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Minocycline is Cytoprotective in Human Corneal Endothelial Cells and Induces Anti-Apoptotic Bcl-2 and XIAP

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Abstract:

Introduction: Loss of corneal endothelial cells is one major factor limiting transplant clearness and survival after keratoplasty. Beside others, apoptosis due to cellular stress is responsible for this decrease. This study investigates possible anti-apoptotic and cytoprotective effects of Minocycline on a cultured human corneal endothelial cell line (HCEC-SV40) under oxidative stress and TGF-beta.

Methods: Corneal endothelial cells (CEC) were treated with 1µM to 150µM Minocycline. Cell viability and IC50 were evaluated after 48h and after H₂O₂ treatment (tetrazolium-dye-reduction assay and live-dead-assay). Expression of Bcl-2 and XIAP (X-linked inhibitor of apoptosis) and their mRNA were assessed by RT-PCR and Western-Blot analysis after treatment with Minocycline alone and consecutive incubation with 200µM H₂O₂ and TGF-beta-2. A quantitative detection of histone-associated DNA fragmentation by ELISA was performed.

Results: Minocycline concentrations from 1µM to 50µM showed no toxic effects on CECs. Pre-treatment with 10-40µM Minocycline led to an increase in viability after H₂O₂ treatment. In addition, Minocycline pre-treatment attenuated the increase of histone-associated DNA fragmentation after treatment with H₂O₂ and TGF-beta-2 significantly. When CECs cells were treated with Minocycline and then consecutively with H₂O₂ or TGF-beta-2, RT-PCR and Western-Blot analysis yielded an overexpression of Bcl-2 and XIAP.

Conclusion: In this study Minocycline prevented apoptotic-cell-death in cultured CECs *in vitro*. Our results suggest, that Minocycline might offer cytoprotective properties that might help to prevent loss of corneal endothelial cells *in vivo*.

Introduction

Corneal transparency is a major prerequisite for normal vision. The corneal endothelium is comprised of a single layer of cells of neural crest origin, maintains osmotic homeostasis of the cornea and is essential for ensuring corneal clearness ¹. Therefore, a healthy endothelium is essential. Unlike in other mammalian species, capacity of the human corneal endothelium for regeneration after injury is severely limited ^{2 3}. In general, wound repair and cell loss are achieved by the enlargement and migration of the surrounding cells ^{2 3}. The normal endothelial cell count is about 3000 cells/mm² in young adults and decreases to approximately 2000 during a lifetime ⁴. Even if there is no exact threshold, a cell density less than 400-800 cells/mm² often produces corneal opacity through inadequate corneal hydration ¹. Corneal opacities due to endothelial cell loss are commonly irreversible. In such cases, keratoplasty is often the only therapeutic option to improve vision ⁵.

One major quality-criterion for donor corneae for transplantation is the endothelial cell count and the success of corneal transplantation depends at least in parts on a healthy endothelium of the donor cornea ⁵. It has been demonstrated that the deterioration of the corneal endothelium in donor corneas for transplantation occurs more frequently with increasing length of organ culture time and leads to cell loss ^{6 7}. This results in an increased susceptibility of cells.

Reactive oxygen species (ROS) intermediates and oxidative stress are considered to induce apoptotic cell death and have been implicated in the progression of aging ⁸. Among various causes, oxidant species and oxidative stress are considered to be cytotoxic, and it has been suggested that they are responsible for corneal cell death ⁹. There is evidence that this loss of endothelial cells is at least partly due to apoptosis ¹⁰.

Apart from that, many models have shown that apoptotic cell death is a result of changes in environmental stimuli, such as growth factors. The Transforming Growth Factor beta (TGF- β) family includes three multifunctional proteins, TGF- β 1, TGF- β 2 and TGF- β 3, that are found in aqueous humor ¹¹. TGF- β is involved in regulating cell differentiation, cell proliferation and other cell functions ¹². Altered levels of TGF- β have been reported in several ocular conditions and pathologies such as uveitis, glaucoma or diabetes and are considered to be pro-apoptotic ^{11 13-15}. In addition, it has been demonstrated that the activity of TGF- β is related to the production of hydrogen peroxide and induces oxidative stress in lens epithelial cells ¹⁶. TGF- β 2, the isoform of TGF- β most expressed in human eyes, reportedly inhibits the proliferation of corneal endothelial cells ^{17 18}. Furthermore, TGF- β 2 stimulates prostaglandin synthesis in corneal endothelium and inhibits its proliferation in a dose-dependent manner ¹⁸.

Minocycline is a semi-synthetic, second-generation tetracycline. It is cheap, widely available and has proven its safety and its beneficial antimicrobial and anti-inflammatory actions for many years ¹⁹. Besides, Minocycline has recently been demonstrated to have cytoprotective

properties in several cellular systems, and models for neurodegenerative and retinal disease ²⁰⁻²³ . This study evaluates whether Minocycline has toxic effects at various concentrations on cultivated human corneal endothelial cells (CECs). In addition, we investigated the effects of Minocycline, regarding viability, induction of cellular death and apoptosis in CECs. To elucidate potential protective and anti-apoptotic effects of Minocycline, the expression of two anti-apoptotic proteins, Bcl-2 and XIAP (X-linked inhibitor of apoptosis), and their mRNA was quantified.

Methods

Minocycline was obtained as lyophilized powder (Sigma-Aldrich) and reconstituted with sterile water to obtain a 1mM stock-solution. This stock-solution was then further diluted in a 1:1 mixture of M199/F12 medium (M199/F12; Biochrom, Cambridge, UK) to obtain the final concentrations of 1µM, 5µM, 7.5µM, 10µM, 15µM, 20µM, 40µM, 50µM, 75µM, 100µM, and 150µM.

