

# Idebenone Prevents Oxidative Stress, Cell Death and Senescence of Retinal Pigment Epithelium Cells by Stabilizing BAX/Bcl-2 Ratio

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## Key Words

Age-related macular degeneration · Oxidative stress · Apoptosis · Retinal pigment epithelium cells · Cell death

## Abstract

**Purpose:** Age-related macular degeneration (AMD) is one of the leading causes of blindness. Degeneration of the retinal pigment epithelium (RPE) is pathognomonic for the disease, and oxidative stress plays an important role in the pathogenesis of this disease. This study investigates potential anti-apoptotic and cytoprotective effects of idebenone on cultured RPE cells (ARPE-19) under conditions of oxidative stress. **Methods:** ARPE-19 cells were treated with 1–100  $\mu\text{M}$  idebenone. Cell viability (MTT assay), induction of intracellular reactive oxygen species (ROS) and histone-associated DNA fragments in mono- and oligonucleosomes, expression of proapoptotic BAX and antiapoptotic Bcl-2 as well as senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal) activity were investigated under exposure to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). **Results:** Idebenone concentrations from 1 to 20  $\mu\text{M}$  showed no toxic effects on ARPE-19 cells. When cells were treated with  $\text{H}_2\text{O}_2$ , pretreatment with 5, 7.5, 10, and 20  $\mu\text{M}$  idebenone led to a significant increase in the viability of ARPE-19 cells. In addition, idebenone pretreatment significantly attenuated the induction of SA- $\beta$ -Gal and intracellular ROS as well as the amount of histone-associated DNA frag-

ments after treatment with  $\text{H}_2\text{O}_2$ . The reduction of proapoptotic BAX and the elevation of antiapoptotic Bcl-2 under idebenone show that this process is rather mediated by inhibiting  $\text{H}_2\text{O}_2$ -induced apoptosis, not necrosis. **Conclusion:** In this study, idebenone increased survival of ARPE-19 cells and reduced cell death, senescence, and oxidative stress by stabilizing the BAX/Bcl-2 ratio.

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## Introduction

Age-related macular degeneration (AMD) is the main reason for severe vision loss in the elderly population worldwide [1–3]. Both dry and wet forms of AMD exist. About 10% of AMD patients suffer from wet AMD which may result in rapid loss of central vision due to choroidal neovascularization in the central retina [3]. The other 90% of patients suffer from dry forms, such as geographic atrophy (GA), in which visual acuity usually decreases gradually [3]. Nevertheless, due to the large number of patients affected, the gradual decrease in central vision is a major source of visual impairment in the elderly population. GA is characterized by the atrophy of retinal pigment epithelium (RPE) cells and consecutive photoreceptor degeneration [3]. At present, there is no satisfactory treatment for GA and dry AMD [3].

AMD is a multifactorial disease, and the pathogenesis is still not completely understood. However, beside others, increased cumulative light exposure, smoking, hemodynamic abnormalities, and inflammation have been implicated as risk factors [3].

There is substantial evidence that all these risk factors have a common denominator: abnormal increased production of reactive oxygen species (ROS) [4]. In this context, it is of importance that RPE cells are highly susceptible to oxidative stress [5]. One reason for this is that RPE cells contain numerous intracellular photoreactive compounds, including lipofuscin, melanin, and others, absorbing especially in the blue spectral range and thereby promoting the formation of oxygen radicals [6]. In addition, numerous oxygen radical-producing enzymes as well as the high content of fatty acids and the high metabolism rate of the RPE cells contribute to a metabolic imbalance and to an oxidized status [7]. Moreover, regenerating all-*trans*-retinal from the vision circle and fatty acids for the metabolism of photoreceptor outer segments and the nutritional function of the RPE for the neuroretinal tissue result in an extremely high metabolic activity of RPE cells [8]. As a result, RPE cells have to cope with a highly increased intracellular ROS production [8].

To protect RPE cells from oxidative stress-induced cellular damage, antioxidant supplementation has been proposed as supporting treatment in certain forms of dry AMD [9]. Nevertheless, to date, there is still no satisfactory treatment for GA.

Idebenone is a derivate of coenzyme Q10, but with a ten-fold higher antioxidant capacity. As a component of the respiratory chain it acts as a proton and electron carrier from complex I and II to complex III. The precise mechanisms of its antioxidative and cytoprotective effects are not completely understood, but a number of experimental and clinical studies demonstrated (neuro-)cytoprotective effects in degenerative diseases such as Friedreich's ataxia [10, 11] or Leber's hereditary optic neuropathy [12]. In addition, effects on antiapoptotic protein expression have been described [13–15].

The present study evaluates the effect of idebenone on cultured human RPE cells under conditions of oxidative stress.

## Methods

Idebenone was generously provided by Santhera Pharmaceuticals (Liestal, Switzerland). The compound was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Mo., USA) and diluted with DMSO and the cell culture medium to the desired

concentration, with a final DMSO concentration of 0.1% (v/v) for in vitro studies. DMSO was added to cultures at 0.1% (v/v) as a solvent control.

### Ethics

The methods of securing human tissue were humane, included proper consent and approval, complied with the Declaration of Helsinki, and were approved by the local ethics committee.

