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Prevention of corneal keratocyte apoptosis after argon fluoride excimer laser irradiation with the free radical scavenger ubiquinone Q10

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PURPOSE. *To assess* in vitro *the potential of the free radical scavenger ubiquinone Q10 in preventing keratocyte apoptosis after argon fluoride (ArF) excimer laser irradiation.*
METHODS. *Cultured rabbit keratocytes were irradiated at very low single-pulse laser fluences. The cumulative effects generated by three total fluence doses between 12 and 45 mJ/cm², representative of single-pulse subablative doses during photorefractive keratectomy (PRK) in humans, were evaluated. We employed the following parameters to compare pretreated (10 µM ubiquinone Q10) and untreated samples: 1) number and morphology of living cells by Trypan blue test and ultramicroscopy, respectively; 2) level of free-radical formation assessed by malonaldehyde quantitation; 3) cellular energy level evaluated by ATP assay.*
RESULTS. *Excimer laser irradiation kills cultured keratocytes by inducing apoptosis. The effect increases with the cumulative fluence dose. In the samples pretreated with ubiquinone Q10 there were significantly fewer cumulative apoptotic events than in the untreated ones. Quantitative analysis of malonaldehyde cellular levels suggested this protective action of ubiquinone Q10 was connected with its ability to scavenge laser-generated free radicals. ATP assay also confirmed that it raised cellular energy levels.*
CONCLUSIONS. *The treatment of corneal keratocytes with relatively low concentrations of ubiquinone Q10 can prevent apoptosis after ArF excimer laser irradiation. If these findings are confirmed on human keratocytes this treatment could be usefully exploited in the PRK surgical procedure. That might lead to a reduction in the occurrence of haze and curvature regression triggered by programmed cell death. (Eur J Ophthalmol 2000; 10: 32-8)*

KEY WORDS: *Corneal keratocyte apoptosis, Argon fluoride excimer laser, Ubiquinone Q10, PRK, LASIK*

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INTRODUCTION

Excimer laser photorefractive keratectomy (PRK) is a safe, effective, and predictable surgical treatment for myopia (1, 2), astigmatism (3) and hyperopia (4). Only a few complications have been reported so far, mainly related to high curvature corrections. The main undesired effect is the formation of a subepithelial scar which can cause significant light scattering; it can be clinically recognized as a stromal haze (5). This

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PRK follow-up effect is the consequence of the wound healing response that involves keratocytes and epithelial cells, and leads eventually to the formation of new material – collagen, proteoglycans, hyaluronic acid, etc. – which may cause visual impairment and regression of the curvature correction.

The dynamics of the healing response involves various factors, intraoperative and postoperative, which can make it more aggressive. For example, the risk increases with higher correction, i.e. when a larger amount of tissue is removed by laser ablation (1), with the roughness and irregularity of the exposed surface (6), and in cases of extensive postoperative ultraviolet exposure.

In the last few years, several pathogenic mechanisms have been postulated to explain haze formation (6, 7). Wilson et al (8-10) indicated keratocyte apoptosis as one of the principal events leading to wound healing and tissue reaction after PRK, thus resulting in haze formation and regression of the refractive correction. In this case, the generation of free radicals, particularly reactive oxygen species (ROS) (11) has been indicated as the cause of apoptosis (12). Experimental investigations have already shown that the occurrence of haze in rabbits after PRK (13) can be reduced by employing dimethylsulfoxide and superoxide dismutase, the first being a specific scavenger for (·OH) and the second for ($\cdot O^{2-}$) ROS.

To control excessive wound healing pharmacologically after corneal laser surgery, several drugs have been proposed and tested in laboratory investigations and clinical trials on humans; these include corticosteroids (14,15) and antimetabolites (16). Both these drug categories have been employed to modulate cellular activity and proliferation during wound healing, on the assumption that keratocytes and epithelial cells were already triggered for wound healing (10). The results of these studies have been controversial, so there is still no clear evidence that these drugs really control the postoperative tissue reaction.

