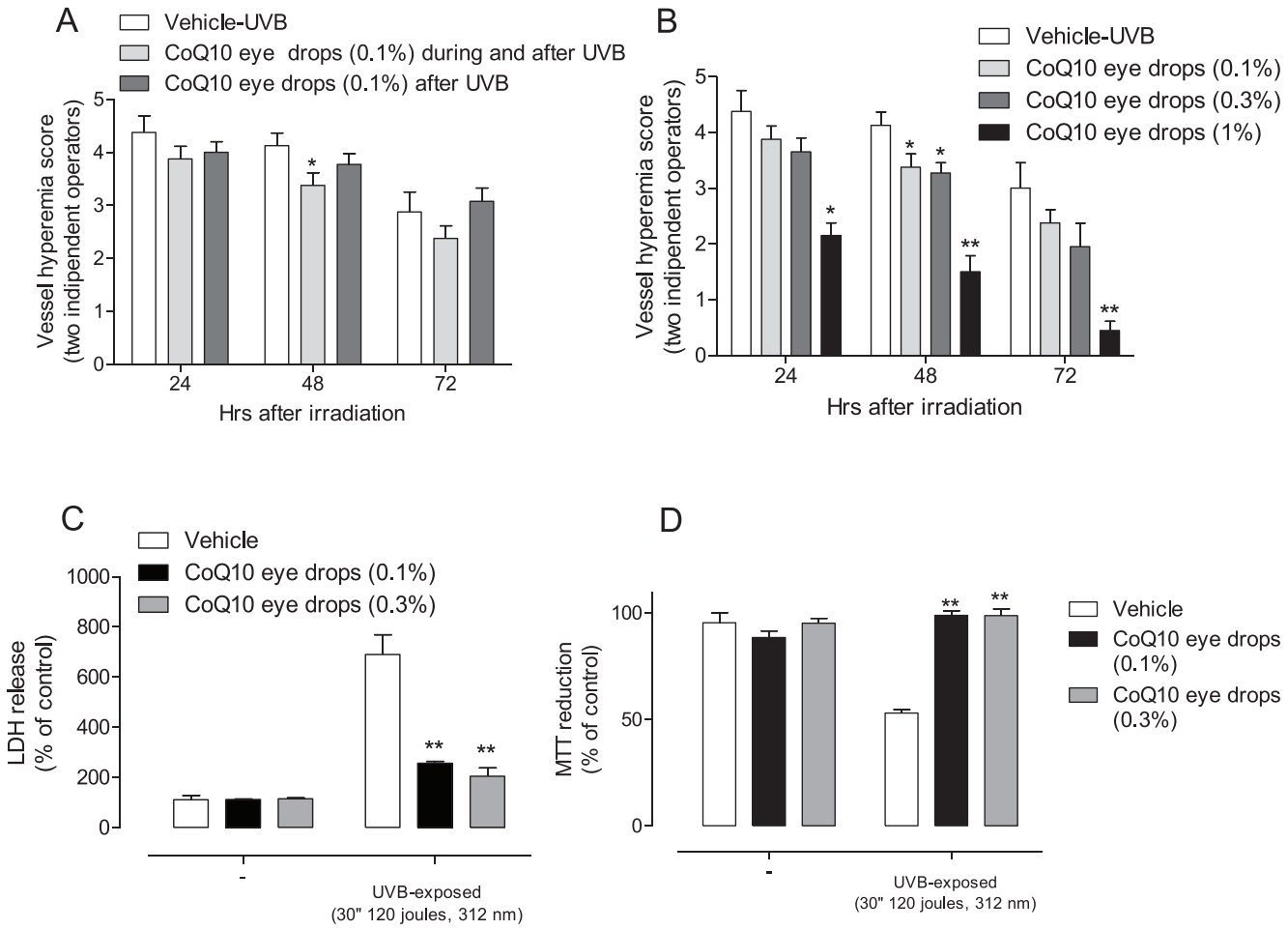

Figure 1. The CoQ10 ophthalmic solution reduces damages caused by UVB exposure in vivo and in vitro. Five rabbit eyes were exposed to UVB radiation and treated with vehicle alone (during and after exposure), or CoQ10 eye drops during and after UVB exposure or only after the insult. Vessel hyperemia was evaluated by blind operators at the indicated time after exposure (**A**). The protective effect of different CoQ10 concentration on UVB-induced vessel hyperemia was evaluated (**B**). The HCE cells were exposed to UVB (312 nM) in presence of vehicle or eye drops containing different CoQ10 concentrations. Cell survival and mitochondrial function were evaluated 24 hours after the insult by means of LDH release (**C**) and MTT reduction (**D**), respectively. *Error bars*: represent the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, ANOVA plus Tukey's post hoc test.

was used to separate the eyelids after applying 0.5% proparacaine hydrochloride (Alcaine; Alcon, Fort Worth, TX, USA). Five New Zealand male rabbits (12 weeks; Harlan) were used for every treatment group. According to the groups, an 8.0-mm Trephine blade (Katena, Denville, NJ, USA) was placed and fixed firmly on the corneal surface with gentle pressure. Few drops of absolute ethanol were put into the well and left in place for 30 seconds, then absorbed with a dry cellulose sponge and the eye was irrigated thoroughly. The epithelium was peeled using a Crescent blade along the boundary. All procedures of epithelial removal were performed on the right eyes of experimental animals. According to the treatment group, CoQ10 ophthalmic solution or vehicle alone was instilled in rabbit eye immediately after surgery and for the following three days every 8 hours (2 drops per eye).