### ARPE-19 Cell Culture

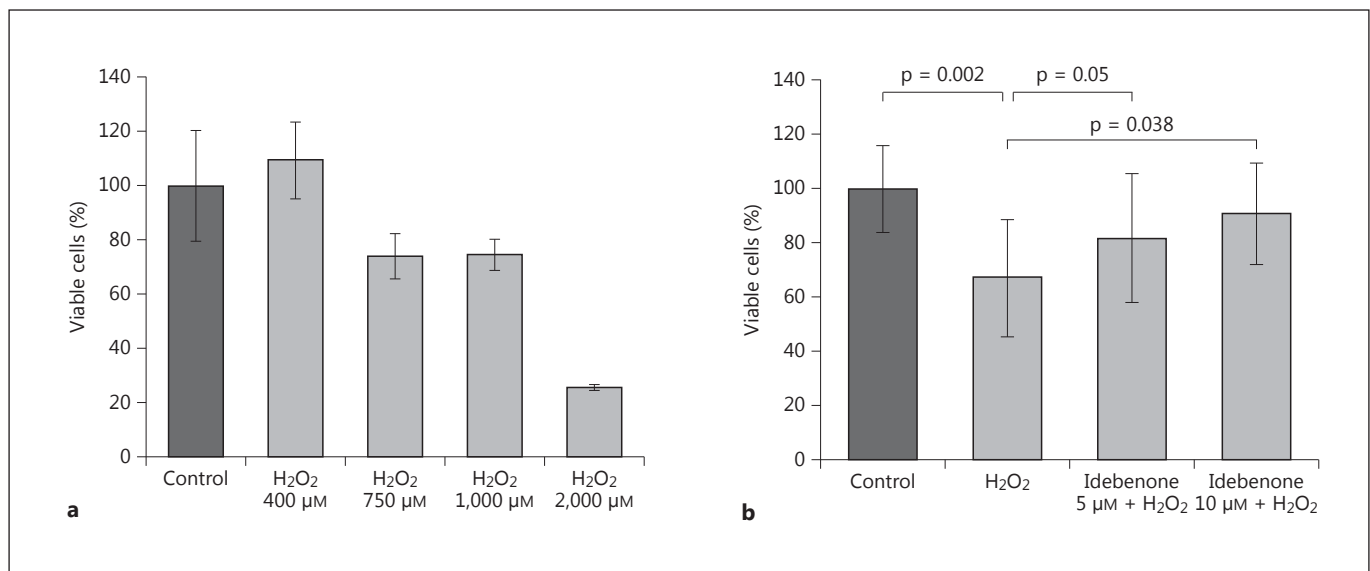
ARPE-19 cells, a human RPE cell line, were purchased from ATCC (Manassas, Va., USA) and grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/Ham's F-12), supplemented with 10% FCS, 50 IU penicillin/ml and 50 µg streptomycin/ml (Biochrom) at 37°C and 5% CO<sub>2</sub> as described previously [16]. The cell culture medium was changed every 48 h. 100% confluence was achieved after 72 h of growing. Only confluent ARPE-19 cells were used for the experiments.

### Cell Culture Treatment

For the tetrazolium dye-reduction assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)], ARPE-19 cells were seeded in 12-well tissue culture plates upon confluence for 72 h and then kept in serum-free conditions (i.e. cell culture medium without FCS, other factors kept constant) for 24 h. Afterwards, they were exposed to various idebenone concentrations (1, 2.5, 5, 7.5, 10, 20, 50, 75, 100, and 150 µM) for 48 h. To investigate the effects of idebenone on ARPE-19 cells regarding oxidative stress, we used idebenone concentrations of 5 and 10 µM, which showed the strongest effects in preliminary studies. Regarding ROS, senescence-associated β-galactosidase (SA-β-Gal), histone-associated DNA fragments, and BAX and Bcl-2 concentrations of 5 and 7.5 µM were chosen because, here, the antioxidative effect was most intense with a secure distance from the toxic dose. Idebenone has been applied as a pretreatment because as a coenzyme Q10 analogue it acts as an electron carrier in the respiratory chain [17]. Therefore, it is able to immediately eliminate newly built radicals and may be more effective than when applied afterwards. A previous study showed stronger effects with pre- than with post-treatment [18]. Also, we [19] and others [20–22] showed promising results with pretreatment; therefore, we chose this regimen again for the present study.

### Exposure to Hydrogen Peroxide

To test the effects of idebenone under conditions of oxidative stress, ARPE-19 cells were exposed to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). In preliminary studies of our laboratory, several concentrations of H<sub>2</sub>O<sub>2</sub> between 400 and 2,000 µM and exposure times were tested, and the concentration of 750 µM H<sub>2</sub>O<sub>2</sub> and a 2-hour treatment were chosen in order to produce an adequate degree of oxidative stress without a too strong cell injury in untreated ARPE-19 cells. A diagrammed presentation of this can be found in figure 1a. To investigate the effect of idebenone on H<sub>2</sub>O<sub>2</sub>-induced cellular damage, cells were seeded upon confluence in 12-well plates as described above and then exposed to the abovementioned different concentrations of idebenone for 48 h, but 750 µM H<sub>2</sub>O<sub>2</sub> was added for the last 2 h (i.e. for 46 h idebenone only; for 2 h idebenone plus H<sub>2</sub>O<sub>2</sub>). Then, the serum-free medium containing H<sub>2</sub>O<sub>2</sub> and idebenone was removed by carefully rinsing the cells with serum-free medium three times. After another 24 h of incubation with serum-



**Fig. 1. a** Cell viability under a 2-hour treatment with different concentrations of H<sub>2</sub>O<sub>2</sub>. A clear decrease in viability was achieved by exposure to 750 μM H<sub>2</sub>O<sub>2</sub>. **b** Treatment with H<sub>2</sub>O<sub>2</sub> led to a significant decrease in cell viability. Pretreatment with 5 and 10 μM idebenone significantly attenuated the decrease in viability after exposure to H<sub>2</sub>O<sub>2</sub>.

free medium, MTT assay, quantitative detection of histone-associated DNA fragments in mono- and dinucleosomes, and CM-H2DCFDA staining and SA-β-Gal staining were investigated. In addition, RT-PCR analysis and Western blotting were performed.