The present study set out to investigate the possible protective role of the coenzyme ubiquinone Q10 against keratocyte apoptosis after ArF excimer laser irradiation. We are currently testing ubiquinone Q10 as a potential inhibitor of haze as it is a well-known free-radical scavenger which also plays a key role in the mitochondrial respiratory chain (17-20).

MATERIALS AND METHODS

Cell culture. A rabbit corneal keratocyte line (RCE cells) was cultured in glass coverslips and used for the *in vitro* experiments. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/ HAM'S nutrient mixture F12 1/1, supplemented with 15% fetal calf serum, 2 mM glutamine, 5 µg/ml insulin, 10 ng/ml EGF and 50 IU/ml penicillin, in a humidified atmosphere of 5% CO_2 at 37°C. Living cells were counted by the Trypan blue exclusion method.

Cell treatments. Semiconfluent RCE cell cultures were pretreated for 2 hours with 10 µM ubiquinone Q10 dissolved in 0.04% Lutrol F107, used as vehicle. Cells pretreated with vehicle alone were employed as control.

After removal of culture medium and washing with sterile PBS, the cell cultures were irradiated with an ArF excimer laser (193 nm). So as to cover the whole culture area we expanded the laser beam up to 10 cm spot diameter. Total fluence doses of 12 mJ/cm^2, 24 mJ/cm^2, and 45 mJ/cm^2 were obtained by applying a given number of pulses of about 0.22 mJ/pulse. Culture medium was then added again and the cells were cultured for a further period in order to assess the effects of treatment as the overall time course or at different end-points.

Counting living cells and apoptotic death events. Living cells were counted by the Trypan blue exclusion method. Cumulative apoptotic events were scored by time-lapse videomicroscopy using a Zeiss inverted phase-contrast microscope equipped with a 10× objective, Panasonic CCD cameras and JVC BR9030 time-lapse video recorders. After cell detachment from the substrate, apoptotic deaths were counted when the cells had shrunk completely and blebbing had started.

Quantification of cellular malonaldehyde. Malonaldehyde levels, as an indication of free-radical formation, were quantified in cellular acid extracts by a spectrophotometric thiobarbituric acid-based method, previously described (21).

Quantification of ATP. After the laser treatments, RCE cells were collected by centrifugation in 15-ml tubes. Pellets of about 2x10^5 cells were resuspended in distilled water, then the tubes were immediately placed in a boiling water bath for 5 minutes. The samples were then cooled at room temperature and

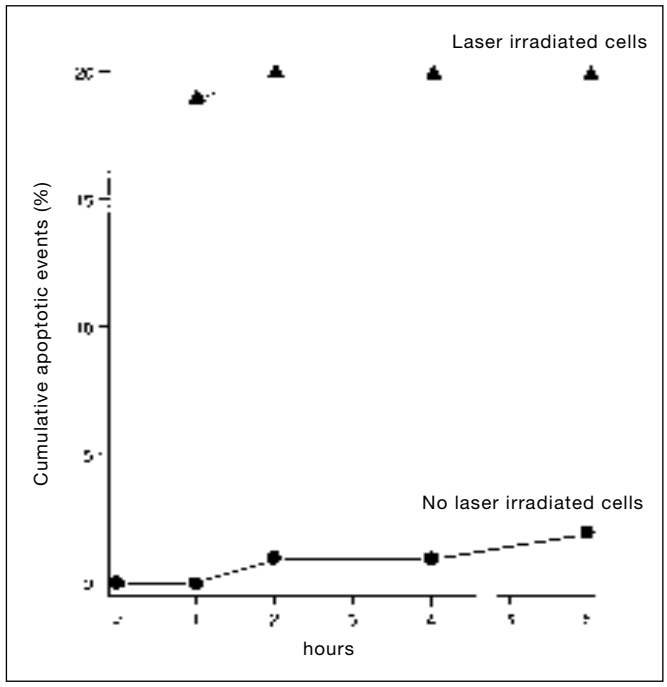


Fig. 1 - *Time-course of cumulative apoptotic death events scored at time-lapse videomicroscopy, in RCE cells irradiated with a fluence of 12 mJ/cm².*