Human Corneal Endothelial Cells

Immortalized, cultured human CECs from a well established cell line (HCEC-SV40) were provided by Prof. J. Bednarz (Hamburg, Germany) ²⁴. CECs were grown in a 1:1 mixture of M199/F12 medium (M199/F12; Biochrom, Cambridge, UK) supplemented with 5% FCS and without addition of antibiotics.

Cell Culture Treatment

For all cell culture experiments, CECs (2×10^5 cells/well) were seeded in 35-mm diameter tissue culture dishes and exposed to Minocycline concentrations (1µM to 150µM). For exposure to the various concentrations of Minocycline, the CECs were kept for 24 hours in serum-free conditions. After the cells were washed with PBS, they were incubated for 24h with various concentrations of Minocycline dissolved in a 1:1 mixture of M199/F12 medium for CECs. Then the serum-free medium containing Minocycline was removed by carefully rinsing the cells with

serum-free medium three times. After another 24h hours of incubation with serum-free medium, cell viability assay, RNA-isolation, or protein extraction was performed.

For testing the various concentrations of Minocycline under conditions of oxidative stress, the cells were treated for 24h with the two tested concentrations of Minocycline (15 μ M and 20 μ M), and then 200 μ M H₂O₂ was added for four hours.

In unpublished pre-work of our laboratory several concentrations of H₂O₂ and times of treatment were tested and the concentration of 200 μ M H₂O₂ and a four hour treatment was chosen in order to produce an adequate degree of cell injury in untreated CECs.

Then the serum-free medium containing H₂O₂ and Minocycline was removed by carefully rinsing the cells with serum-free medium three times. After another 24 hours of incubation with serum-free medium, cell viability assay, RNA-isolation, or protein extraction was performed.

For testing Minocycline in the presence of TGF- β 2, cells were treated for 24h with the various concentrations of Minocycline (15 μ M and 20 μ M) and then 1ng/mL TGF- β 2 was added. After another 24 hours of incubation with Minocycline and TGF- β 2, RNA-isolation or protein extraction was performed.

MTT Assay

Tetrazolium dye-reduction assay (MTT; 3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyl tetrazolium bromide) was used to determine the cell

survival rate. The MTT assay was performed as described in the literature by Mosmann²⁵ with some modifications. The medium was removed, cells were washed with PBS, and 2000 μ L/well MTT solution (1.5 mL MTT stock, 2 mg/mL in PBS, plus 28.5 mL DMEM) was added. CECs were incubated at 37°C for 1 hour. Formazan crystals that formed were dissolved by the addition of dimethylsulfoxide (DMSO; 1000 μ L/well). Absorption was measured by a scanning multiwell spectrophotometer at 550 nm (Molecular Probes, Garching, Germany). The results were expressed as the mean percentage of the control viability. Experiments were performed in triplicate and repeated three times. CECs from the same passage, incubated with balanced saline without the addition of other substances, served as the control. For all experiments, stationary cell cultures were used. The MTT test, as performed in our study, is a well-established test for assessment of cell viability.

Live–Dead Cell-Viability Assay

Confluent CECs were prepared and treated as described above. Cell viability was quantified based on a two-color fluorescence assay. The nuclei of nonviable cells appear red because of staining by the membrane-impermeable dye propidium iodide (Sigma-Aldrich); whereas, the nuclei of all cells were stained with the membrane-permeable dye Hoechst 33342 (InterGen; Purchase, NY; USA). Confluent cultures of CECs growing on coverslips in 35-mm tissue culture dishes were exposed to the various concentrations of Minocycline as described for the MTT assay. For the

evaluation of cell viability, the cells were washed in PBS and incubated with 2.0 µg/mL propidium iodide and 1.0 µg/mL Hoechst 33342 for 20 minutes at 37°C. Subsequently, the cells were analyzed with an epifluorescence microscope (Aristoplan; Leitz, Wetzlar, Germany). The labeled nuclei were then counted in fluorescence photomicrographs, and dead cells were expressed as a percentage of the total nuclei in the field. Data (mean ± SD) is based on the sampling of 6-10 photomicrographs per condition in three independent experiments performed in duplicate. The percentage of dead cells was scored by counting at least 500 cells in fluorescence photomicrographs of representative fields. CECs of the same passage served as the control.

Detection of apoptosis

Apoptosis is characterized by membrane blebbing, condensation of cytoplasm and the activation of endogenous endonucleases. This leads to internucleosomal cleavage of DNA and the generation of mono- and oligonucleosomes, which are tightly complexed with histones. To quantify apoptosis in CECs quantitative detection of histone-associated DNA fragments in mono- and oligonucleosomes, a marker for apoptosis, was performed by an enzyme-linked immunosorbent assay (ELISA). CECs were treated with Minocycline and 200µM H₂O₂ or Tgf-beta2 as described before. Cells were then collected from culture dishes and subjected to a cell death detection ELISA (Roche Applied Science, Indianapolis, IN, USA).

RNA Isolation and Real-Time PCR

The total RNA for Bcl-2 and XIAP detection was isolated from 35-mm tissue culture dishes by the guanidium thiocyanate-phenol-chloroform extraction method. Quantification of Bcl-2 and XIAP mRNA was performed by RT-PCR using a LightCycler Instrument (LightCycler System, Roche Diagnostics, Germany) according to previous descriptions ²⁶. The levels of Bcl-2 and XIAP mRNA were determined as a relative ratio (RR), which was calculated by dividing the level of Bcl-2 and XIAP mRNA by the level of the 18S rRNA housekeeping gene in the same samples. All experiments were performed at least in triplicate and repeated three times.