#### MTT Assay

The MTT assay was used to determine metabolic activity of ARPE-19 cells. The MTT assay is a well-established test for an indirect measurement of cell viability by determining the activity of the mitochondrial succinate dehydrogenase and NAD(P)H metabolism. Elevated NAD(P)H as a marker of increased cellular metabolic activity results in an increased reduction of MTT substance. It was performed as described in the literature by Mosmann [23], with some modifications [16, 24]. The medium was removed, cells were washed with PBS, and 1,000 ml/well MTT solution (1.5 ml MTT stock, 2 mg/ml in PBS, plus 28.5 ml DMEM) was added. ARPE-19 cells were incubated at 37°C for 1 h. The formazan crystals that formed were dissolved by the addition of DMSO (700 ml/well). Absorption was measured by a scanning multiwell spectrophotometer (Molecular Probes, Garching, Germany) at 550 nm. Results are expressed as the mean percentage of control extinction. Experiments were performed in triplicate and repeated three times. Untreated ARPE-19 cells of the same passage served as the control. Values of each sample were normalized to a 'blank' containing DMSO only.

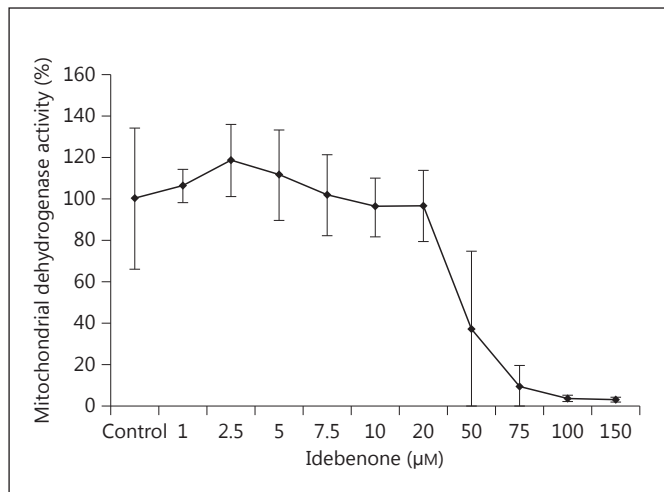
#### Detection of SA-β-Gal Activity

The proportion of ARPE-19 cells positive for SA-β-Gal activity was determined as described by Dimri et al. [25]. Briefly, treated ARPE-19 cells were washed twice with PBS and fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS (pH 6.0) at room

temperature for 4 min. The cells were then washed twice with PBS and incubated for 8 h at 37°C with freshly prepared SA-β-Gal staining solution [1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 40 mM citric acid-sodium phosphate solution (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl<sub>2</sub> diluted in PBS] in darkness. Subsequently, the SA-β-Gal staining solution was removed, and cells were washed with PBS, examined for the development of blue color, and photographed at low magnification (×200) using a light microscope. The amount of SA-β-Gal-positive cells was counted in 5 high-power fields per experiment at ×200 magnification. The results are expressed as the mean average percentage ± SD of six experiments using ARPE-19 cultures from four donors.

#### Detection of Intracellular ROS

Intracellular ROS production was detected by the method described by Nishikawa et al. [26] with some modifications [27]. RPE cells cultured in the presence of 5 and 7.5 μM idebenone were treated with 750 μM H<sub>2</sub>O<sub>2</sub> as described above. Then, the cells were supplemented with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA; Molecular Probes, Eugene, Oreg., USA) dissolved in Krebs-Ringer bicarbonate buffer (135 mM NaCl, 3.6 mM KCl, 10 mM HEPES, 5 mM NaHCO<sub>3</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, and 0.5 mM MgCl<sub>2</sub>, pH 7.4) to a final concentration of 10 μM CM-H2DCFDA for 30 min and incubated with DMEM/F12 medium for 2 h. Subsequently, CM-H2DCFDA staining solution was removed, cells were washed with PBS and analyzed under an epifluorescence microscope (Aristoplan). The degree of fluorescence was determined as relative fluorescence compared to controls.



**Fig. 2.** Toxicity of idebenone. Up to a concentration of 20  $\mu\text{M}$ , idebenone caused no decrease in the viability of ARPE-19 cells.

**Table 1.** Primers used for RT-PCR

Target	Length	Position	AT, °C	GC, %	Sequence
Bcl-2	19	3465–3483	60	58	agaggtcacgggggcta
	20	3505–3524	60	50	ccaggttaacaaaacccaca
BAX	18	526–543	59	61	caagaccagggtggttg
	18	592–609	59	56	cactcccgccacaagat

AT = Annealing temperature; GC = guanine-cytosine content.