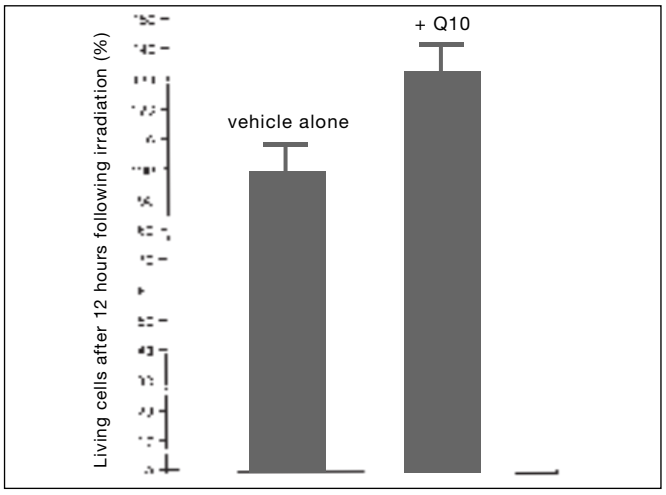


Fig. 2 - *Effect of a 2 hour pretreatment with ubiquinone Q10 (10 μM) on the number of living RCE cells evaluated 12 hours after 24 mJ/cm² excimer laser irradiation. Data are means ± SEM of 3 determinations (p>0.005).*

stored at -20°C for later measurements. ATP in the extracts was quantified by a bioluminescence assay with an ATP determination kit (Molecular Probes, Oregon, USA), using a liquid scintillation analyzers set (Camberra Packard, UK) for bioluminescence analysis, according to the manufacturer's instructions.

RESULTS

The quantification of apoptotic events by time-lapse videomicroscopy showed that laser irradiation at 193 nm was a strong trigger of programmed cell death (Fig. 1). The effect was already clear at a cumulative fluence dose of 12 mJ/cm². The cellular malonaldehyde levels 2 hours after irradiation indicated the photochemical nature of the response, or at least suggested a close correlation between free radical generation by laser irradiation and cell damage. For instance, in samples irradiated with a cumulative fluence dose of 24 mJ/cm², cells produced 12 pg/cell, compared with 0.5 pg/cell of the non-irradiated ones. The malonaldehyde response increased with total fluence dose, whereas the cellular ATP level was reduced as was the number of living cells at the single observation times.

The results using 24 mJ/cm² as cumulative fluence of laser irradiation were as follows. There were changes in the previous after ubiquinone Q10 pretreatment, clearly evidencing its protective action. Thus, the quantification of living cells 12 hours after laser irradiation indicated that ubiquinone Q10 (10 μM) significantly increased their number - by about 35% compared to the controls treated with vehicle alone (Fig. 2). As shown in Figure 3, cellular malonaldehyde levels 2 hours after irradiation in the samples pretreated with ubiquinone Q10 were likewise reduced by a factor of 2.5 compared with the control.

The protective role was also evident in terms of ATP energy levels, since ubiquinone Q10 largely attenuated the loss of energy induced by UV laser irradiation (Fig. 4).

Plates observed by time-lapse videomicroscopy showed significant differences in ubiquinone Q10 treated and untreated samples. Untreated cells were mostly adherent to the substrate (Fig. 5a); irradiated cells were less numerous, detached from the substrate, and shrunken (Fig. 5b).

Ubiquinone Q10 prevented this structural damage induced by the ArF excimer laser (Fig. 5c).