Primer used for RT-PCR

Target	Length	Position	AT(°C)	%GC	Sequence
Bcl-2	19	3465 - 3483	60	58	agaggtcacgggggctaata
	20	3505 - 3524	60	50	ccaggtaacaaaacCCcaca
XIAP	25	846 - 870	60	36	tttgggacatggatatactcagtt
	25	910 - 934	59	40	agcactttactttatcaccttcacc

Protein Extraction and Western Blot Analysis of Bcl-2 and XIAP

CECs grown on 35-mm tissue culture dishes were washed twice with ice-cold PBS, collected, and lysed in RIPA cell lysis buffer. After centrifugation for 30 min at 19,000g in a microfuge (5810R; Eppendorf, Hamburg, Germany) in the cold, the supernatant was transferred to fresh tubes and stored at -70 °C for future use. The protein content was

measured by the bicinchoninic acid (BCA) protein assay (Pierce; Rockford, IL; USA). Denatured proteins (1.2 mg) were separated under reducing conditions by electrophoresis using a 5% SDS-Polyacrylamide stacking gel and a 12% SDS-polyacrylamide separating gel, transferred with semi-dry blotting onto a polyvinyl difluoride membrane (Roche), and probed with a mouse anti-Bcl-2-antibody or a mouse anti-XIAP-antibody as described previously ²⁶. Chemiluminescence was detected with an imager (LAS-1000; RayTest) and generated light units (LU). The exposure times ranged between 1 and 10 min. All experiments were performed at least in triplicate. Quantification of the chemiluminescence signal by densitometry was performed by using a specific computer software (AIDA; RayTest; Straubenhardt, Germany).

Statistic Evaluation and Expression of Results

All data were analyzed using SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). For all statistical tests, $p < 0.05$ was considered significant. Results of the MTT assay are presented as mean ($\pm$ SD) units of absorbance. Ten individual samples per group were measured in triplicate. Results of the RT-PCR are presented as mean ($\pm$ SD) ratios of the investigated mRNA and 18S rRNA, all experiments were performed in triplicate and repeated three times. Western blot analysis was performed analogously; experiments were performed at least in triplicate in CEC cultures. Analysis of variance with Bonferroni correction in post-hoc testing was applied to investigate statistical significance of differences. To

evaluate the effects of treatment with the various concentrations of Minocycline again univariate ANOVA modelling was performed considering the factors TGF beta treatment and H₂O₂ concentration. Post-hoc testing for significant factors applied Bonferroni correction.

Results

Testing Concentrations of Minocycline in Cultured CECs

No gross abnormalities could be detected in CECs with phase-contrast microscopy for Minocycline concentrations up to 75 μ M (data not shown). The number of cells, counted in phase-contrast microscopy, correlated well with the results of the MTT test.

When CECs that were pre-treated with 15 μ M or 20 μ M Minocycline, were treated with 200 μ M H₂O₂, cells showed only very few signs of toxicity. Control cells treated with 200 μ M H₂O₂, that had no Minocycline pre-treatment, showed pronounced morphological signs of toxicity such as abnormal shape and appearance, cellular lysis, and destruction (data not shown).

MTT-Assay

Minocycline concentrations between 1 μ M and 50 μ M showed no significant toxic effects on CECs (24 hour exposure). No significant decrease in cellular viability was detected compared to the control. Concentrations between 75 μ M ($p=0.696$) and 100 μ M ($p<=0.001$) Minocycline led to a dose-dependent reduction of viability. The concentration of Minocycline that inhibited viability by 50% (IC₅₀) was determined from the dose-response curves and for 24-hour application was approximately 100 μ M (± 6.8 95% CI) for CECs. The tested

Minocycline concentrations of 150 μ M reduced the number of viable cells to less than 30% (Fig. 1).

When cells were treated with 200 μ M H₂O₂ only, a significant decrease in viability was detected, compared to the untreated control cells ($p \leq 0.001$). In contrast, cells that were treated with Minocycline in concentrations between 10 μ M and 40 μ M and then subsequently treated with 200 μ M H₂O₂ showed no significant decrease in cell viability compared to cells that were treated with Minocycline concentrations between 10 μ M and 40 μ M only. When cells were treated with Minocycline in concentrations between 50 μ M and 150 μ M and then subsequently treated with 200 μ M H₂O₂, a pronounced, dose-dependent decrease in the viability of cells was detected. (Fig. 1)

Live/Dead Assay for Corneal Endothelial Cells

Minocycline concentrations between 1 μ M and 75 μ M did not show any significant effect on cell viability on CECs, neither after 24 hours treatment, nor after additional treatment with 200 μ M H₂O₂ for 4 hours. When CECs were treated with Minocycline in a concentration of 20 μ M, no increase of the percentage of non-viable cells could be detected ($p=1.0$). In contrast, pre-treatment with 20 μ M Minocycline did reduce the number of death cells when treated with 200 μ M H₂O₂ for 4 hours ($p \leq 0.001$) compared to controls (Fig. 2). High concentrations of Minocycline ($>75\mu$ M)

induced a marked, dose-dependent reduction of viable CECs (data not shown).

Detection of apoptosis

To investigate the effect of Minocycline on apoptosis due to 200 μ M H₂O₂ or Tgf-beta2 exposure in CECs, a quantitative detection of histone-associated DNA fragments in mono- and oligonucleosomes was conducted by using an ELISA method.

In our experimental set-up both, 200 μ M H₂O₂ ($p \leq 0.001$) and Tgf-beta2 ($p = 0.031$) led to a significant increase of histone-associated DNA fragments in cultured CECs. In contrast, when cells were pretreated with Minocycline for 24 hours and then exposed to 200 μ M H₂O₂ or Tgf-beta2, Minocycline treatment decreased the amount of histone-associated DNA fragments significantly, compared to those cells that were treated with H₂O₂ or Tgf-beta2 alone (Fig. 3).

Expression of Bcl-2 and XIAP mRNA in CECs

Bcl-2 and XIAP messenger RNA expression was detected in every sample. All detected mRNA levels of Bcl-2 and XIAP were normalized to those of 18S rRNA, and values are expressed as the relative ratio (RR) of Bcl-2/18S or XIAP/18S. The findings indicate that treatment with Minocycline leads to an increased mRNA expression of both Bcl-2 and XIAP. In contrast, TGF- β 2 and H₂O₂ treatment significantly decreased mRNA expression of Bcl-2 and XIAP in CECs.

