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LABORATORY STUDY

Melatonin and amfenac modulate calcium entry, apoptosis, and oxidative stress in ARPE-19 cell culture exposed to blue light irradiation (405 nm)

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Abstract

Purpose **Under conditions of oxidative stress, cell apoptosis is triggered through the mitochondrial intrinsic pathway. Increased levels of reactive oxygen species (ROS) are linked to excess cell loss and mediate the initiation of apoptosis in a diverse range of cell types. The aims of this study were to assess intracellular Ca^{2+} release, ROS production, and caspase-3, and -9 activation in ARPE-19 cells during the blue light-mediated cell death, and to examine a potential protective effect of melatonin and amfenac, in the apoptotic cascade.**

Methods **ARPE-19 cells were cultured in their medium. First, MTT tests were performed to determine the protective effects of amfenac and melatonin. Cells were then exposed to blue light irradiation in an incubator. Intracellular Ca^{2+} release experiments, mitochondrial membrane depolarization, apoptosis assay, glutathione (GSH), glutathione peroxidase (GSH-Px), and ROS experiments were done according to the method stated in the Materials and methods section.**

Results **Cell death was clearly associated with increased levels of ROS production, as measured by 2′,7′-dichlorofluorescein fluorescence, and associated increase in Ca^{2+} levels, as measured by Fura-2-AM. Blue light-induced cell death was associated with an increased level of caspase-3 and 9, suggesting mediation *via* the apoptotic pathway. Cell death was also associated with mitochondrial depolarization. Melatonin was shown to delay these three steps.**

Conclusion **Melatonin, amfenac, and their combination protect ARPE-19 cells against blue light-triggered ROS accumulation and caspase-3 and -9 activation. The antiapoptotic effect of melatonin and amfenac at doses inhibiting caspase synthesis modified Ca^{2+} release and prevented excessive ROS production, suggesting a new therapeutic approach to age-related macular degeneration.**

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Introduction

Age-related macular degeneration (AMD) is one of the most common causes of progressive blindness in elderly individuals in developed countries.[1] The pathogenesis of AMD is not well-understood and there is no efficient prevention for this disease yet. Some studies indicate that long term exposure to light may initiate AMD.[2–5] It has been well-known that human retina can be damaged by visible light. Against this energy, retina is protected by the cornea and lens which can absorb ultraviolet light below 400 nm. Visible spectrum's components, which initiate cellular dysfunction and several cell death mechanisms, can be absorbed by biological chromophores (formed by rhodopsin, which intermediates in the

photoreceptor outer segment) in retinal pigment epithelial cells (RPE). The blue region of the light spectrum (400–500 nm) has high energy and is able to infiltrate cells and also the organelles.[6] This region of the spectrum also has been reported as a damaging component for retinal tissue. Blue light has been shown to induce production of reactive oxygen species (ROS) in RPE cells,[7] triggering apoptosis.[8]

The indole melatonin (N-acetyl-5-methoxytryptoamine) is a neurohormone that has crucial roles in the regulation of many physiological events.[9] It is a highly lipophilic molecule that can easily pass through the cell membranes and reach subcellular compartments.[10] Production of ocular melatonin is initiated by photoreceptors in the retina.[11] Melatonin is able to protect tissues from the damaging effects of ROS by both scavenging free radicals and increasing the activity of antioxidant defense mechanisms.[12] The protective effect of melatonin is mediated by its interaction with a family of G-protein coupled receptors.[13] Increasing evidence demonstrates melatonin deficiency has a key role in the pathogenesis of AMD and in the present useful approach for melatonin in preventing this disease. However, the mechanisms by which melatonin may affect the pathophysiology of the retina is not well-understood today.