#### Detection of Histone-Associated DNA Fragments in Mono- and Oligonucleosomes

Apoptosis is characterized by membrane blebbing, condensation of cytoplasm, and activation of endogenous endonucleases. This leads to internucleosomal cleavage of DNA and the generation of mono- and oligonucleosomes that are tightly complexed with histones. To quantify apoptosis in ARPE-19 cells, detection of histone-associated DNA fragments in mono- and oligonucleosomes was performed by ELISA. ARPE-19 cells were treated with idebenone (5 and 7.5  $\mu\text{M}$ ) as described above. Cells were then collected from culture plates and subjected to a cell death detection ELISA (Roche Applied Science, Indianapolis, Ind., USA) according to the manufacturer's recommendations [27]. The extinction produced by histone-associated DNA fragments was measured with the ELISA reader.  $\text{H}_2\text{O}_2$ -treated cells produce a much higher extinction detected by ELISA than control cells or cells treated with idebenone only. But even without oxidative stress, a certain amount of histone-associated DNA fragments can be measured. To eliminate this 'natural'  $\text{H}_2\text{O}_2$ -independent apoptosis, it is recommended by the manufacturer to examine the relative increase in histone-associated DNA fragments caused by  $\text{H}_2\text{O}_2$  treatment additionally

to idebenone or sham treatment. This increase is called 'enrichment factor' and equals the value of extinction of  $\text{H}_2\text{O}_2$ -treated cells divided by the value of extinction of untreated cells.

#### RNA Isolation and Real-Time PCR for BAX and Bcl-2

RNA isolation by using the guanidium thiocyanate-phenol-chloroform extraction method and RT-PCR using the LightCycler System (Roche Diagnostics, Mannheim, Germany) was performed as described previously [24, 28]. Quantitation of Bcl-2 and BAX mRNA was performed with specific primers (table 1). Primers and probes were selected by the ProbeFinder software version 2.04. All primers and probes were designed to cross intron-exon boundaries in order to avoid amplification of genomic DNA. All PCR products were sequenced to ensure product validity. To normalize for differences in the amount of total RNA added to each reaction, 18S rRNA was simultaneously processed in the same sample as an internal control. The levels of Bcl-2 and BAX mRNA were determined as the relative ratio, which was calculated by dividing the level of Bcl-2 or BAX mRNA by the level of the 18S rRNA house-keeping gene in the same samples. Ratios are expressed in a decimal format. All experiments were performed at least in triplicate and repeated three times.

#### Statistical Analysis

Data was analyzed using SPSS 22 for Windows (SPSS Inc., Chicago, Ill., USA). Quantitative results are presented as means  $\pm$  standard deviations. The effect of various idebenone concentrations on cell viability under oxidative stress was statistically tested using the Mann-Whitney test for two independent samples without normal distribution. Statistical comparisons among the different concentrations of idebenone (each repeated three times and measured in triplicate) in testing of ROS, of histone-associated DNA fragments, and of expression of BAX and Bcl-2 and of SA- $\beta$ -Gal were also performed with the Mann-Whitney test. For all statistical tests,  $p < 0.05$  was considered significant.

## Results

### Cell Viability

ARPE-19 cells were treated with 1, 2.5, 5, 7.5, 10, 20, 50, 75, 100, and 150  $\mu\text{M}$  idebenone for 48 h to investigate potential toxic effects of this substance on the cells. Cells treated with concentrations between 1 and 20  $\mu\text{M}$  showed no difference in intracellular mitochondrial dehydrogenase activity, an indirect measure for cellular viability, in the MTT assay. An idebenone concentration of 50, 75, 100, and 150  $\mu\text{M}$  led to a dose-dependent decrease in intracellular mitochondrial dehydrogenase activity (fig. 2).

### Effect of Idebenone on $\text{H}_2\text{O}_2$ -Induced Decrease in ARPE-19 Cell Viability

Results of our preliminary studies with various concentrations of  $\text{H}_2\text{O}_2$  at an exposure time of 2 h are shown in figure 1a. This time span was found to be the best in previous experiments with different exposure times (data



not shown). 750  $\mu\text{M}$  appeared to be the proper concentration to gain a decrease in cell viability due to oxidative stress without disturbing the cell metabolism too much. This  $\text{H}_2\text{O}_2$  treatment led to a significant decrease in intracellular mitochondrial dehydrogenase activity, an indirect measure for cellular viability, of ARPE-19 cells in the MTT assay [viability of 100% in control cells vs.  $67 \pm 21\%$  in  $\text{H}_2\text{O}_2$ -treated cells ( $p = 0.002$ )]. When pretreated with 5 and 10  $\mu\text{M}$  idebenone, cells showed a viability of  $81 \pm 23$  and  $91 \pm 18\%$ , respectively. So, pretreatment with idebenone led to a significantly higher viability despite the exposure to  $\text{H}_2\text{O}_2$  ( $p = 0.05$  for 5  $\mu\text{M}$  and  $p = 0.038$  for 10  $\mu\text{M}$  idebenone; fig. 1b).