The cells treated with both Minocycline and with TGF- β 2 or H₂O₂ seemed to compensate the decrease of Bcl-2 and XIAP mRNA expression by TGF- β 2 and H₂O₂ reaching mRNA levels of Bcl-2 and XIAP comparable to those of the untreated controls or even higher. In addition the cells pre-treated with Minocycline showed significantly higher levels of Bcl-2 and XIAP mRNA in CECs than cells treated with TGF- β 2 or H₂O₂ only (Fig. 4).

Protein-Expression of Bcl-2 and XIAP in Human CECs

To verify that the decrease of Bcl-2 and XIAP under TGF- β 2 and H₂O₂ treatment and the minocycline-induced increase of Bcl-2 and XIAP in mRNA transcription translates into increased protein synthesis, whole cellular protein extracts whole cellular protein extracts of untreated CECs as the control, cells treated with Minocycline concentrations alone, and cells consecutively treated with TGF- β 2 or 200 μ M H₂O₂. were analyzed by Western blotting. An even protein load in each lane was confirmed by the staining of the polyvinyl difluoride membranes with Coomassie Brilliant Blue after the blotting procedure.

Both treatment with 200 μ M H₂O₂ and TGF- β 2 treatment decreased Bcl-2 and XIAP expression of CECs compared to the control. CECs that were pre-treated with 20 μ M Minocycline and then treated with 200 μ M H₂O₂ showed a significantly lower decrease of both, Bcl-2 and XIAP expression compared to the control than those cells that were treated only with H₂O₂. The same effect was detected for TGF- β 2 treatment. CECs that

were pre-treated with 20 μ M Minocycline showed a significant increased expression of Bcl-2 and XIAP compared to the control. (Fig. 5).

Discussion

Deterioration of the corneal endothelium can lead to endothelial cell loss and irreversible opacification of the cornea. Such loss is even more serious as in humans the capacity of corneal endothelial cell regeneration is limited^{2 3}. Therefore, one major prerequisite for corneal clearness is the endothelial cell count.

Apoptosis is one important mechanism of corneal endothelial cell loss, both in physiological and pathological conditions¹⁰. *In vitro* investigations of bovine corneal endothelial cells have demonstrated that endothelial cell apoptosis is mediated by oxidative stress and that apoptosis is dependent on local environmental factors^{27 28}. The aim of our study was to elucidate whether the tetracycline antibiotic Minocycline offers properties to prevent apoptotic cell death and consecutive CEC loss in a cultured human corneal endothelial cell line *in vitro*. The investigated cell line does not only offer morphological characteristics of the physiological corneal endothelium *in vivo*, but also possesses functional properties, as the capacity to regulate stromal hydration²⁹. The presented data might be useful to prevent endothelial cell loss of cultured donor corneas for transplantation and under certain conditions *in vivo*.

Minocycline is a well-established and safe drug and has proven its antimicrobial and anti-inflammatory qualities for many years¹⁹. In addition to its antibiotic and anti-inflammatory activity, it has shown cytoprotective characteristics against various stimuli, including oxidative stress, ischemia and cytokines^{22 30-32}. The cytoprotective effects of

Minocycline have been suggested to be anti-inflammatory but also anti-apoptotic. Recent reports have demonstrated Minocycline's antioxidant properties and its influence on caspases and nitric oxide synthase activity³³. Its inhibiting effect on the release of apoptotic proteins, such as cytochrome c and Smac/Diablo, and its induction of anti-apoptotic proteins, such as Bcl-2 or XIAP, have been described in several cellular systems^{20 22}.

Our results demonstrate that Minocycline protects cultured corneal endothelial cells from oxidative stress. We were able to show that oxidative stress due to hydrogen peroxide leads to an increase of cell death and apoptosis in the investigated human CEC line. This induction of apoptotic cell death went along with a decrease of two anti-apoptotic proteins – Bcl-2 and XIAP. In contrast, we were able to show that Minocycline is able to attenuate this induction of cell death and that at least one cytoprotective mechanism of Minocycline is its induction of these anti-apoptotic proteins (Bcl-2 and XIAP).

Bcl-2 is generally considered to be a custodian of mitochondrial functional integrity as it stabilizes the mitochondrial membrane against the release of cytochrome c. Through its inhibitory effect on caspase activation, Bcl-2 is able to interrupt apoptosis³⁴. It has been shown that Bcl-2 mediates cell survival and decreases apoptosis in human retinal pigment epithelial cells (RPE)³⁵ and other ocular and non-ocular cell lines^{36 37}. The X-linked inhibitor of apoptosis (XIAP) is another potent and well-characterized anti-apoptotic protein. It is related to the tumor suppressor

protein p35, which suppresses the activity of the apoptosis-promoting key caspases 3, 7, and 9³⁸. It has been demonstrated that the over-expression of XIAP protects injured neuronal cells and photoreceptors from further compromise in vivo^{39 40}.

The growth-factor TGF- β is expressed by almost every cell. Altered levels of TGF- β are associated with several ocular pathologies including glaucoma.¹⁴ It has been demonstrated that both acute and chronic glaucoma can lead to endothelial cell loss and that preoperative glaucoma is a major risk factor for increased endothelial cell loss following keratoplasty^{41 42}. So another important finding of our investigations is that TGF- β 2, the isoform of TGF- β most expressed in human eyes, induced apoptotic cell death in human CECs. This corresponds well to the apoptotic effects of TGF- β described previously in many cellular systems¹⁴. This induction of apoptosis was accompanied by a reduction of Bcl-2 and XIAP levels in CECs. In contrast, we were able to show that Minocycline increases Bcl-2 and XIAP expression on both the RNA level and the protein level, not only under standard conditions but also under conditions of oxidative stress and increased levels of TGF- β 2.