Rapid change in intracellular calcium ion ($[Ca^{2+}]_i$) is one of the ubiquitous intracellular signaling mechanism that controls numerous cellular functions, from fertilization to gene expression, contraction, or secretion. Hence, cytosolic Ca^{2+} concentrations are susceptible to rapid and localized increases, which are achieved *via* the calcium ions' exchange through the cell membrane or *via* release from the endoplasmic reticulum through specialized ion channels.[14] The proapoptotic effects of Ca^{2+} are mediated by a diverse range of Ca^{2+}-sensitive factors that are compartmentalized in various intracellular organelles.[15] If the free $[Ca^{2+}]_i$ increases due to the degeneration of cation channel activity, physiologic cell functions will be lost.[16,17] Excessive Ca^{2+} load to the cytosol may induce apoptosis by stimulating the release of apoptosis-promoting factors.

Apoptosis or programmed cell death mechanism is controlled mainly by two major pathways: the extrinsic pathway, in which cell membrane receptors trigger the apoptotic process; and the intrinsic pathway, in which mitochondria has a crucial role. Numerous reports suggest that the oxidative stress caused dysregulated homeostasis of $[Ca^{2+}]_i$ is accompanied by alterations in the apoptotic behavior of cell types. Amfenac, is a member of the nonsteroidal anti-inflammatory drugs (NSAIDs) class, and is intended for the prevention and treatment of pain and inflammation. Amfenac has the ability to reduce cyclooxygenase 1 and 2 (COX-1 and COX-2) enzymes.

In the current study, we aimed to investigate the probable protective effects of amfenac, melatonin, and their combination on the *in vitro* response of RPE cells to a nonlethal dose of blue light.

Materials and methods

Chemicals

All chemicals (cumene hydroperoxide, KOH, NaOH, thiobarbituric acid, 1,1,3,3-tetraethoxypropane, 5,5-dithiobis-2 nitrobenzoic acid, tris-hydroxymethyl-aminomethane, glutathione, butylhydroxytoluol, Triton X-100, and ethylene glycol-bis [2-aminoethyl-ether]-N,N,N,N-tetraacetic acid (EGTA)) were obtained from Sigma-Aldrich (St Louis, MO, USA) and all organic solvents (n-hexane, ethyl alcohol) were purchased from Merck (Darmstadt, Germany). Fura-2 acetoxymethyl ester was purchased from Invitrogen (Carlsbad, CA, USA). All reagents were of analytical grade. All reagents except the phosphate buffers were prepared daily and stored at $+4\,^{\circ}C$. Reagents were equilibrated at room temperature for half an hour before an analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at $+4\,^{\circ}C$ for 1 month. APOPercentage assay kit was purchased from Biocolor (Belfast, Northern Ireland, UK).

Study groups

Group I was the control group, ARPE-19 cells were incubated for 24 h in their medium (37 °C and 5% CO_2).

Group II was Blue light group, ARPE-19 cells were exposed to 405 nm wavelength blue light during 24 h (37 °C and 5% CO_2).

Group III was amfenac group, ARPE-19 cells were incubated with 1 μM amfenac for 24 h, according to the results that were obtained from MTT test (37 °C and 5% CO_2).

Group IV was melatonin group and ARPE-19 cells were incubated with 200 μM melatonin for 3 days, according to results that were obtained from MTT test (37 °C and 5% CO_2).

Group V was blue light + amfenac group and ARPE-19 cells were first supplemented with 1 μM amfenac and then exposed to the blue light for 24 h (37 °C and 5% CO_2).

Group VI was blue light + melatonin group and ARPE-19 cells were first supplemented with 200 μM melatonin for 2 days and then exposed to the blue light for 24 h (37 °C and 5% CO_2).

Group VII was blue light + amfenac + melatonin group and ARPE-19 cells were first supplemented with 200 μM melatonin for 2 days, then added 1 μM amfenac to the

medium, followed by exposure to blue light for 24 h (37 °C and 5% CO_2).

Cell culture

Human RPE cell line ARPE-19 (ATCC, Manassas, VA, USA)[18] was grown in a mixture of a medium containing 1 : 1 ratio of Dulbecco's modified eagle medium and Ham's F12 medium supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany) and 1% penicillin–streptomycin combination (Biochrom) according to the manufacturer's instructions. Cells were used at passages 3–10.