#### Expression of SA- $\beta$ -Gal

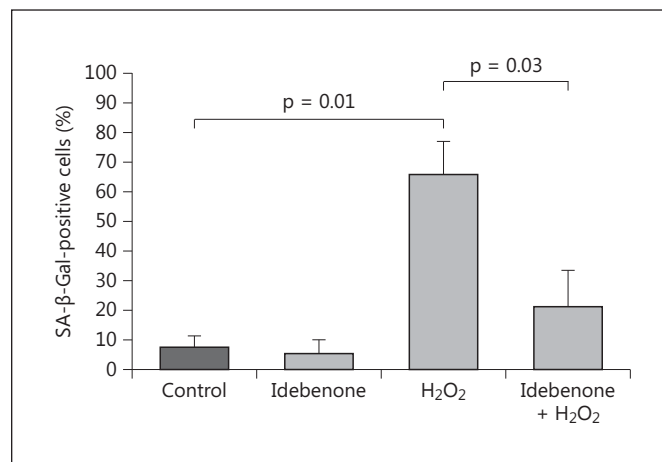
SA- $\beta$ -Gal is a well-known marker for cell aging, established especially in research regarding skin aging. In the present study,  $\text{H}_2\text{O}_2$  treatment led to a significant increase in the expression of SA- $\beta$ -Gal ( $p = 0.01$ ). When pretreated with 5  $\mu\text{M}$  idebenone, the increase in SA- $\beta$ -Gal expression mediated by  $\text{H}_2\text{O}_2$  was significantly ameliorated ( $p = 0.03$ ; fig. 3, 4).

#### Expression of Intracellular ROS

There was no difference between the expression of intracellular ROS in idebenone-treated and control cells. In contrast,  $\text{H}_2\text{O}_2$ -treated cells showed a significantly higher expression of intracellular ROS ( $p = 0.001$ ). Pretreatment with idebenone significantly reduced the expression of intracellular ROS in  $\text{H}_2\text{O}_2$ -treated cells ( $p = 0.01$ ; fig. 4, 5).

#### Detection of Histone-Associated DNA Fragments in Mono- and Oligosomes

Detection of histone-associated DNA fragments was performed by ELISA to investigate a possible antiapoptotic effect of idebenone on ARPE-19 cells. The raw data of extinction read by ELISA are presented in figure 6a. To make the effect of  $\text{H}_2\text{O}_2$  and the protection by idebenone better visible, the data are expressed as enrichment factor as described above, i.e. the multiplication of apoptosis due to  $\text{H}_2\text{O}_2$ , and displayed in figure 6b. Here, treatment with 750  $\mu\text{M}$   $\text{H}_2\text{O}_2$  led to a significant increase in histone-associated DNA fragments corresponding with a significant increase in apoptosis in control and idebenone-treated cells ( $p = 0.0001$ ). The factor by which  $\text{H}_2\text{O}_2$  treatment elevated the expression of histone-associated DNA fragments compared to cells without oxidative stress was  $12.8 \pm 4.1$  in control cells,  $10.4 \pm 3.7$  in cells pretreated with 5  $\mu\text{M}$  idebenone, and  $6.9 \pm 1.8$  in cells pretreated with 7.5  $\mu\text{M}$  idebenone (fig. 6a). Figure 6b shows this 'enrich-

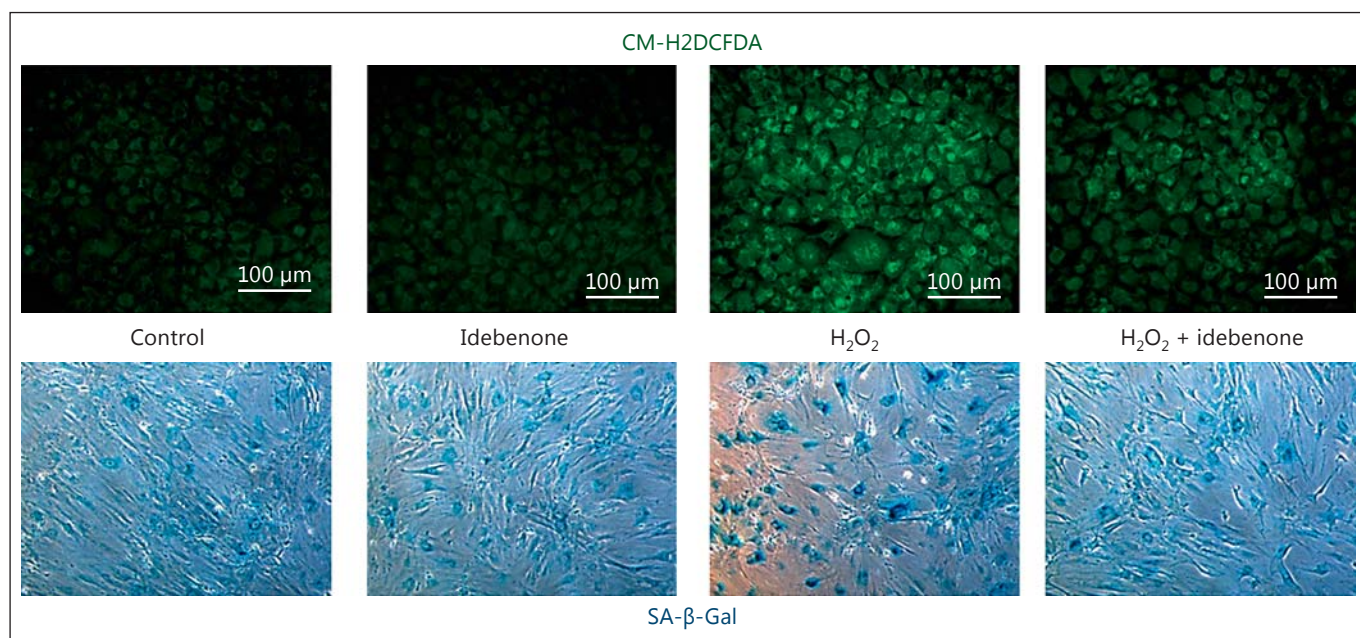


**Fig. 3.**  $\text{H}_2\text{O}_2$  treatment led to a significant increase in the amount of SA- $\beta$ -Gal-positive ARPE-19 cells. Pretreatment with 5  $\mu\text{M}$  idebenone significantly lowered this increase with  $\text{H}_2\text{O}_2$ .

