Effect of Tempol, a Membrane-Permeable Free Radical Scavenger, on *In Vitro* Model of Eye Inflammation on Rabbit Corneal Cells

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Abstract

Purpose: Inflammatory corneal diseases such as bacterial keratitis provoke severe injury to the visual functions and physical structure, leading to opaqueness, wounding, damage to the cornea, and even long-lasting vision loss. Usually antioxidant substances have been of great attention as candidate therapies in the management of keratitis in both humans and animals. Based on the findings, the aim of our research was to examine the effects of Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), a membrane-permeable free radical scavenger with exclusive antioxidant properties, on *in vitro* model of eye inflammation of rabbit corneal cells stimulated with lipopolysaccharide (LPS) (Seruminstitute Rabbit Cornea).

Methods: The cells were pretreated with Tempol and incubated with LPS for 24 h. LPS stimulation triggered increased cellular mortality, oxidative stress, cytokine levels expression of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6, and also enhanced prostaglandin E₂ (PGE₂) levels and cyclooxygenase-2 (COX-2) expression.

Results: Pretreatment with Tempol (3 mM) significantly increased cell viability and antioxidant activity as well as decreased reactive oxygen species production, cytokines, PGE₂ levels, and COX-2 expression.

Conclusions: Taken together, Tempol could be a new therapeutic strategy for management of ocular inflammatory disorders for clinical and veterinary use.

Keywords: inflammation, oxidative stress, rabbit corneal, Tempol, keratitis

Introduction

INFLAMMATORY CORNEAL DISEASES provoke severe injury to the visual functions and physical structure, leading to opaqueness, wounding, damage to the cornea, and also long-lasting loss of vision.

Viruses and bacteria represent the most communal infective agents that distress the ocular surface and cause inflammatory damage. When subjected to the insults, the corneal immune response is activated, with the liberation of cytokines and inflammatory cells infiltration.¹ Bacterial keratitis is an important ocular infection and one of the key causes of visual disability in the world.²

Animals other than humans are possible victims of bacterial keratitis. For example, infective bovine keratoconjunctivitis, normally instigated by *Moraxella bovis*, is the

main healthiness difficulty in cattle. *Pseudomonas* also caused keratitis in dogs and horses. Cats could also develop bacterial keratitis, but less than dogs. Several other mammals can acquire keratitis, underlining the incidence of bacteria capable to produce eye pathogenesis.³

During keratitis, stimulated inflammatory cells release inflammatory cytokines, such as interleukin-6 (IL-6), IL-1 β , and tumor necrosis factor- α (TNF- α), which then provoke the necrosis of keratocytes and degradation of collagen in stroma. This could result in the reduction of corneal stroma and successive scarring creation.⁴ Several studies reported that corneal injury during inflammatory condition is connected principally to the polymorphonuclear neutrophil (PMN) infiltration in the corneal tissue with the relief of proteolytic enzymes, arachidonic acid, and reactive oxygen species (ROS).^{5,6}

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In keratitis, inflammation frequently persists and lapses long after the bacteria were removed, causing numerous injuries to the structure and cornea function. While bacteria and virus could be limited by antibiotics, the immune system, when stimulated, has to be controlled due to pathogen products, for example, lipopolysaccharide (LPS).¹ Antioxidant treatment may reduce the inflammatory damage and could have a conspicuous function in the therapy of corneal inflammation. The combination with voriconazole and antioxidant epigallocatechin gallate reduces fungal keratitis in rabbits.⁷ Bucolo et al.⁸ demonstrated that taurine (TAU), small organic molecules, significantly reduced ROS production in corneal [Seruminstitute Rabbit Cornea (SIRC)] cells after oxidative stress.

Furthermore, several experimental results showed that both pirenexine and melatonin prevent the formation of superoxide and oxidative effect in cell culture and entire rabbit corneas submitted to exposure of UV-B.⁹ Taken together, the antioxidant substances have been of pronounced interest as potential therapies for the treatment of keratitis in both humans and animals. In this regard, Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl), a member of a family of nitroxide compounds, has been considerably studied in numerous animal models such as renal ischemia/reperfusion, inflammation induced by zymosan injection, brain injury, and colitis.^{10–14} In addition, regarding ocular pathologies, Tempol protects against cataract, retinal damage, and glaucoma.^{15–17}

Based on the data obtained from the scientific literature, the goal of our study was to study the effects of Tempol, a membrane-permeable free radical scavenger with unique antioxidant properties,^{18,19} on *in vitro* model of eye inflammation of rabbit corneal SIRC line cells, which exhibits several qualities of corneal keratocytes^{20,21} stimulated with LPS (a surrogate for bacterial infection).