Results

CoQ10-Containing Ophthalmic Solution Reduces UVB Exposure Damage Both In Vivo and In Vitro

First, we evaluated the protective effect of CoQ10 ophthalmic solution adopting different treatment schedules. Five rabbit eyes were treated with the eyes drop (CoQ10 0.1%) during and after UVB exposure or only after the insult. Interestingly, we found that the vessel hyperemia was significantly decreased after 48 hours from exposure with the treatment during and after UVB irradiation (Fig. 1A) compared to those with vehicle alone (during and after exposure). Therefore, we tested the effects of different CoQ10 concentrations on UVB-induced hyperemia. Treatments with higher concentrations of CoQ10 (0.3% and 1%) led to dose-dependent protective effect, which already was detectable after 24 hours from UVB exposure and lasted even after 72 hours (Fig. 1B). To confirm this in vivo finding, we performed a similar experiment by exposing HCE cells to UVB radiation. Survival rate (Fig. 1B) and mitochondrial function (Fig. 1C) were evaluated by means of LDH release and MTT reduction potential, respectively. As shown in Figures 1B and 1C, we observed an increase in LDH release and a decrease in MTT reduction in HCE cells exposed to UVB radiation. Again, treating cells during UVB exposure with CoQ10 ophthalmic solution led to a significant decrease in LDH release (Fig. 1C) and an increase in MTT reduction that reached a maximum when CoQ10 ophthalmic solution was supplemented with 0.3% of CoQ10 (Fig. 1D).

