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Research Article

Therapeutic Potential of Water-Soluble Resveratrol-Loaded Polyethylene Glycol Monostearate Micelles in a Mouse Model of Dry Eye Disease

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Abstract

Background: Dry eye disease (DED) is a multifactorial ocular surface condition that causes damage and discomfort, significantly impairing the quality of life of patients. Recently, phytochemicals such as trans-resveratrol (Res) have attracted growing interest due to their potential therapeutic effects against the DED-related pathophysiology. To enable the therapeutic application of water-insoluble Res, this study aimed to evaluate the efficacy of a previously developed water-soluble micelle formulation (stPEG/Res) containing polyethylene glycol monostearate (stPEG) in treating DED. **Methods:** The antioxidant activity of stPEG/Res was assessed using the 2,2-diphenyl-1-picrylhydrazyl radical-scavenging assay, and its cytotoxicity was evaluated in a human corneal epithelial cell line. Furthermore, an *in vivo* efficacy study was performed using a mouse model of DED induced by lacrimal gland excision. Tear volume, corneal histopathological changes, inflammatory responses, and apoptosis in the cornea were evaluated. **Results:** The stPEG/Res formulation exhibited antioxidant activity and a cytotoxicity profile against corneal epithelial cells comparable to that of free Res. Furthermore, in the mouse model of DED, stPEG/Res alleviated dry eye symptoms not by restoring tear volume, but by suppressing inflammation and apoptosis in the cornea. **Conclusions:** These findings indicate that stPEG/Res has potential as a novel therapeutic option for corneal disorders associated with DED.

Keywords: dry eye syndromes; resveratrol; micelles; drug delivery systems; cornea; inflammation; apoptosis

1. Introduction

Dry eye disease (DED) is a chronic condition that causes damage and discomfort to the ocular surface due to abnormalities in tear production and quality. When severe, it results in visual impairment and a marked reduction in quality of life [1,2]. Epidemiological studies indicate that DED affects a substantial portion of the population—ranging from approximately 5% to 50% worldwide depending on diagnostic criteria—with higher prevalence in older adults [3]. Current treatments, including artificial tears, punctal plugs, and anti-inflammatory agents, can alleviate symptoms; however, there remains an unmet need for novel therapies that address both the inflammatory and oxidative aspects of DED pathology [4].

The pathology of DED involves multiple molecular mechanisms, including oxidative stress, inflammation, and apoptosis; therefore, therapeutic approaches targeting these processes are necessary [5]. Trans-resveratrol (Res), a polyphenolic compound found in grapes and red wine, possesses several pharmacological activities, including antioxidant and anti-inflammatory effects [6]. These properties suggest that Res may ameliorate DED pathology, and several studies have reported that Res-based eye drops protect the ocular surface [7,8,9].

However, the major obstacle to the clinical use of Res is its very poor water solubility. In water, its solubility is less than 0.05 mg/mL, which makes formulation difficult and limits bioavailability [10]. Consequently, systemic or topical delivery of Res often requires high doses or harsh solvents, and ocular formulations of Res have not yet been successful.

To overcome this limitation, a water-soluble micellar formulation of Res was developed using a polyethylene glycol (PEG)-based surfactant, PEG monostearate (stPEG) [11]. Its hydrophilic–lipophilic balance can be tuned by adjusting the PEG chain length, enabling optimal solubilization of hydrophobic drugs. In previous studies, stPEG micelles were used to solubilize lipophilic antioxidants (Res, α-tocopherol, and coenzyme Q10) while maintaining their biological activity [12,13]. Intravenous administration of water-soluble Res micelles (stPEG/Res), composed of stPEG with 40 PEG repeats and Res, significantly improved neurological and motor outcomes in a mouse model of intracerebral hemorrhage [11].

The success of the previous study indicates that stPEG/Res can effectively deliver bioactive Res *in vivo*. Moreover, stPEG is a surfactant used in the approved eye drop Papilock Mini and has attracted attention as a safe and effective ocular drug delivery carrier with high intracellular delivery capability [14].

Considering these findings, we hypothesized that stPEG/Res may be beneficial for treating ocular surface diseases. In particular, the water-soluble stPEG/Res eye drop enables localized delivery of Res to the cornea and conjunctiva without the use of toxic solvents. The nanoscale micelle-forming capability of stPEG is expected to enhance corneal penetration and intracellular delivery of Res while minimizing irritation. Therefore, this study evaluated the stPEG/Res formulation *in vitro* and in an established DED mouse model. The objective was to determine whether topical administration of stPEG/Res can suppress inflammation on the ocular surface.

2. Materials and Methods

2.1 Materials

Res, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and resazurin were obtained from Tokyo Chemical Industry (Tokyo, Japan). Fetal bovine serum (FBS) and normal goat serum were obtained from Japan Bioserum (Fukuyama, Japan) and Vector Laboratories (Burlingame, CA, USA), respectively. Medetomidine, midazolam, and butorphanol tartrate were obtained from Kyoritsu Seiyaku (Tokyo, Japan), Alfresa (Osaka, Japan), and Meiji Seika Pharma (Tokyo, Japan), respectively. Povidone-iodine was obtained from Mundipharma (Tokyo, Japan). Mayer's hematoxylin solution and 0.5% aqueous Eosin Y solution were purchased from Merck Millipore (Burlington, MA, USA). Purified mouse anti-iNOS/NOS type II antibody (RRID: AB_397718) was purchased from BD Biosciences (Franklin Lakes, NJ, USA; Cat# 610328). Goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody Alexa Fluor 488 (RRID: AB_2576217), goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody Alexa Fluor 594 (RRID: AB_2534091), and proteinase K were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The kit used for the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was obtained from Takara Bio Inc. (Shiga, Japan). stPEG and rabbit anti-Iba1 antibody (RRID: AB_839504) were sourced from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), which also supplied the remaining general reagents and solvents. Reagents not described above were used as received at the highest purity grade available from commercial suppliers.