Methods

Cell culture and experimental design

SIRC is the used cell line (ATCC_CCL-60) with fibroblast morphology. Based on ATCC history, the SIRC cell line was originated by M. Volkert of the Statens Seruminstitut, Copenhagen, Denmark, from the cornea of a normal rabbit in 1957. Little is documented concerning the history of this cell line for about the first 400 passages. This cell line is aneuploid, with a modal number of 66 chromosomes. A previous paper described for the first time the expression of a mix of epithelial and fibroblastic markers in SIRC cells, suggesting a hybrid description of this cell line. In fact, these cells present both epithelial (occludin) and fibroblastic (lumican, α -SMA, and vimentin) markers.²² In addition, among the cytokeratins, SIRC cells express CK3 and CK12 cornea-specific markers of epithelium.²³ Eagle's minimum essential medium mixed with 10% fetal bovine serum was used for cell culture, in which the cells were cultured at 37°C and 5% CO₂. Subsequently, they were also placed in 6-well plates 24 h before the experimentation at 1.2×10^5 cells/well density in 2 mL (medium). This was changed daily, and the cells were preserved until subconfluence. The cell line was used within the space of 5 passages *in vitro*, starting from fresh frozen batches, to avoid phenotypic drift events.

The SIRCs were treated at different concentrations (1, 3, 6, and 10 mM) of Tempol (dissolved in 1% of DMSO) to

ascertain the high dilutions with less toxicity on cell viability. One percent DMSO was used as control. After that, when we found the high concentration with less toxicity, the cells were preincubated in serum-free medium with 3 mM of Tempol for 1 h (3 mM was selected based on the cell viability test) and then excited with LPS (from *Escherichia coli* 1.0 μ g/mL) for 24 h for all experiments.

Cell viability assay

Cell viability was calculated as described in a previous study.²⁴ The cells were incubated at 37°C with 0.2 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) for 1 h. Cell viability was calculated at 550 nm optical density (OD550) using a microplate reader.²⁴

Western blot analysis

Western blot analysis was done as previously defined²⁴ with specific primary anticyclooxygenase-2 (COX2) (1:500) [Santa Cruz Biotechnology (N-20) Cat# sc-1746, RRI-D:AB_627284] and secondary antibody (1:2,000; Jackson ImmunoResearch, West Grove, PA). The β -actin (1:500, Cat# sc-69879, RRID:AB_1119529; Santa Cruz Biotechnology) was considered as control. A chemiluminescence detection system reagent was used.²⁴

Cytokine production

The proinflammatory levels of cytokines such as TNF- α , IL-6, and IL-1 β were assessed by Elisa kit. TNF- α , IL-6, and IL-1 β ELISA assays were performed according to manufacturer's details (Cat# DY5670, DY7464, DY7984; R&D System, United Kingdom).

Intracellular ROS determination

To better study the effect of Tempol on both oxidative stress and antioxidant capacity of Tempol on SIRC cells stimulated with LPS, we analyzed the ROS content. Intracellular ROS was revealed using the total ROS detection kit as previously displayed.²⁵

Prostaglandin E2 level

To better examine the inflammatory response related to LPS stimulation, we also assessed the prostaglandin E2 (PGE₂) production in supernatants of cells incubated for 24 h. PGE₂ levels were designed in culture medium by a competitive binding ELISA according to the manufacturer's instructions (ab133021; Abcam, Inc., Cambridge, MA).

Superoxide dismutase activity

In the cells, superoxide dismutase (SOD) was calculated by nitro blue tetrazolium (NBT) reaction as previously suggested.²⁶ The reaction mixture, with 0.2 mL of supernatant, 2.4 mL of 50 mM Na₂CO₃, 0.1 mL of 3 mM EDTA, 0.1 mL of 3 mM xanthine, 0.1 mL of 0.8 mM NBT, and 0.1 mL of xanthine oxidase (XO), was saturated with O₂ and incubated for 30 min at 24°C. The inhibition of NBT reduction in each sample was calculated spectrophotometrically at 470 nm. The SOD content, expressed as mU/mg protein, was measured by relating to inhibition by the SOD

standard detected at the same time. The detection limit was 0.4 mU of total activity for assay.

Materials

All compounds were obtained from Sigma-Aldrich Company. All other reagents were of topmost money-making grade obtainable. All stock solutions were prearranged in nonpyrogenic saline (0.9% NaCl; Baxter, Milan, Italy).

Statistical calculation

All values were articulated as mean $\pm$ standard error of the mean of *N* observations. A *P* value of <0.05 was deliberated significant. The outcomes were examined by one-way ANOVA, followed by a Bonferroni *post hoc* test for multiple assessments.