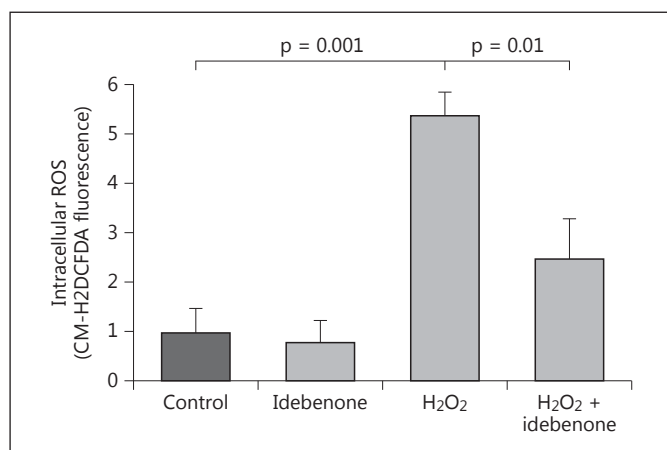
ment factor' normalized to the control group (100%). Pretreatment with 7.5  $\mu\text{M}$  idebenone led to a significantly lower enrichment factor ( $55.9 \pm 16.8\%$ ;  $p = 0.001$ ) compared to control cells; 5  $\mu\text{M}$  idebenone showed this by trend ( $79.2 \pm 21.9\%$ ;  $p = 0.058$ ), and the difference between idebenone 5 and 7.5  $\mu\text{M}$  was significant ( $p = 0.001$ ).

#### mRNA Expression of BAX and Bcl-2

In order to confirm that the detected increase in histone-associated DNA fragments is caused by apoptosis, not by necrosis, we quantified the mRNA expression of the proapoptotic protein BAX and the antiapoptotic protein Bcl-2. As both need to be in a steady balance to allow normal cell survival, we also calculated the BAX/Bcl-2 ratio. After normalization to the control group, expression of BAX was 100% in control cells,  $136 \pm 28\%$  in  $\text{H}_2\text{O}_2$ -treated cells,  $115 \pm 9\%$  in cells treated with  $\text{H}_2\text{O}_2$  and 5  $\mu\text{M}$  idebenone, and  $103 \pm 35\%$  in cells treated with  $\text{H}_2\text{O}_2$  and 7.5  $\mu\text{M}$  idebenone. Expression of Bcl-2 was 100% in control cells,  $70 \pm 20\%$  in  $\text{H}_2\text{O}_2$ -treated cells,  $83 \pm 49\%$  in cells treated with  $\text{H}_2\text{O}_2$  and 5  $\mu\text{M}$  idebenone, and  $115 \pm 57\%$  in cells treated with  $\text{H}_2\text{O}_2$  and 7.5  $\mu\text{M}$  idebenone. Therefore, the BAX/Bcl-2 ratio was 100,  $211 \pm 86$ ,  $170 \pm 83$ , and  $112 \pm 67\%$ , respectively. So, exposure of ARPE-19 cells to  $\text{H}_2\text{O}_2$  led to a significant increase in the proapoptotic BAX ( $p = 0.037$ ), to a significant decrease in the antiapoptotic Bcl-2 ( $p = 0.034$ ), and to a subsequent significant increase in the BAX/Bcl-2 ratio ( $p = 0.037$ ; fig. 7). Pretreatment with idebenone led to a dose-dependent decrease in proapoptotic BAX and an increase in antiapop-



**Fig. 4.** Treatment with  $\text{H}_2\text{O}_2$  increased the expression of intracellular ROS (upper row) and SA- $\beta$ -Gal (lower row) in ARPE-19 cells. Pretreatment with 5  $\mu\text{M}$  idebenone markedly inhibited this increase with  $\text{H}_2\text{O}_2$ .