Exposure of ARPE-19 cells to blue light

Illumination of blue light was produced by LED-based system generating 405 nm blue light at an output power of 1 Mw/cm^2 (Conrad Electronic GmbH, Hirschau, Germany). LED arrays were developed with the help of Electronics and Communication Engineering Faculty of Süleyman Demirel University. Cells were excited in their own flasks (TPP, Trasadingen, Switzerland) for different number of hours, as stated in the Materials and methods section.

Calcium $[Ca^{2+}]_i$ determination by fluorescent dye

Cells were loaded with Fura-2 by incubation with 4 μM Fura-2 acetoxymethyl ester (Fura-2/AM) for 30 min at room temperature according to a procedure published elsewhere.[19] Once loaded, the cells were washed and gently resuspended in Na-HEPES solution containing (in mM): NaCl, 140; KCl, 4.7; $CaCl_2$, 1.2; $MgCl_2$, 1.1; glucose, 10; and HEPES, 10 (pH 7.4). The seven groups were exposed to H_2O_2 for stimulating $[Ca^{2+}]_i$ release. Fluorescence was recorded from 2 ml aliquots of magnetically stirred cellular suspension (2×10^6 cells/ml) at 37 °C using a spectrofluorometer (Cary Eclipse, Varian Inc., Sydney, Australia) with excitation wavelengths of 340 and 380 nM and emission at 505 nM. Changes in $[Ca^{2+}]_i$ were monitored by using the fura-2 340/380 nM fluorescence ratio and were calibrated according to the method of Grynkiewicz *et al*.[20] Ca^{2+} release was estimated using the integral of the rise in $[Ca^{2+}]_i$ for 150 s after addition of H_2O_2.[21] Ca^{2+} release is expressed nM, taking a sample every second (nM/s) as previously described.[19]

Measurement of ROS-sensitive fluorescence

Cells were loaded with 2 μm dihydrorhodamine-123 (DHR-123) by incubation at 37 °C for 30 min as previously described.[22] This probe is a nonfluorescent cell-permeable compound. Once inside the cell, it turns fluorescent upon oxidation to yield rhodamine-123 (Rh-123), fluorescence being proportional to ROS generation. The fluorescence intensity of Rh-123 was measured in an automatic microplate reader (Tecan Infinite M200, Grödig, Austria). Excitation was set at 488 nm and emission at 543 nm. Treatments were carried out in triplicate. Data are presented as fold increase over the pretreatment level (experimental/control).

Measurement of lipid peroxidation level

Lipid peroxidation levels in the ARPE-19 cell lines were measured with the thiobarbituric acid reaction by the method of Placer *et al*.[23] The quantification of thiobarbituric acid-reactive substances was determined by comparing the absorption to the standard curve of malondialdehyde (MDA) equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3 tetramethoxypropane.

Reduced GSH, GSH-Px, and protein assay

The GSH content of the ARPE-19 cells was measured at 412 nM using the method of Sedlak and Lindsay.[24] GSH-Px activities of ARPE-19 cells were measured spectrophotometrically at 37 °C and 412 nM according to the Lawrence and Burk method.[25] The protein content in the ARPE-19 cells was measured by method of Lowry *et al*[26] with bovine serum albumin as the standard.

Measurement of mitochondrial membrane potential

Cells were incubated with 1 ml JC-1 for 15 min at 37 °C as previously described.[22] The cationic dye, JC-1, exhibits potential-dependent accumulation in mitochondria. It indicates mitochondrial depolarization by a decrease in the red-to-green fluorescence intensity ratio. After incubation with JC-1, the dye was removed, and the cells were washed in PBS. The green JC-1 signal was measured at the excitation wavelength of 485 nm and the emission wavelength of 535 nm, and the red signal, at the excitation wavelength of 540 nm and the emission wavelength of 590 nm. Fluorescence changes were analyzed using a fluorescence spectrophotometer (Tecan Infinite M200). Treatments were carried out in triplicate. Data are presented as emission ratios (590/535). Changes in mitochondrial membrane potential were quantified as the integral of the decrease in JC-1 fluorescence ratio.