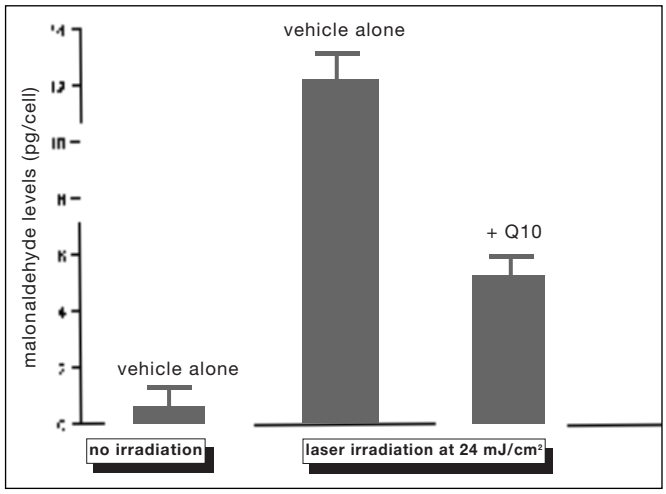


Fig. 3 - *Spectrophotometric quantification of cellular malonaldehyde in basal conditions or after 2 hours pretreatment with vehicle alone (Lutrol) or 10 µM ubiquinone Q10, followed by 24 mJ/cm² irradiation. Data are means ± SEM of 3 determinations (p>0.001).*

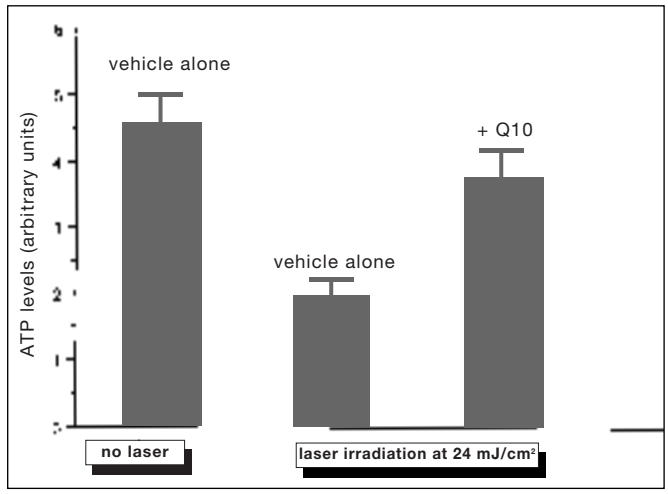
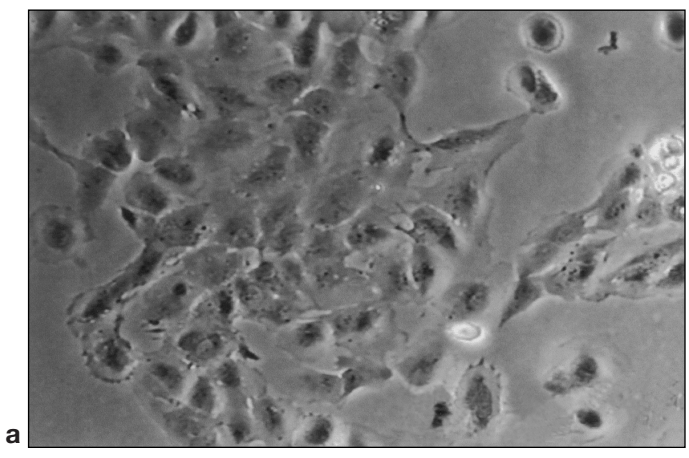
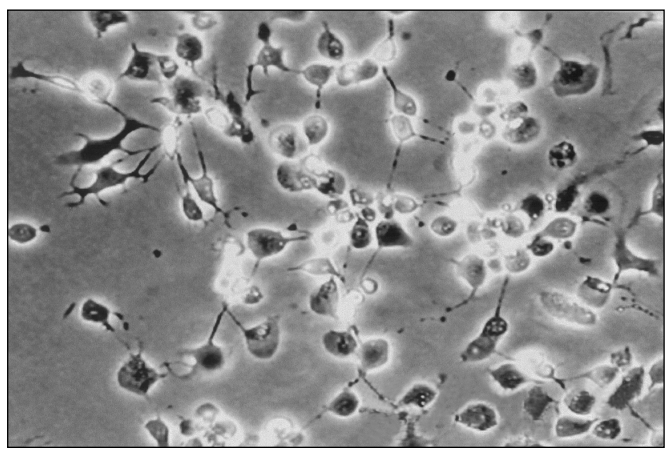