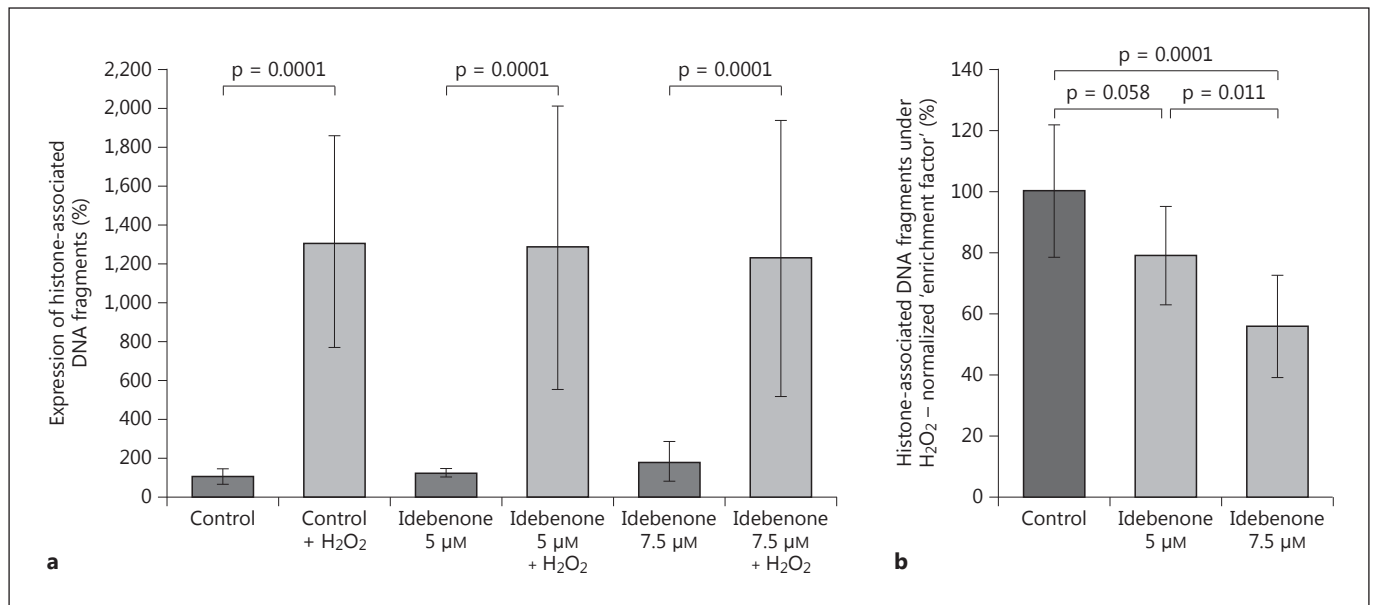
**Fig. 5.** There was no difference in the expression of ROS between idebenone-treated cells and controls.  $\text{H}_2\text{O}_2$  treatment led to a significant increase in fluorescence due to intracellular ROS. Pretreatment with 5  $\mu\text{M}$  idebenone significantly attenuated this increase in ROS fluorescence.

totic Bcl-2 under oxidative stress, which resulted in a normalization of the elevated BAX/Bcl-2 ratio. These results were clearly recognizable as shown in figure 7, but due to the relatively high standard deviation they were not statistically significant at a p level of 0.05.

## Discussion

To date, not all mechanisms involved in the development and progression of AMD to retinal pigment epithelial atrophy and consecutive photoreceptor degeneration are completely understood. Nonetheless, oxidative stress seems to be a central mechanism through which known risk factors, e.g. advanced age, smoking, or UV light, have an impact on retinal cell death [4]. In addition, there seems to be no doubt that induction of oxidative stress and oxygen radicals promote cellular apoptosis.

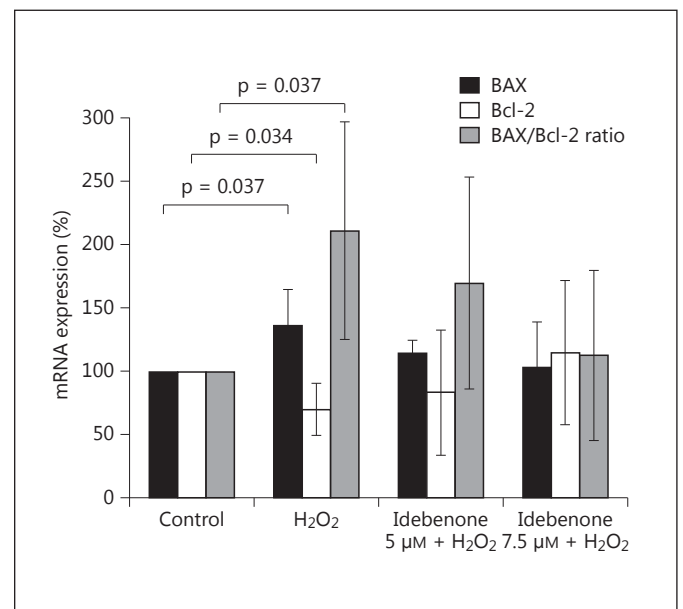
Apoptosis is a cell-autonomous mechanism that is genetically controlled by the dying cell, resulting in the activation of tumor suppressor proteins [29] and apoptosis-initiating caspases [30]. A number of studies found that apoptosis plays an important role in retinal cell death, both in physiological and pathological conditions, and particularly in AMD [5, 31–33]. Dunaief et al. [34], for example, demonstrated in vivo the involvement of apoptosis in RPE cell death at the margins of GA in dry AMD eyes, and Hinton et al. [31] showed an involvement of apoptosis in choroidal neovascularization membranes of patients suffering from neovascular AMD. Numerous in vitro investigations revealed that retinal cell apoptosis is mediated by oxidative stress [5, 35, 36]. In addition, apoptosis due to oxidative stress is characterized not only by



**Fig. 6. a** Gross expression of histone-associated DNA fragments in the different treatment groups. H<sub>2</sub>O<sub>2</sub> treatment significantly increased this expression. **b** Elevated expression of histone-associated DNA fragments in control ARPE-19 cells due to H<sub>2</sub>O<sub>2</sub> treatment, normalized to control. Pretreatment with 5 and 7.5 μM idebenone significantly attenuated apoptosis following H<sub>2</sub>O<sub>2</sub> treatment resulting in a significantly lower expression of histone-associated DNA fragments.

elevated caspase levels, but also by an increase in the proapoptotic protein BAX and a decrease in the antiapoptotic protein Bcl-2 [27, 37–39]. BAX activates caspases by upregulating the tumor suppressor gene p53 [40, 41], and Bcl-2 inhibits caspases by stabilizing the permeability of the mitochondrial membrane [41]. An imbalanced relation of these proteins puts the cells in a proapoptotic state. Former studies showed this for several cell types [37–39] and especially for retinal cells [20, 42].