Results

Tempol effect on cell viability

The high concentration of 6 and 10 mM is able to diminish the cell viability by 44% and 16%, respectively, while the

FIG. 1. Effect of Tempol on cell viability. Cell viability was assessed 24 h after treatment at the indicated concentrations (1, 3, 6, and 10 mM) of Tempol with and without LPS stimulation (A, B). Values are given as mean $\pm$ SEM of *n* = 10 wells for each group in duplicate. Each assay was repeated 3 times in different days. Data are representative of 3 independent experiments; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus Ctr; ###*P* < 0.001 versus LPS. LPS, lipopolysaccharide; SEM, standard error of the mean.

concentrations of 1 and 3 mM exhibited no toxic effects on cell viability, which is $\sim 91\%$ and 92% . Moreover, in our study, the cells were stimulated with LPS to produce inflammatory response, and also pretreated with Tempol. The findings acquired reported a relevant protective effect against LPS-induced inflammatory process in cells pretreated with Tempol only at 3 mM (77%), whereas pretreatment at other concentrations showed no significant protection (Fig. 1A, B). Since the concentrations of 6 and 10 mM produced a high reduction of the cell viability, both concentrations were not employed in additional experiments. Based on this, we decided to perform all experiments with 3 mM Tempol.

Tempol effect on ROS production and SOD activity

The untreated control group discharged low levels of ROS, while LPS stimulation considerably increased ROS content (Fig. 2A). Pretreatment with Tempol at 3 mM significantly

FIG. 2. Effect of Tempol on cell ROS production and SOD activity. The figure shows the levels of ROS content (A) and antioxidant SOD activity (B). Values are given as mean $\pm$ SEM of *n* = 10 wells for each group in duplicate. Each assay was repeated 3 times in different days. Data are representative of 3 independent experiments; ****P* < 0.001 versus Ctr; ##*P* < 0.01 versus LPS, ###*P* < 0.001 versus LPS. ROS, reactive oxygen species; SOD, superoxide dismutase.

decreased ROS levels (Fig. 2A). Moreover, SOD activity was calculated. LPS stimulation diminished SOD activity in SIRC cells (Fig. 2B). The concentration of 3 mM of Tempol is able to prevent the decrease in SOD activity (Fig. 2B).

Effect of Tempol on $TNF-\alpha$, $IL-1\beta$, and $IL-6$ levels

A significant increase in the production of $TNF-\alpha$, $IL-6$, and $IL-1\beta$ was analyzed after LPS stimulation (Fig. 3A–C). Nevertheless, pretreatment with Tempol at 3 mM significantly reduced all cytokine levels (Fig. 3A–C).

Effect of Tempol on PGE_2 levels and COX-2 expression

Tempol in SIRC cells (deprived of LPS) had no relevant effect on PGE_2 production (Fig. 4A). As estimated, LPS induced a significant growth in PGE_2 production compared with control (Fig. 4A). Tempol treatment in LPS-stimulated SIRC produced a substantial reduction in PGE_2 levels expression (Fig. 4A). In this regard, COX-2 expression in SIRC (Fig. 4B) after LPS stimulation was also measured. On the contrary, LPS-stimulated SIRC significantly expressed inducible COX-2 matched to control (without LPS) (Fig. 4B). Tempol treatment (3 mM) significantly diminished the LPS-SIRC COX-2 expression (Fig. 4B).

Discussion

Corneal inflammation causes a substantial harm to vision with scraping and hole produced by bacterial keratitis or pathogen products, for example LPS.²⁷ LPS, the main element of the cell barrier of gram-negative bacteria, is a fundamental feature in generating abundant cytokines and triggering inflammatory response in many tissues, such as cornea.² During the inflammation associated with infectious keratitis, PMN aggregation takes place in the cornea. The activation of PMN causes a “respiratory burst” that together with the activation of arachidonic acid cascade results in considerable release of different free radicals in the inflamed cornea, which act as potent oxidizing agents causing protein alteration, DNA degeneration, and lipid peroxidation of the cell membrane. Antioxidant substances have been investigated in diverse models of experimental keratitis demonstrating a protective response.⁵ The role nowadays recognized to oxidative stress in the advance of inflammatory disorders underlines the importance of antioxidant therapy in optimizing the use of treatments for these pathologies.

Among numerous antioxidants offered, Tempol is a membrane-permeable, catalytic, metal-independent antioxidant. It is an interesting compound because it activates the metabolism of O_2^- similar to the activity of SOD, and is considered in fact a mimetic of SOD. However, Tempol abolishes the metabolism of reactive oxygen and nitrogen species, and increases the antioxidant action of the catalase.