Fig. 4 - *Bioluminescence-based quantification of ATP cellular levels determined in the same conditions as Figure 3. Data are means ± SD of 2 determinations.*





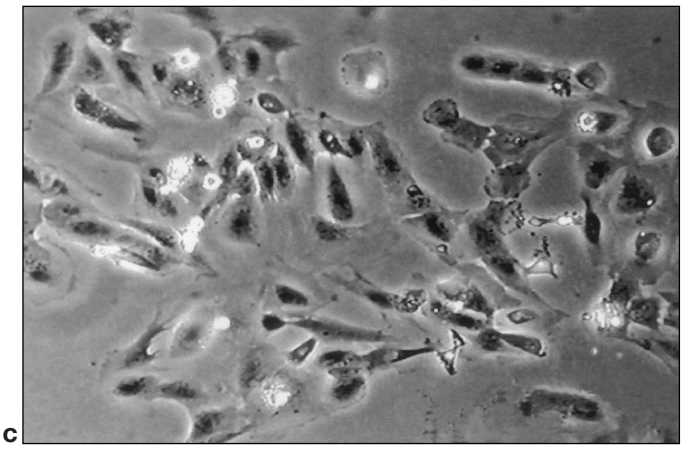


Fig. 5 - *Typical microscopic appearance of plates of RCE cells in basal conditions* ***(a)*** *or irradiated with 24 mJ/cm² cumulative fluence doses of ArF excimer laser radiation after a 2 hour pretreatment with vehicle alone (Lutrol)* ***(b)*** *or 10 µM ubiquinone Q10* ***(c)****. Plates were observed 24 hours after irradiation.*

DISCUSSION

The high photon energy of UV radiation at 193 nm (6.4 eV) can cause the direct breakage of organic molecules by dissociating bonds like C-N and C-C. The generation of hydroxyl radical (·OH) by cleavage of the water molecule has also been demonstrated and

quantified through electron spin resonance spectroscopy (12). Thus ArF excimer laser irradiation can generate many species of free radicals well below the ablation threshold (46 mJ/cm^2) (22, 23), the photodissociation being a single photon effect.

On the other hand, this feature is what mainly underlines the very high optical absorption of the biotissue in the UV spectral region and the ultrashort ablation rate usefully exploited in PRK surgery.

Among the large variety of free radicals that can be generated ROS are the most cytotoxic, besides being able to break down proteoglycans and collagen by direct cleavage of the covalent bonds. We investigated evaluated free radical generation on the basis of their ability to stimulate malonaldehyde production, then their cytotoxicity after ArF excimer laser irradiation of RCE cells, and the possibility of reducing it with ubiquinone Q10.

Time-lapse videomicroscopy clearly indicated that programmed cell death, the last stage of cellular injury, is the main death pathway induced by this irradiation in cultured keratocytes, as shown by a dynamic score.

Compared to cells pretreated with vehicle (Lutrol) alone, ubiquinone Q10 significantly boosted the number of living cells by lowering the number of apoptotic events.