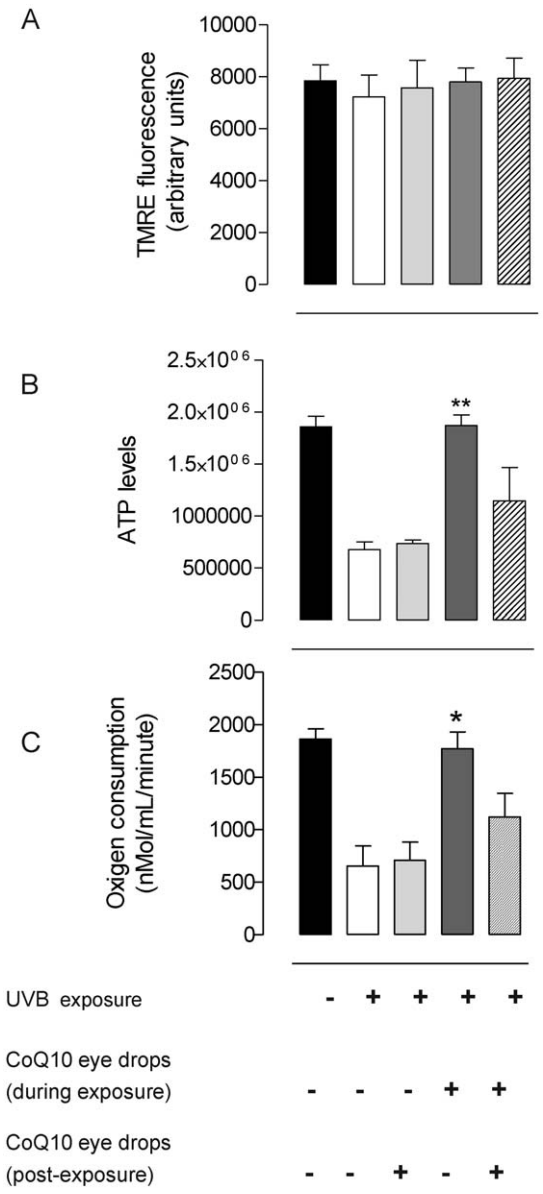

FIGURE 2. The CoQ10 ophthalmic solution preserves mitochondrial efficiency during UVB exposure. In HCE cells exposed or not to UVB radiation and treated as indicated in the graphs mitochondrial function was assessed (24 hours after the insult) by means of TMRE fluorescence (**A**), ATP production (**B**), and oxygen consumption (**C**). *Error bars*: represent the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, ANOVA plus Tukey's post hoc test.

CoQ10-Containig Eye Drops Preserve Mitochondrial Efficiency During UVB Exposure

We next wondered whether the protective effect of CoQ10 ophthalmic solution was due to its direct effect on mitochondrial functions. To this aim, HCE cells were treated during and/or after UVB irradiation with CoQ10 ophthalmic solution or vehicle alone, followed by analysis of mitochondrial bioenergetics. Despite the fact that UVB exposure did not affect mitochondrial membrane potential (Fig. 2A), we observed a significant decrease in cellular oxygen consumption and ATP production (Figs. 2B, 2C) after irradiation. Notably, ATP decline was completely prevented when cells were treated during UVB exposure (Fig. 2B). Accordingly, oxygen consumption was maintained at control levels in treated cells (Fig. 2C). However, treatment lost its efficacy when it was administered after UVB exposure (Figs. 2B, 2C). Again, when cells were incubated with the CoQ10-containing eye drops during and after UVB irradiation, the treatment showed only a slight increasing tendency (Figs. 2B, 2C).

CoQ10-Containing Ophthalmic Solution Increases Wound Healing After Corneal Epithelium Removal

The effect of CoQ10 ophthalmic solution was finally evaluated in a model of rabbit corneal wound healing. Rabbit eyes (five per group) were treated with vehicle alone or with CoQ10 ophthalmic solution. Immediately after surgery, no differences were appreciable between vehicle or treated animals (Fig. 3A). Interestingly, already after 12 hours from surgery animals treated with CoQ10-containing ophthalmic solution showed a marked reduction in damaged area compared to vehicle-treated animals and this significant difference lasted until 48 hours after corneal epithelium removal (Figs. 3A, 3B).

DISCUSSION

Numerous studies reported beneficial effects of CoQ10 implementation in pathological conditions related to mitochondrial dysfunction with a subsequent intensive generation of free radicals. The CoQ10 acts like an antioxidant and free radical scavengers after iatrogenic damage and has a protecting role versus the harmful effects of free radicals. Moreover, in ophthalmology, several studies have been published on its possible capital role in corneal wound healing and reduction of keratocyte apoptosis after refractive surgery. To our knowledge, this is the first study trying to unveil the mechanism of CoQ10 efficacy during corneal wound healing process.