FIG. 3. Effect of Tempol on cytokines levels. The figure shows the cytokine levels of $TNF-\alpha$ (A), $IL-1\beta$ (B), and $IL-6$ (C). Values are given as mean $\pm$ SEM of $n = 10$ wells for each group in duplicate. Each assay was repeated 3 times in different days. Data are representative of 3 independent experiments; *** $P < 0.001$ versus Ctr; ### $P < 0.001$ versus LPS. IL, interleukin; ND, not detectable; TNF, tumor necrosis factor.

FIG. 4. Effect of Tempol on PGE₂ levels and COX-2 expression. The figure shows PGE₂ levels (A) and COX-2 expression (B). A representative blot is shown (B). Densitometric analysis of protein expression of 3 gel pictures obtained in 3 different experiments, normalized to β -actin (B) *** P <0.001 versus Ctr; ### P <0.001 versus LPS. COX-2, cyclooxygenase-2; PGE₂, prostaglandin E₂.

Tempol is able to increase the bioavailability of NO and catalytically removes the peroxynitrite species (ONOO⁻), and has been studied in many models of oxidative stress.^{28,29} Thus, radical-induced oxidative stress and inflammation can be modulated by Tempol (TP)-based nitroxide drugs to prevent or treat vascular, ocular, and other pathological conditions and aging-associated disease.³⁰

For these reasons, the aim of our research was to study the effects of Tempol on *in vitro* model of eye inflammation in SIRC cells. In this study, SIRC cell line was used because it presents a morphology that is more coherent with a fibroblastic cell phenotype, comparable with corneal keratocytes.²² Here, we confirmed that Tempol was able to respond to the inflammatory process and oxidative stress caused by LPS in rabbit corneal cells. Moreover, LPS-stimulated SIRC cells showed improved ROS production and diminished cell viability and antioxidant SOD activity. Treatment with Tempol significantly diminished intracellular ROS levels as well as enhanced both cell viability and antioxidant activity. These results are in accord with previous *in vitro* study in which Tempol attenuated PC12 cells damage encouraged by hypoxia with increased cell viability, inhibited oxidative stress, and normalized SOD activity.³¹ The protective effect of Tempol derivatives against retinal damage induced by light has previously been observed in rats.¹⁶

Therefore, Tempol had a significant influence on the cellular skeleton of retinal ganglion cells in methanol-poisoned rats.³² The enrollment of lymphocytes at sites of inflammation is caused by the action of proinflammatory cytokines IL-1 β or TNF- α . Prostaglandins (PGs) have a major contribution to intraocular damage. Several studies in dogs have reported the quantification of PGE₂ levels as a

measure of intraocular inflammation.³³ Furthermore, a significant increase in gene expression of COX-2, IL- β , and IL-8 has been found in an experimental model of uveitis and in human ARPE-19 cells activated by LPS.³⁴ A previous study on keratitis also showed an increase in the expression of COX-2 in the canine cornea.³⁵ Furthermore, mice subdued to hostile conditions showed elevated levels of COX-2, PGE synthase mRNA levels on the ocular surface.³⁴ In this regard, in our research, SIRC excited by LPS have significantly increased levels of TNF- α , IL-1, and IL-6, expression of COX-2, and excessive levels of PGE₂. The administration of Tempol significantly reduced the induction of COX-2 production. Furthermore, the treatment with Tempol in SIRC activated by LPS has elicited a critical decrease in both the cytokines and the PGE₂ levels. Our data are also in agreement with other reports in which Tempol was successful in causing lens epithelial DNA injury and cataract development in x-ray subjected rabbits¹⁵ as well as was able to reduce proinflammatory cytokines, including IL-1 and TNF- α , in ocular hypertensive eyes.¹⁷

In summary, in our study we described that Tempol was able to reduce eye inflammation in *in vitro* model of SIRC cells stimulated with LPS. This research exhibited that Tempol was able to reduce oxidative stress, proinflammatory cytokine levels such as TNF, IL-1, and IL-6, and COX-2 protein that has significant roles in the keratitis disorder, and could exemplify a new therapeutic strategy for management of ocular inflammatory disorders in both humans and animals.

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Authors' Contributions

RC and DI made contribution to conception and design, acquisition of data, interpretation of data, and drafting of the article; EG, MC, and RS were involved in acquisition of data and analysis; DB, SC, and RDP revised critically the article. All authors have read and approved the final article.

Author Disclosure Statement

No competing financial interests exist.

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