Because of its antibiotic and anti-inflammatory actions, minocycline is widely used and has proven its safety as an oral antibiotic for many years^{19 43}. In our *in vitro* study, treatment of CEC with Minocycline in concentrations up to 50 μ M under standard conditions did not reveal significant toxicity. However, after treatment with hydrogen peroxide,

doses higher than 40 μ M led to a significant decreased viability of cells. In our experimental setting Minocycline concentrations of 20 μ M revealed anti-apoptotic effects. This would correspond to an estimated dose of approximately 750mg/day in human adults, which is undoubtedly a high dosage^{19 44 45}. On the other hand, previous reports have shown that daily tetracyclin doses even higher than 2000mg are still relatively safe in healthy pregnant women¹⁹. Our results from cell-culture experiments seem to indicate that Minocycline protects the corneal endothelium from two potential apoptotic stimuli. In addition, Minocycline increases the expression of two anti-apoptotic protein in cultured CECs. Even if the therapeutic range of minocycline in our setting was limited, it might be worthwhile to further investigate minocycline's anti-apoptotic and cytoprotective effects on the corneal endothelium.

The CECs we used for our experiments have demonstrated morphological characteristics of the physiological corneal endothelium *in vivo*, but also possesses functional properties, such as the capacity to regulate stromal hydration²⁹. Nevertheless, one major limitation of our study might be, that the cells we used for our experiments were from an immortalized, cultured cell line with all its limitations and our results cannot be directly transferred *in vivo*. In addition, it is not clear that the cytoprotective effect of Minocycline seen after short-term exposure also occurs after long-term exposure.

Nevertheless, our findings indicate that Minocycline might offer cytoprotective properties that could help to increase corneal endothelial cell survival and resistance to stress as it occurs under certain pathologic conditions and ocular disease ⁹. The anti-apoptotic, cytoprotective effects of Minocycline described here may provide a basis to prevent corneal endothelial cell loss under such pathologic conditions and help to make these cells less susceptible to cellular stress. Indeed, further investigations are necessary to corroborate this data, but our *in vitro* findings suggest, that Minocycline might offer properties that could potentially prevent corneal endothelial cell loss *in vivo*.

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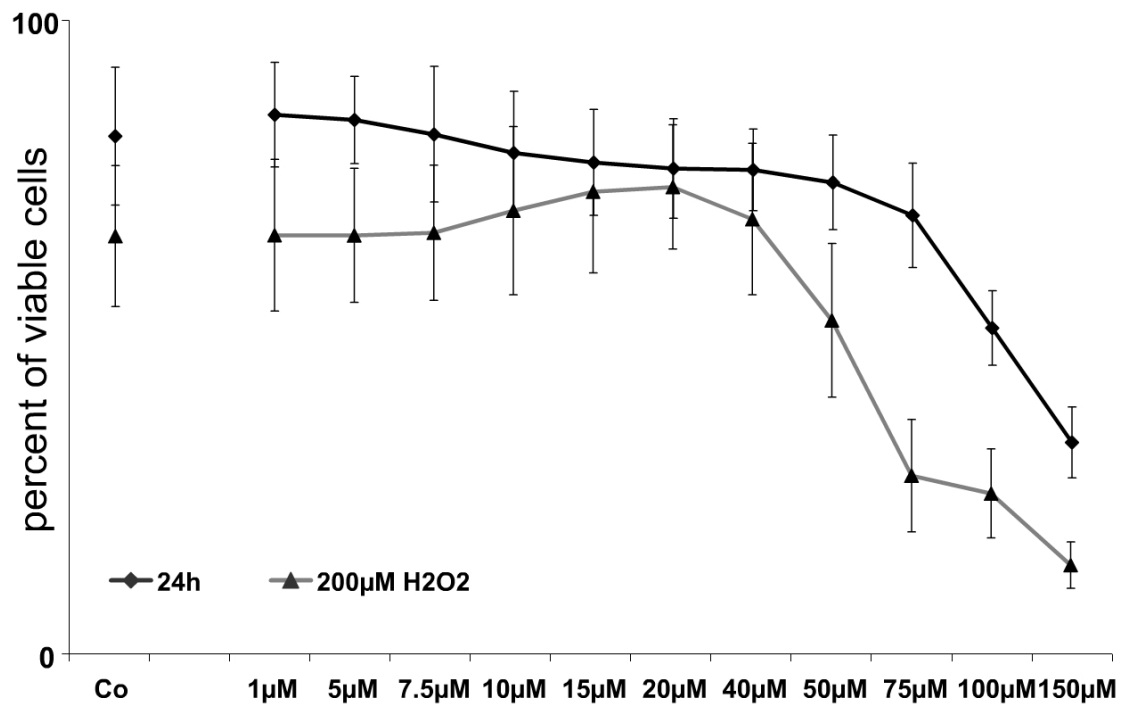


Fig. 1:

The viability of CECs after 24 hour treatment with the concentrations of minocycline investigated (black curve, diamonds) and additionally treated with 200µM H₂O₂ (grey curve, triangles), measured by a colorimetric test (MTT). X-axis: concentrations of minocycline tested. Error bars, SD.