In the present work we evaluated the potential protective effects of CoQ10 ophthalmic solution on different epithelial stressing conditions. First, we studied the protective effect of CoQ10 ophthalmic solution on UVB-induced cell damage, adopting an in vivo and an in vitro approach. Interestingly, we found that when rabbit eyes were exposed to UVB radiation in the presence of the CoQ10 ophthalmic solution, the resulting damage was reduced. To confirm that the observed effect was really due to the properties of CoQ10, we tested in the same model, ophthalmic solutions containing different CoQ10 concentrations and found that higher coenzyme concentration led to a better outcome after UVB irradiation. Intriguingly, the same results were obtained in vitro, using cultured HCE cells. Even in this case the presence of CoQ10 ophthalmic solution during the exposure to UVB radiation led to a better survival rate, evaluated by release ratio of lactate dehydrogenase and a better mitochondrial function evaluated as the capacity of these organelles to reduce the substrate MTT. To better clarify the possible molecular mechanism underlying the observed protective effects, we assessed whether different metabolic parameters were affected in cells exposed to UVB radiation and treated or not with the CoQ10 ophthalmic solution. First, we evaluated the mitochondrial membrane potential as this parameter usually is decreased when the functioning rate of the organelle is reduced, and we found that the mitochondrial potential was unaffected either by UVB exposure or CoQ10 treatment. Interestingly, we also observed a decrease in mitochondrial ATP production and in oxygen consumption in cells exposed to UVB radiation. A possible explanation for these apparently conflicting results is that mitochondrial membrane potential is due to a proton gradient, resulting from proton extrusion by respiratory complexes and proton entrance through the ATP synthase. Accordingly, a reduction of respiratory rate (leading to reduced oxygen consumption) is