Previously cited literature suggested testing this coenzyme in the present application, and a quick summary is in order to outline the specific mechanism of its protective function. Ubiquinone Q10 plays a key role in the mitochondrial respiratory chain as a component of the inner mitochondrial membrane complexes III and I and, consequently, in the oxidative metabolism chain-associated ATP production. However, Q10 is spread widely within the cell, being also present in endoplasmic reticulum, in plasma membranes and in Golgi apparatus, acting as an antioxidant molecule and free-radical scavenger (19). Ubiquinone Q10 has been reported to be a gatekeeper and modulator of the permeability transition pore (PTP), a mitochondrial inner membrane conductance channel (24, 25). Opening the PTP increases inner mitochondrial membrane permeability, causing the collapse of the proton-motive force, disruption of ionic homeostasis and massive dephosphorylation of ATP, an early sequence of events in the apoptotic pathway (26-30). This leads to extrusion of cytochrome-c from the mitochondrion to cytoplasm and its binding to Apaf-1, which triggers the caspase cascade (27, 31-33). By stopping the opening of the PTP induced by Ca^{2+} overload (34,35), ubiquinone Q10 appears to be a potential mitochondrial inhibitor of apoptotic signal transduction, and presents functional analogies with the antiapoptotic oncogene *bcl-2*.

With respect to our application, the quantification of cellular malonaldehyde suggests that the antiapoptotic effects of ubiquinone Q10 after ArF laser irradiation can be attributed to its inhibiting free radical production. Nevertheless the possibility of these effects being more specifically due to its regulatory function on mitochondrial PTP is not to be excluded.

CONCLUSIONS

The present study shows that *in vitro* ubiquinone Q10 prevented apoptosis of RCE cells after excimer laser irradiation. It appears reasonable – though more research is obviously required – to expect similar results for human keratocytes, in which case this free radical scavenger in the PRK surgical procedure might help reduce the incidence of haze formation and curvature regression.