Apoptosis assay

The APOPercentage assay (Biocolor Ltd., Belfast, Northern Ireland, UK) was performed according to the manufacturer's instructions and elsewhere.[27] The APOPercentage assay is a dye-uptake assay, which stains

only the apoptotic cells with a red dye. When the membrane of apoptotic cell loses its asymmetry, the APOPercentage dye is actively transported into cells, staining apoptotic cells red, thus allowing detection of apoptosis by spectrophotometer.[27]

Determination of moderate incubation doses of amfenac and melatonin by cell viability (MTT) assay

Cell viability was evaluated by the MTT assay on the basis of the ability of viable cells to convert a water-soluble, yellow tetrazolium salt into a water-insoluble, purple formazan product. The enzymatic reduction of the tetrazolium salt happens only in living, metabolically active cells but not in dead cells. ARPE-19 cells were seeded in 25 cm^2 flasks at a density of 2×10^6/tube and subsequently exposed to several concentrations of amfenac (10 nM–100 μM) and melatonin (50 μM–1 mM) at different incubation times (1–48 h for amfenac and 12 h—5 days for melatonin) at 37°C. After the treatments, the medium was removed and MTT was added to each tube and then incubated for 90 min at 37 °C in a shaking water bath. The supernatant was discarded and DMSO was added to dissolve the formazan crystals. Treatments were carried out in duplicate. Optical density was measured in automatic microplate reader (Tecan Infinite M200) at 490 and 650 nm (as reference wavelength) and presented as the fold increase over the pretreatment level (experimental/control).

Assay for caspase activities

To determine caspase-3 and -9 activities, ARPE-19 cells were sonicated and cell lysates were incubated with 2 ml of substrate solution (20 mm HEPES (pH 7.4), 2 mm EDTA, 0.1% CHAPS, 5 mm DTT and 8.25 μM of caspase substrate) for 1 h at 37 °C, as previously described.[19] The activities of caspase-3 and -9 were calculated from the cleavage of the respective specific fluorogenic substrate (AC-DEVD-AMC for caspase-3 and AC-LEHDAMC for caspase-9). Substrate cleavage was measured with a fluorescence spectrophotometer with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Preliminary experiments confirmed that caspase-3 or -9 substrate cleaving was not detected in the presence of the inhibitors of caspase-3 or -9, DEVD-CMK or z-LEHDFMK, respectively. The data were calculated as fluorescence units per milligram of protein.

Statistical analysis

Data are expressed as means ± SEM of the number of determinations. Statistical significance was analysed by using the SPSS packet program (9.05, SPSS, Chicago, IL, USA). To compare the effects of different treatments, statistical significance was calculated by Mann–Whitney U test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Determination of doses of amfenac and melatonin on cell viability of ARPE-19 cells

The dosage and time period of both amfenac and melatonin was determined according to the MTT results shown in Figures 1 and 2, respectively. Cells were incubated with different dosage and time periods of both chemicals as stated in the Materials and methods section. Our main criterion was to determine the best dosage and the time period to observe 50% fold decrease in cell

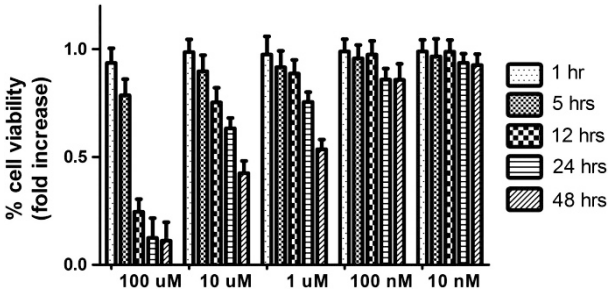

Figure 1 Effects of different time and concentrations of amfenac on ARPE-19 cell viability. Viable cells were determined by cell viability test (MTT). Cell viability is expressed as the percentage of control. Data are expressed as mean ± SD ($n = 6$). There was statistical significance at five different concentrations of amfenac between 100 μm and 10 nM. The toxic effect of amfenac on MTT started at 100 μM at 12 h incubation. Hence, we determined to use amfenac in 1 μM dosage during 24 h.