In this study, we were able to show that exposure of ARPE-19 cells to oxidative stress leads to a decrease in the viability of RPE cells and an increased formation of intracellular ROS. This decrease in viability is caused by RPE cell apoptosis, not necrosis, as shown by the significantly elevated expression of histone-associated DNA fragments and the elevated BAX/Bcl-2 ratio. In addition, the strongly elevated expression of SA-β-Gal indicates promotion of cellular aging under H<sub>2</sub>O<sub>2</sub>. SA-β-Gal is a well-established marker for cellular aging, mainly known from dermatological studies. It represents the enzymatic level of cell senescence with expansion of the lysosomal compartment [43]. It is only expressed by senescent, not presenescent cells, e.g. fibroblasts and keratinocytes [25, 44]. Also, in the eye, SA-β-Gal is elevated in senescent



**Fig. 7.** mRNA expression of BAX and Bcl-2 and the BAX/Bcl-2 ratio in control cells, H<sub>2</sub>O<sub>2</sub>-treated cells, and idebenone- plus H<sub>2</sub>O<sub>2</sub>-treated cells. H<sub>2</sub>O<sub>2</sub> significantly elevated the BAX/Bcl-2 ratio, which indicates a disturbed balance and a shift into a proapoptotic state. Idebenone pretreatment prior to H<sub>2</sub>O<sub>2</sub> treatment attenuated this shift.

cells [19]. So, the results of this study underline the pivotal role of oxidative stress in aging and apoptosis of RPE cells.

While antioxidants are used to potentially prevent the progression of early and intermediate stages of AMD, still, in the majority of patients, the disease progresses over time and no effective treatment exists to stop apoptotic RPE cell death in advanced nonneovascular AMD [45].

Idebenone is a synthetic analogue of ubiquinone with a ten-fold higher potency. It acts as an electron carrier between the complexes of the respiratory chain and, therefore, influences the electric balance within the mitochondrion [15]. Furthermore, it inhibits lipid peroxidation and stabilizes the mitochondrial membrane via this pathway [17, 46]. This underlines potential antiapoptotic as well as antioxidant effects of idebenone.

In the present study, ARPE-19 cells pretreated with idebenone showed significantly less formation of intracellular ROS after exposure to  $H_2O_2$ . This indicates the potential of idebenone to ameliorate the cytotoxic effects of oxidative stress on RPE cells as a potential approach to prevent progression of dry AMD. In addition, cells pretreated with idebenone showed significantly better viability under oxidative stress.

An important finding of our study is that idebenone inhibited an increase in SA- $\beta$ -Gal in RPE cells under the conditions of oxidative stress. As mentioned above, the SA- $\beta$ -Gal enzyme is strongly correlated with ageing processes. This may indicate an antiaging effect of idebenone.

Previous studies demonstrated antiapoptotic effects of idebenone on several cell types [19, 47–49]. To date, the use of idebenone and research regarding idebenone are focused on its protective effects on neuronal cells and tissues. The first studies evaluated effects on the murine brain under hypoxia [50]; later on, it became a clinical treatment for patients with Friedreich's ataxia [10] and Leber's hereditary optic neuropathy [12]. Our recently published study showed its antiapoptotic, antioxidative, and antiaging effects on primary optic nerve head astrocytes in terms of glaucoma treatment [19]. Rather few studies evaluated other tissues, such as kidney, and also showed promising antioxidative effects [51]. AMD is an important oxidative stress-related eye disease whose treatment options are still not exhausted [3]. The epithelial tissue of the retina plays an important role in the pathogenesis of this disease [3]. Therefore, one aim of our study was to evaluate the effects of idebenone on RPE cell apoptosis after exposure to oxidative stress.  $H_2O_2$  treat-

ment led to a significant increase in apoptosis, which could be demonstrated in terms of significantly more histone-associated DNA fragments and an elevated BAX/Bcl-2 ratio due to an increase in the proapoptotic BAX and a decrease in the antiapoptotic Bcl-2. Idebenone significantly reduced histone-associated DNA fragments in a dose-dependent manner and led to a normalization of the BAX/Bcl-2 ratio. These findings indicate a protective impact of idebenone on RPE cell apoptosis after exposure to oxidative stress. Thus, the present study contributes to the evaluation of idebenone as a protective agent in non-neuronal tissue.

We are aware that we did not provide confirming data from an animal model. However, many in vivo studies showed much potential of idebenone against oxidative stress in the eye [12, 52, 53] and other organs [13, 21, 54]. Antioxidants already are a major pillar of AMD therapy, but they are still not fully satisfactory. Therefore, idebenone as an agent which can prevent optic nerve head astrocytes and retinal ganglion cells from apoptosis and which can successfully treat Leber's hereditary optic neuropathy is worth to be further investigated regarding AMD therapy. The present study makes a small contribution to this objective.

## Conclusion

Our experiments show substantial cytoprotective effects of idebenone mediated by inhibition of oxidative stress and protection of RPE cells from cellular aging processes. This leads to a significant inhibition of oxidative stress-related apoptosis of RPE cells. Therefore, our in vitro results imply that idebenone may have the required properties of a potential future therapeutic agent of AMD in vivo.

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## Disclosure Statement

The authors have no financial or competing interests concerning the present study.



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