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REFERENCES

1. Brancato R, Tavola A, Carones F, et al. Excimer laser photorefractive keratectomy for myopia: results in 1165 eyes. Italian Study Group. Refract Corneal Surg 1993; 9: 95-104.
2. Brancato R, Carones F. Indications, results and complications of refractive corneal surgery with lasers. Curr Opin Ophthalmol 1994; 5: 90-7.
3. Carones F, Brancato R, Morico A, Venturi E, Gobbi PG. Compound myopic astigmatism correction using a mask in-the-rail excimer laser delivery system. Eur J Ophthalmol 1996; 6: 221-33.
4. Carones F, Gobbi PG, Vigo L, Brancato R. Photorefractive keratectomy for hyperopia: long-term nonlinear and vector analysis of refractive outcome. Ophthalmology 1999; 106: 1976-83.
5. Krauss JM, Puliafito CA, Steinert R. Laser interactions with the cornea. Surv Ophthalmol 1986; 3: 37-53.
6. Campos M, Raman S, Lee M, McDonnell PJ. Keratocyte loss after different methods of de-epithelialization. Ophthalmology 1994; 101: 890-4.
7. Lohmann CP, Gartry DS, Kerr Muir MG, Timberlake GT, Fitzke FW, Marshall J. Corneal haze after excimer laser refractive surgery: objective measurements and functional implications. Eur J Ophthalmol 1991; 1: 173-80.
8. Wilson SE. Programmed cell death, wound healing, and laser refractive surgical procedures: Molecular-cell biology for corneal surgeron. J Refract Surg 1997; 13: 171-5.
9. Wilson SE, Kim WJ. Keratocyte apoptosis: implications on corneal would healing, tissue organization and disease. Invest Ophthalmol Vis Sci 1998; 39: 220-6.
10. Helena MC, Baerveldt F, Kim WJ, Wilson SE. Keratocyte apoptosis after corneal surgery. Invest Ophthalmol Vis Sci 1998; 39: 276-83.
11. Pettit GH, Ediger MN, Hahn DW, Landry RJ, Weiblinger RP, Morehouse KM. Electron paramagnetic resonance spectroscopy of free radicals in corneal tissue following excimer laser irradiation. Lasers Surg Med 1996; 18: 367-72.
12. Shimmura S, Masumizu T, Nakai Y, et al. Excimer laser-induced hydroxyl radical formation and keratocyte death *in vitro*. Invest Ophthalmol Vis Sci 1999; 40: 1245-9.
13. Jain S, Hahn Tw, McCally R, Azar DT. Antioxidants reduced corneal light scattering after excimer keratectomy in rabbits. Lasers Surg Med 1995; 17: 160-5.
14. Gartry D, Kerr Muir MG, Lohmann CP, Marshall J. The effect of topical corticosteroids on refractive outcome and corneal haze photorefractive keratectomy. A prospective randomized doule-blind trial. Arch Ophthalmol 1992; 110: 944-52.
15. Carones F, Brancato R, Venturi E, Scialdone A, Bertuzzi A, Tavola A. Efficacy of corticosteroids in reversing regression after myopic photorefractive keratectomy. Refract Corneal Surg 1993; 9 (suppl) S52-6.
16. Majmudar PA, Forstot SL, Dennis RF et al. Topical mitomycin-c for subepithelial fibrosis after refractive corneal surgery. Ophthalmology 2000;107: 89-94.
17. Frei B, Kim MC and Bruce NA. Ubiquinol-10 is an effective lipid-soluble antioxidant at physiological concentrations. Proc Natl Acad Sci 1990; 87: 4879-83.
18. Beyer RE. An analysis of the role of coenzyme Q in free radical generation and as antioxidant. Biochem Cell Biol 1992; 70: 390-403.
19. Lenaz G, Fato R, Castelluccio C, et al. The function of coenzyme Q in mitochondria. Clin Invest 1993; 71: 66-70.
20. Lenaz G, Gavazzoni M, Genova ML, et al. Oxidative stress, antioxidant defences and ageing. Biofactors 1998; 8: 195-204.
21. Novelli GP, Adembri C, Gandini E, et al. Vitamin E protects human skeletal muscle from damage during surgical ischemia-reperfusion. Am J Surg 1997; 173: 206-9.
22. Srinivasan R, Suttcliffe E. Dynamics of the ultraviolet laser ablation of corneal tissue. Am J Ophthalmol 1987; 103: 470-1.
23. Puliafito CA, Steinert RG, Deutsch TF, Hillenkamp F, Dehn EJ, Adler CM. Excimer laser ablation of the cornea and lens. Experimental studies. Ophthalmology 1985; 92: 741-8.
24. Bernardi P, Broekemeier KM, Pfeiffer DR. Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane. J Bioenerg Biomembr 1994; 26: 509-17.
25. Zoratti M, Szabo I. The mitochondrial permeability transition. Biochim Biophys Acta 1995; 1241: 139-76.
26. Kowaltowski AJ, Casilho RF, Vercesi AE. Ca(2+)-induced mitochondrial membrane permeabilization: role of coenzyme Q redox state. Am J Physiol 1995; 269: 141-7.
27. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998; 281: 1309-12.
28. Imberti R, Nieminen AL, Herman B, Lemasters J. Mitochondrial and glycolytic dysfunction in lethal injury to hepatocytes by t-butylhydroperoxide: protection by fructose, cyclosporin A and trifluoperazine. J Pharmacol Exp Ther 1993; 265: 392-400.
29. Pastorino JG, Snyder JW, Serroni A, Hoek JB, Farber JL. Cyclosporin and carnitine prevent the anoxic death of cultured hepatocytes by inhibiting the mitochondrial permeability transition. J Biol Chem 1993; 268: 13791-8.
30. Zamzami N, Marchetti P, Castedo M, et al. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med 1995; 182: 367-77.
31. Salvesen G, Dixit VM. Caspases: intracellular signalling by proteolysis. Cell 1997; 91: 443-6.

32. Schwartz SM. Cell death and the caspase cascade. Circulation 1998; 97: 227-9.
33. Thornberry NA, Lazebnik Y. Caspases: Enemies within. Science 1998; 281: 1312-6.
34. Fontaine E, Eriksson O, Ichas F, Bernardi P. Regulation of the permeability transition pore in skeletal muscle mitochondria. Modulation by lectron flow through the respiratory chain complex i. J Biol Chem 1998; 273: 12662-8.
35. Fontaine E, François I, Bernardi P. A ubiquinone-binding site regulates the mitochondrial permeability transition pore. J Biol Chem 1998; 273: 25734-40.