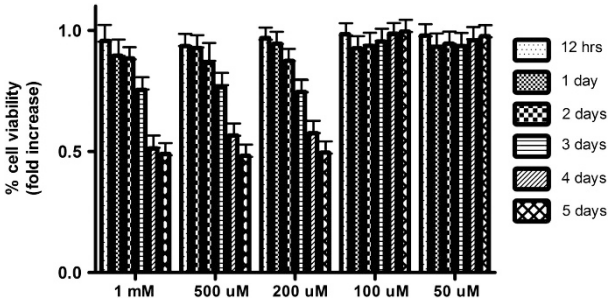

Figure 2 Effects of different time and concentrations of melatonin on ARPE-19 cell viability. Viable cells were determined by cell viability test (MTT). Cell viability is expressed as the percentage of control. Data are expressed as mean ± SD ($n = 6$). There was statistical significance at five different concentrations of melatonin between 1 mM and 50 μM. Hence, we determined to use melatonin in 200 μM dosage during 3 days.

viability compared to the control group ($P<0.001$). We found that amfenac shows its therapeutic effect at a dosage of 1 μM for 24 h. Melatonin shows its best protective effect at 200 μM dosage for 3 days of period. After MTT experiments, five more experiments were performed.

Effects of amfenac and melatonin on intracellular Ca^{2+} release in ARPE-19 cells

Blue light induced a significant increase in intracellular Ca^{2+} levels ($P<0.001$) as shown in Figures 3 and 4. Amfenac and melatonin decreased $[Ca^{2+}]_i$ ($P<0.001$; Figures 3 and 4), with a significant additive effect: $[Ca^{2+}]_i$ levels were significantly lower in amfenac and melatonin combination group compared with either group alone ($P<0.05$).

Effects of amfenac and melatonin on lipid peroxidation, GSH, and ROS levels in ARPE-19 cells

The effects of melatonin and amfenac on $[Ca^{2+}]_i$ homeostasis after blue light implementation correlate with increase in lipid peroxidation as indicated by the increase in MDA, intracellular ROS levels and reduction in GSH ($P<0.05$; Table 1). However, melatonin and amfenac supplementations were associated to an increase in GSH levels compared with other groups, and decreased MDA levels ($P<0.05$) (Table 1).

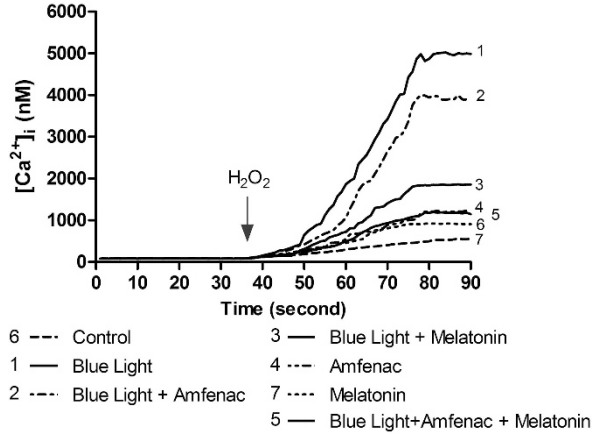

Figure 3 Mobilization of calcium in ARPE-19 cells. ARPE-19 cells were preincubated with the amfenac (1 μM during 24 h) and melatonin (200 μM during 3 days) then exposed to blue light irradiation. Intracellular Ca^{2+} levels were evaluated as described in the Materials and methods section. ARPE-19 cells were then loaded with intracellular fluorescent dye (Fura-2-AM). Fura-2 loaded ARPE-19 cells were stimulated with H_20_2. Traces are representative of six different/separate experiments.