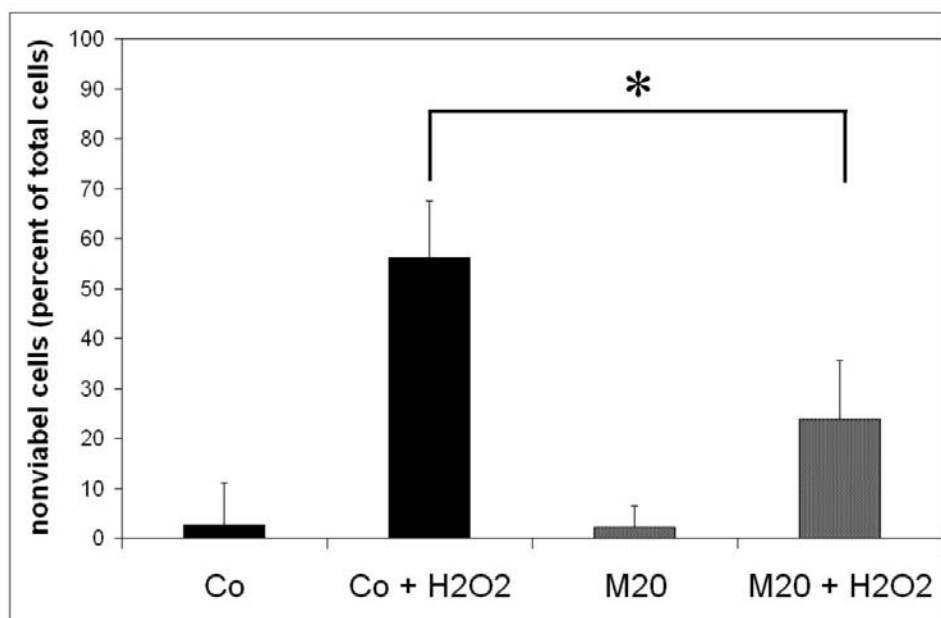
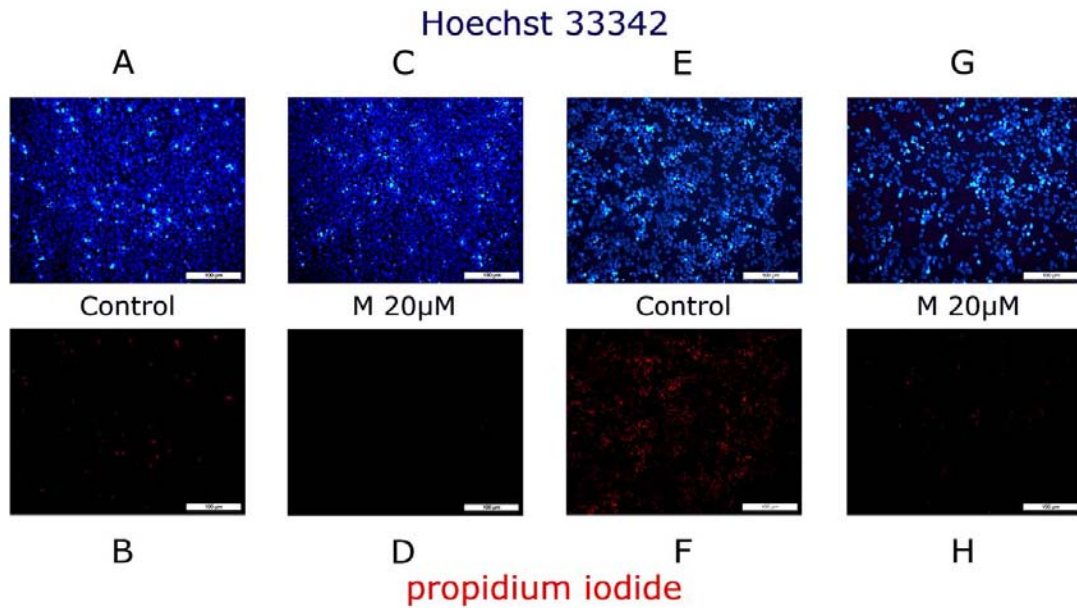


Fig. 2:

CECs were treated for 24 hours with various concentrations of minocycline only or treated with minocycline for 24 hours and 200μM H₂O₂ for 4 hours as described. After exposure of the cells to the substance alone or in combination with oxidative stress, viability was determined by staining all nuclei with Hoechst 33342 and dead cells with propidium iodide. (A)

Representative fluorescence photomicrograph of Hoechst 33342-stained, untreated CECs as the control. (B) Nonviable cells in the corresponding field. (C) Fluorescence photomicrograph of CECs treated with 20 μ M minocycline for 48h and labeled with Hoechst 33342. (D) Nonviable CECs treated with 20 μ M for 48h in the same field. (E) Fluorescence photomicrograph of CECs treated with 200 μ M H₂O₂ only. (F) Nonviable CECs treated with 200 μ M H₂O₂ only in the same field. (G) Fluorescence photomicrograph of CECs treated with minocycline concentrations of 20 μ M for 24h and with 200 μ M H₂O₂ and labeled with Hoechst 33342. (H) Nonviable CECs treated with minocycline concentrations of 20 μ M for 48h and with 200 μ M H₂O₂ in the same field.