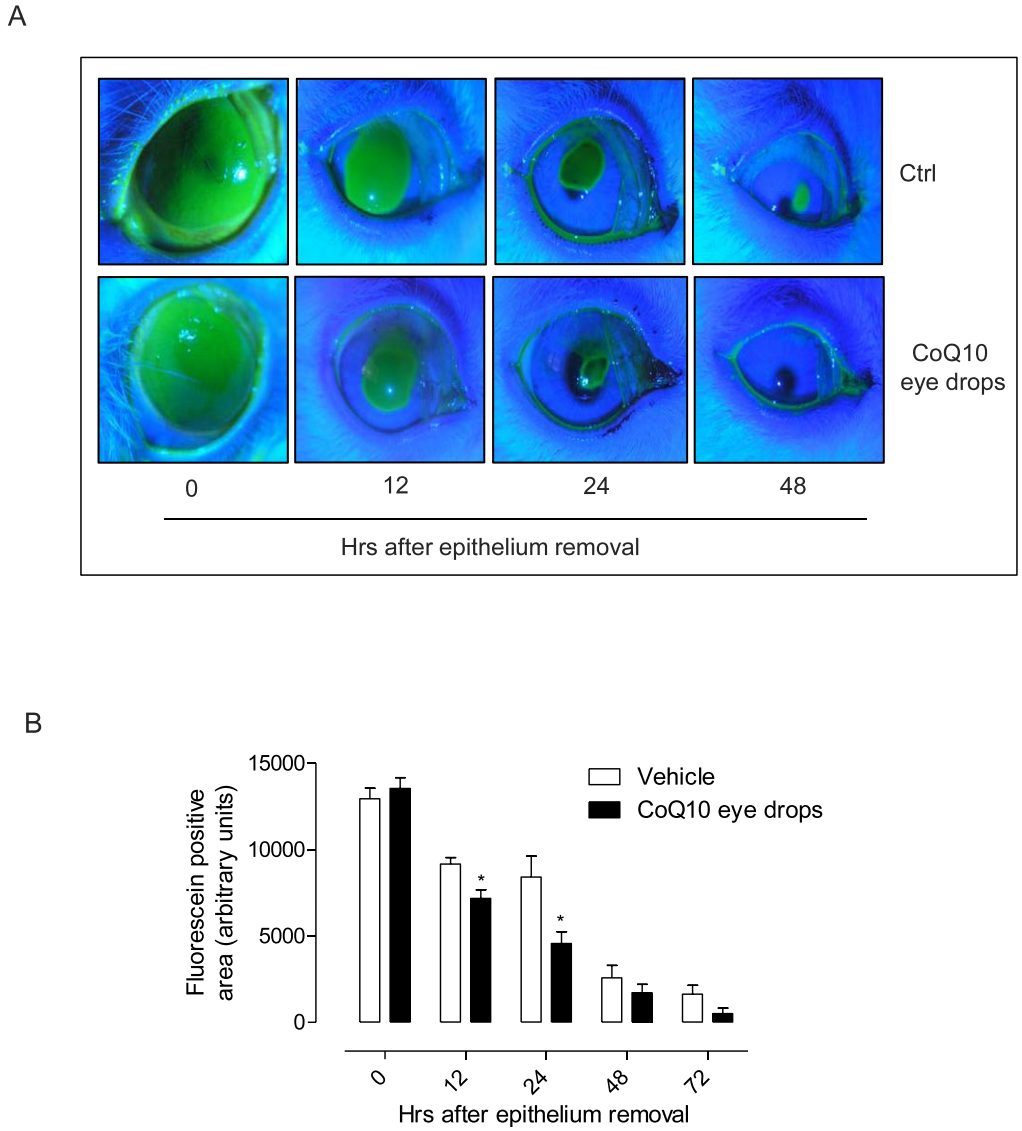

FIGURE 3. The CoQ10 ophthalmic solution increases wound healing after corneal epithelium removal. After mechanical removal of corneal epithelium, eyes of five rabbits were treated with vehicle alone or with CoQ10 ophthalmic solution (3 instillations per day, two drops per eye). Wound healing was evaluated by means of fluorescein staining at different time points (**A**). Fluorescein-positive areas were calculated by means of analytical software (**B**). Images show the results out of three independent experiments (**A**). (**B**) *Error bars*: represent the mean ± SEM of three independent experiments. $^{*}P < 0.05$, ANOVA plus Tukey's post hoc test.

not invariantly related to decreased mitochondrial membrane potential in case reduction of electron flux occurs concomitantly to reduce ATP synthase activity.

Nevertheless these reductions were prevented when CoQ10 ophthalmic solution was added to the culture medium during exposure. We also found that treatment after UVB exposure did not restore the normal ATP production and respiratory functions. In apparent contrast with the protective effect showed by treating cells during exposure, we observed a reduction in the efficacy of the treatment when it was prolonged even after the end of irradiation. This could be due to the procedure used in this experimental setting since to treat the cells, the culture medium was replaced with ophthalmic solution. The lack of medium for prolonged time could be responsible for the effect observed in Figures 2B and 2C.

Finally, since we described an increase in mitochondrial functions in the CoQ10-treated cells, we wondered whether CoQ10-containing ophthalmic solution would be able to improve wound healing in a model of refractive surgery, a process in which higher mitochondrial activity has been reported to be beneficial.[3,4] In line with the finding in HCE cells, we found that CoQ10 ophthalmic solution treatment led to a quicker recovery from epithelium removal under in vivo conditions.

Recently, it has been reported the role of CoQ10 in regulation of mitochondrial induced apoptosis. In particular, CoQ10 acts as free radical scavenger, therefore, controlling the mitochondrial transition pore opening.[7] Furthermore, other mechanisms have been proposed as crucial for beneficial effects mediated by CoQ10 in a variety of conditions. It has been postulated that CoQ10 could be part of the mitochondrial PTP (mPTP) complex functioning as an inhibitor of its opening.[18] Thereby, CoQ10 could prevent apoptosis independently from its role as a potent free radical scavenger.

Nevertheless, since the treatment with CoQ10 ophthalmic solution loses its efficacy when administered after the insult we hypothesized that CoQ10-dependent protection might be

ascribed to its well-known ability to scavenge reactive radicals.[3]

In conclusion, CoQ10 can influence epithelial wound healing by several different mechanisms. CoQ10 can improve the viability of corneal epithelial cells culture and the mitochondrial bioenergetics, enhancing healing rates when administered after corneal lesion.

Acknowledgments

Supported by grants from Regione Toscana Health Projects 2012, and Ente Cassa di Risparmio di Firenze.

Disclosure: **R. Mencucci**, None; **E. Favuzza**, None; **C. Boccalini**, None; **A. Lapucci**, None; **R. Felici**, None; **F. Resta**, None; **A. Chiarugi**, None; **L. Cavone**, None

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