Effects of amfenac and melatonin on apoptosis, caspase-3, and -9 levels in ARPE-19 cells

The effects of melatonin and amfenac on apoptosis levels, caspase-3 and -9 levels after blue light implementation was shown in Table 1. By itself, amfenac and melatonin each decrease the apoptosis ($P<0.05$). However, melatonin and amfenac supplementations were associated with a significant decrease in mitochondrial depolarization levels compared with other groups ($P<0.05$). Caspase-3 and -9 levels were also decreased in a manner relating with apoptosis levels. Amfenac and melatonin significantly decreased ($P<0.05$) apoptosis levels but their combination made the strongest reduction ($P<0.05$) compared with the other groups.

Discussion

Inflammation is a protective mechanism designed to defend the body against endogenous and exogenous antigens. However, chronic inflammation exerts its cellular adverse effects mainly through excessive production of free radicals and depletion of antioxidants. Within the eye, inflammation is a key mediator of a number of common diseases. Aging may be defined as a progressive decline in the physiological functions of an

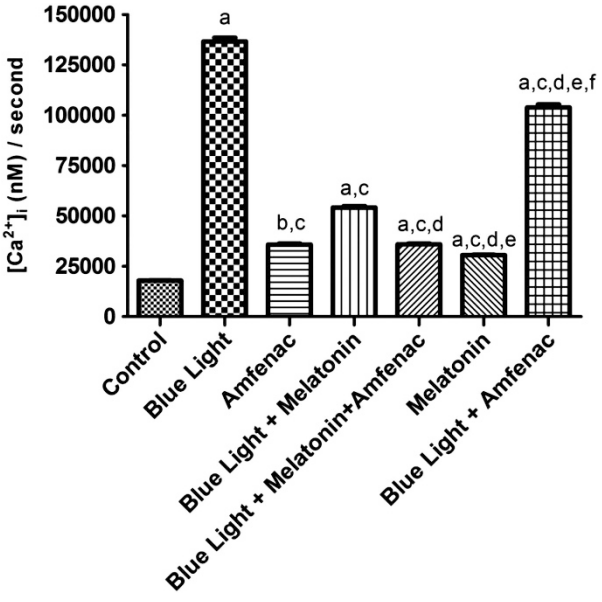

Figure 4 Areas under calcium signaling traces were determined as described under the Materials and methods section and are expressed as fractional changes of emitted fluorescence (F340 and F380). Histograms are representative of six separate experiments. Data are presented as mean ± SD of six independent experiments. [a]$P<0.001$ *vs* control group. [b]$P<0.05$ *vs* control group. [c]$P<0.001$ *vs* blue light group. [d]$P<0.05$ *vs* blue light + melatonin group. [e]$P<0.001$ *vs* melatonin group. [f]$P<0.001$ *vs* blue light + amfenac.

Table 1 Effects of blue light, amfenac, melatonin, and their combinations on lipid peroxidation (LP), glutathione peroxidase (GSH-Px), reduced glutathione (GSH), apoptosis, caspase-3, caspase-9, and ROS levels in ARPE-19 cells. ($n = 8$, mean ± SD), (optical density, O.D.)