(A - H) White scale bar: 100 μ m

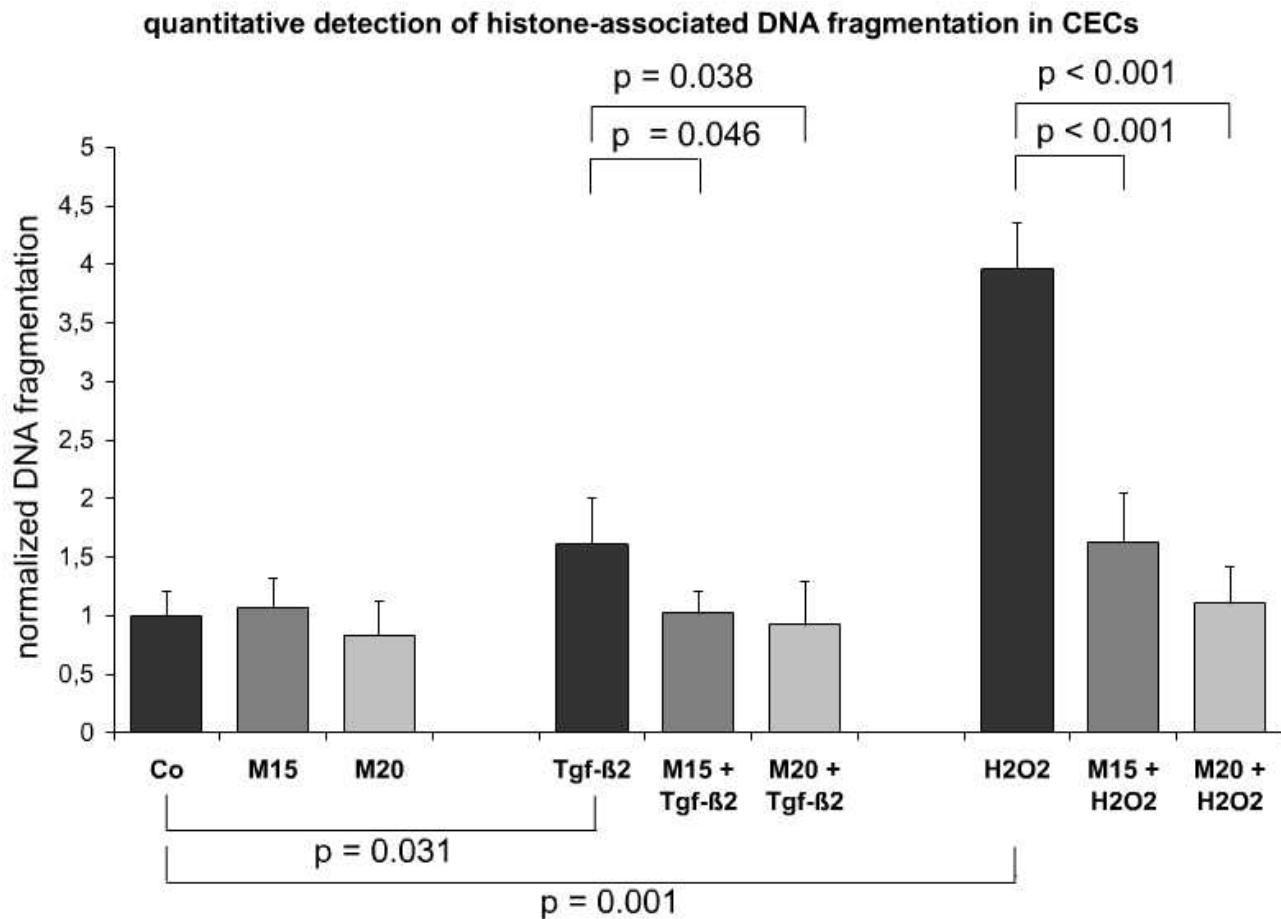


Fig. 3:

Inhibitory effect of Minocycline on Tgf-beta2 and H₂O₂ induced apoptosis in CECs. Histone-associated DNA fragmentation in CECs as investigated by an ELISA method: untreated control (Co), CECs treated with 200μM H₂O₂ (H2O2) or Tgf-beta2 (TGF-β2), after treatment with 15μM and 20μM minocycline (M15 and M20) only, or after Minocycline treatment and additional treatment with 200μM H₂O₂ or Tgf-beta2. Each value was normalized to the mean amount of histone-associated DNA fragments detected in the control. Data values are means ± SD.

Y-axis: ratio of histone-associated DNA fragmentation of each probe normalized to histone-associated DNA fragmentation of the control

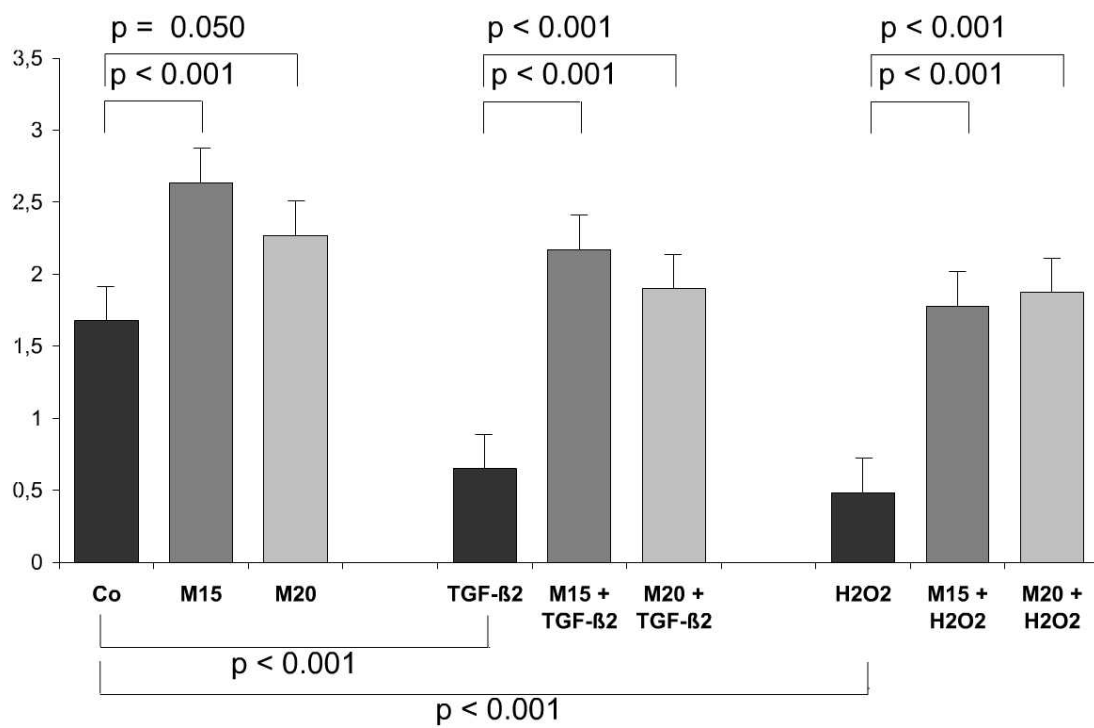
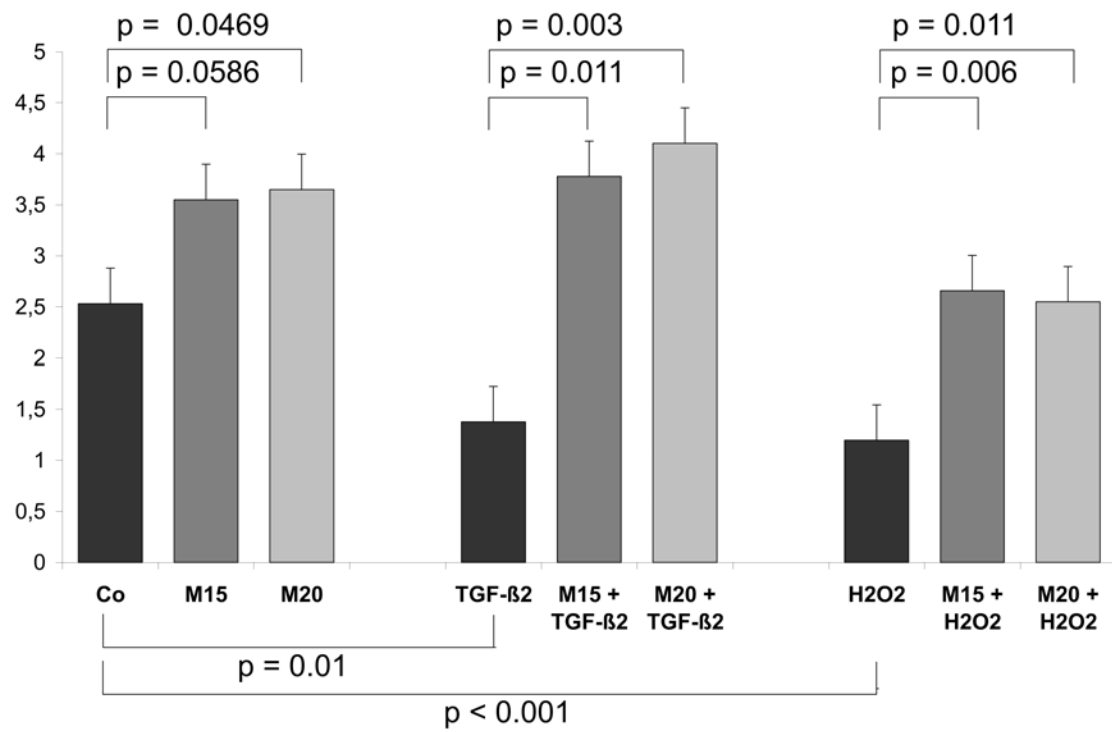