Groups/Parameters	*Control*	*Blue light*	*Amfenac*	*Melatonin*	*Blue light + amfenac*	*Blue light + melatonin*	*Blue light + amfenac + melatonin*
LP (μmol/g of protein)	5.79 ± 0.30	6.57 ± 0.35[a]	5.89 ± 0.09[b]	5.35 ± 0.34[a,b,c]	5.89 ± 0.10[b,c]	5.68 ± 0.46[b,c,d]	5.14 ± 0.29[a,b,c,d,e]
GSH (μmol/g of protein)	6.20 ± 0.72	2.79 ± 0.74[a]	8.53 ± 1.24[a,b]	6.74 ± 0.62[b,c]	6.12 ± 0.61[b,c,d]	4.47 ± 0.69[a,b,d,e]	5.27 ± 0.83[a,b,c,d,e,f]
GSH-Px (IU/g of protein)	15.87 ± 3.97	15.02 ± 1.52	14.92 ± 0.71	14.85 ± 3.24	15.90 ± 1.11	15.11 ± 0.51	14.80 ± 0.85
Apoptosis (O.D.)	4297.33 ± 97.54	7183.5 ± 277.41[a]	4408.5 ± 229.54[b]	3948.67 ± 64.69[a,b]	4529.17 ± 196.34[b,c]	4625.33 ± 192.07[b,c]	4250.33 ± 118.57[b,c,d]
Caspase-3 (O.D.)	4389 ± 124.01	13 009 + 175.57[a]	4 496 531 + 135.41[a,b]	4 055 436 + 141,13[a,b]	11 665 962 + 152.39[a,b,c]	10 349 262 + 167.78[a,b,c]	10 362 429 + 177.13[a,b,c,d]
Caspase-9 (O.D.)	4851 ± 142,36	1 128 343 + 179.91[a]	5 266 246 + 154.28[a,b]	4 925 7054 + 187.23[a,b]	9 231 453 + 158.88[a,b,c]	9 464 301 + 157.48[a,b,c]	8 018 703 + 148.54[a,b,c,d]
ROS (O.D.)	44 131.8 ± 1954.8	71 401.66 ± 1819.86[a]	66 376.33 + 2905.13[a,b]	44 551.5 + 777.34[b,c]	65 951.67 + 3943.37[a,b,d]	63 077.83 + 2630.89[a,b,d]	56 666.67 + 1875.44[a,b,c,d,e,f]

[a] $P < 0.05$ *vs* control group.
[b] $P < 0.05$ *vs* blue light group.
[c] $P < 0.05$ *vs* amfenac group.
[d] $P < 0.05$ *vs* melatonin.
[e] $P < 0.05$ *vs* blue light + amfenac group.
[f] $P < 0.05$ *vs* blue light + melatonin group.

organism after the reproductive phase of its life. AMD is one of the most abundant diseases, which is characterized by inflammation as a result of excessive levels of oxidative stress parameters.[28] Moreover, increased levels of lipid peroxidation triggers phospholipase A2, which makes alterations in cell membranes, stimulates immune cells, leads to interleukin secretion from T cells.[29] Owing to age-dependent decreases in melatonin secretion, tissues are getting more sensitive against oxidative stress. The free radical theory of exposure to light during eye operations proposes that lighting and some related diseases are, at least in part, a consequence of oxidative stress.[3]

Melatonin is well-known to have a higher antioxidant capacity than that any of other antioxidants such as vitamin E, it may also have protective effects on different types of retinal cells including RPE cells and photoreceptors.[30] Osborne *et al*[31] showed that melatonin can protect cultured retinal pigment cells from oxidative damage and cell death induced by ischemia and reperfusion model. Moreover, Liang *et al*[32] demonstrated that melatonin has a critical role on prevention against degeneration in photoreceptors of *rds* mutant mice.[32,33] It is also possible to find certain evidences suggesting that the absence of MT1 receptor causes acceleration in age-related photoreceptor decrement.[34] There have been some studies that explain the relation between low melatonin levels and pathogenesis of AMD.[28]
We observed that, incubation with melatonin causes significantly reduced amount of apoptosis, caspase-3, caspase-9, and ROS levels when compared with blue light group samples.

In our experimental model, blue light exposure triggered excess amount of ROS production. These findings are consistent with previous studies.[7] Our novel finding is that the combination of amfenac, a nonsteroidal anti-inflammatory drug and melatonin, a hormone which acts also as an antioxidant agent has a strong protective effect against this pathophysiologically relevant process. Secretion of melatonin decreases with increasing intensity of the electromagnetic wave. This makes tissues more delicate against ROS products.

Inflammation activates numerous intracellular signaling pathways, including the MAPK-dependent pathways. MAPK family members are also key contributors in the regulation of cyclooxygenase pathways, synthesizing COX-2. COX-2 drives the inflammatory response and is implicated in some mechanisms of cell death.[35] Amfenac amide is a prodrug that is converted to amfenac by intraocular hydrolases. Amfenac inhibits both cyclooxygenase COX-1 and COX-2 activity. Mitochondria consume about 90% of the cellular oxygen and are the most susceptible organelles to oxidative damage. Furthermore, the mitochondria are

a leading contributor to intracellular free radical production.[36] In accordance with this information and with our results, using amfenac with combination of melatonin will be a new protective agent against light-induced oxidative damage in RPE cells. It has been previously reported that selective inhibition of COX-2 also has a preventative role in the pathway of pathological angiogenesis in the cornea, retina, and experimentally-induced tumors.[37,38] Hence, NSAIDs inhibiting the activity of the COX enzymes may be a possible pharmacological target for the treatment of retinal neovascularization. In addition to these data, Neisman and Saito mentioned that amfenac can inhibit diabetes-induced production of ROS in rat retinas. It is very well-known that ROS activates cytosolic phospholipase A2 (cPLA2) and COX-2, which can trigger the production of pro-angiogenic prostaglandins.[39,40] Cytosolic phospholipase A2 (cPLA2) is the enzyme that is responsible for releasing arachidonic acid, a COX substrate, from membrane-derived phospholipids. It was proved that cPLA2 can have a pro-angiogenic effect on retinal cell behaviors. These data strongly support that the activation of cPLA2 and/or COX-2 may prevent the ROS-dependent and consequently result in prostaglandin-induced angiogenic cell behaviors.[41] As increased levels of prostaglandins along with inflammation have an important role in the pathogenesis of the cystoid macula edema,[42] anti-inflammatory drugs, including amfenac have been shown to significantly decrease the inflammation associated with this condition.[43,44] According to our results, we believe that, this effect of amfenac is probably due to the reduction of the synthesis of PGE2 thus resulting in the inhibition of apoptosis by reducing the production of ROS in retina and choroid tissue. Our study showed significant reduction in ROS production ($P<0.05$).

Lengthy light exposure has been shown to induce histological changes to retinal layers associated with a reduction in cellular impedance in a manner that is dependent on elevated ROS levels.[45] Moreover, inflammation by itself may cause an augmented cellular response to ROS products.[36] Numerous studies implicate ROS signaling in apoptosis, although a direct connection between apoptosis and increased levels of intracellular Ca^{2+} levels with blue light has not been established.[28] Melatonin and its metabolites are able to scavenge the free radicals, which counteract apoptosis[46] and Espino *et al*[10] also previously demonstrated that melatonin's antiapoptotic effects in human leukocytes are likely related to its free radical scavenging effects. In the current cell culture model, we showed that oxidative stress triggers increased levels of Ca^{2+}, whereas melatonin supplementation provides cell survival advantage against elicited levels of Ca^{2+} levels. Although intracellular Ca^{2+} has been presented as one of the key regulators of the cell survival, this cation can also trigger apoptosis in response to many pathological conditions.[47] Finally, our results demonstrate that melatonin and amfenac combination is able to strongly protect ARPE-19 cells against oxidative stress triggered-Ca^{2+} dyshomeostasis.

In conclusion, melatonin and amfenac have protective effects against blue light-induced retinal cell death. Both separately, or even more powerfully in combination, melatonin and amfenac are able to delay oxidative stress-mediated increases in $[Ca^{2+}]_i$, apoptosis, and caspase activation in ARPE-19 cells. These findings have therapeutic implications for AMD- and -related inflammatory diseases.

Summary

What was known before

- One of the reasons that underlies in AMD is oxidative stress.
- NSAIDs are generally used to treat AMD.
- Melatonin is a neurohormone and its levels reduce with age. The reduction in its synthesis makes cells more sensitive against oxidative damage.

What this study adds

- Melatonin and amfenac combination protect ARPE-19 cells against oxidative stress triggered-Ca^{2+} dyshomeostasis.
- Melatonin and amfenac combination delays oxidative stress-mediated caspase activation-related cell death.
- This combination may be useful in AMD treatment.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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