Fig. 4:

Bcl-2 and XIAP mRNA expression of CECs after 24 hours treatment with 15 μ M or 20 μ M minocycline (M15 or M20) only or after additional treatment with 200mM H₂O₂ or TGF- β 2, as investigated by quantitative RT-PCR.

x-axis: concentrations of minocycline tested; y-axis: relative ratio (RR) of Bcl-2 mRNA normalized to 18s rRNA expressed in decimal format.

Protein Expression in CECs

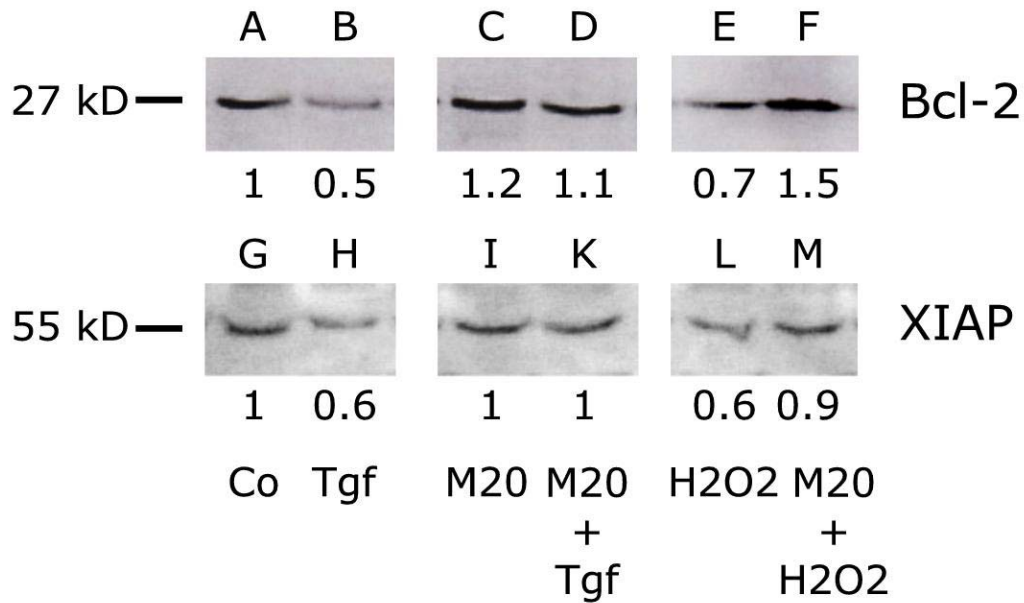


Fig. 5:

Effects of Minocycline-treatment on Bcl-2 and XIAP protein expression. CECs were treated with 20 μM Minocycline for 24 hours only or additionally with TGF-β2 (1 ng/mL) or 200 μM H₂O₂. Western blotting was used to analyze protein expression in the control (A and G) and in treated cell extracts: TGF-β2 (B and H), 20 μM Minocycline (C and I), 20 μM Minocycline and TGF-β2 (D and K), 200 μM H₂O₂ (E and L), and 20 μM Minocycline and 200 μM H₂O₂ (F and M). Protein expression in control cell extracts was set as "1". The values indicate the ratios of treated cell extracts compared to control cell extracts. Ten micrograms of protein were loaded per lane. Even protein load in each lane was confirmed by Coomassie Brilliant Blue staining of the polyvinyl difluoride membranes.