2.2 Cell Culture and Animals

The human corneal epithelial cell line (HCE-T) used in this study (RIKEN RCB2280) was obtained from RIKEN BRC (Tsukuba, Japan). This cell line was originally established by Araki-Sasaki et al. [15] through immortalization with a recombinant SV40-adenovirus vector. The HCE-T cell line was authenticated by human STR profiling. During this study, mycoplasma contamination was examined by indirect DNA fluorochrome staining using 4′,6-diamidino-2-phenylindole (DAPI). No extranuclear punctate DNA staining indicative of mycoplasma contamination was observed, and the HCE-T cells were considered negative for mycoplasma contamination. The HCE-T cell line information was checked using the Cellosaurus database (CVCL_1272), and the cell line was not listed in the ICLAC Register of Misidentified Cell Lines. Cells were grown in 100-mm culture dishes (Corning, NY, USA) using DMEM/F12 supplemented with 5% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL), and were maintained at 37 °C in a humidified incubator under 5% CO_2 and 95% air. Subculturing was performed using 0.25% trypsin–1 mM EDTA solution when the cells reached approximately 80–90% confluence.

Male ddY mice aged four weeks were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). All animals were kept in cages containing sawdust bedding, with food and water available ad libitum. Animal experiments were performed in accordance with the ARRIVE guidelines, Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals used for scientific purposes, and the Fukuyama University guidelines for animal experimentation (approval number: 2025-A-06, approved on October 24, 2025). No predefined inclusion or exclusion criteria were established, and no animals or data points were excluded from the analyses. Mice were randomly allocated to experimental groups by simple random allocation. The surgical procedures, treatment schedule, and measurement procedures were standardized across animals to minimize procedural variability. Outcome assessment and data analysis were performed by investigators blinded to group allocation.

Mice were housed under controlled environmental conditions at 22 ± 1 °C and 50 ± 10% relative humidity. A total of 12 mice were used in the in vivo DED study. The animals were divided into two treatment groups: DED + vehicle group (n = 6 mice) and DED + stPEG/Res group (n = 6 mice). In each mouse, the contralateral eye underwent sham operation and served as the sham-operated control. Histological, immunohistochemical, and TUNEL analyses were performed using sections prepared from the same set of animals. For these analyses, sections from four independent mice per group were evaluated.

2.3 Preparation of stPEG/Res Micelles

stPEG/Res micelles were formulated as described previously [11]. Briefly, 400 mg of stPEG was dissolved in 80 mL of distilled water with continuous stirring. A separate Res stock solution was prepared by dissolving 40 mg of Res in 2 mL of acetone. This stock solution was slowly mixed

with the stPEG solution, and stirring was continued for 3 h at room temperature to allow acetone evaporation. The mixture was then lyophilized under light-protected conditions to yield stPEG/Res powder. The powder was then protected from light and stored at 4 °C. Before use, the powder was reconstituted in distilled water or 5% glucose solution by sonication.

2.4 DPPH Radical-Scavenging Assay

The *in vitro* antioxidant activity was determined by a DPPH radical-scavenging assay based on a previously reported method with minor modifications [16]. In brief, an ethanolic DPPH solution (200 µmol/L) was prepared, and test samples were prepared in ethanol at various Res-equivalent concentrations. Aliquots of each sample (100 µL) and DPPH solution (100 µL) were added to a 96-well plate and reacted for 30 min at 40 °C in the dark. Absorbance at 517 nm was recorded with an Infinite 200 PRO microplate reader (Tecan Japan, Kanagawa, Japan).

For background correction, three control preparations were used: a mixture of ethanol (100 µL) and DPPH solution (100 µL) as blank 1, ethanol alone (200 µL) as blank 2, and a mixture of sample solution (100 µL) and ethanol (100 µL) as the sample blank. DPPH radical-scavenging activity (%) was expressed as follows:

$$\text{Radical-scavenging activity (\%)} = \{[(\text{A\_B1} - \text{A\_B2}) - (\text{A\_S} - \text{A\_SB})]/(\text{A\_B1} - \text{A\_B2})\} \times 100.$$

where A_B1, A_B2, A_S, and A_SB denote the absorbance values of blank 1, blank 2, sample, and sample blank, respectively.

2.5 In Vitro Toxicity

The *in vitro* toxicity of stPEG/Res was evaluated based on its cytotoxicity toward HCE-T cells using the resazurin reduction assay [17]. HCE-T cells were plated at 5×10^4 cells/well in 24-well plates (Violamo, Osaka, Japan) and cultured for 24 h before treatment. After preincubation, cells were exposed to test samples for 24 h. The resazurin assay was then performed by incubating the cells for 4 h with a working solution prepared by mixing resazurin solution (0.1 mg/mL in phosphate-buffered saline [PBS]) and culture medium at a 1:9 ratio. Fluorescence derived from resazurin reduction was recorded with an Infinite 200 PRO microplate reader (Tecan Japan, Kanagawa, Japan) at excitation and emission wavelengths of 544 and 590 nm, respectively.

2.6 DED Mouse Model and Ophthalmic Administration

A DED mouse model was established by exorbital lacrimal gland (ELG) excision [18]. Mice were anesthetized via intraperitoneal injection of a mixed anesthetic solution containing medetomidine, midazolam, and butorphanol tartrate at working concentrations of 0.03, 0.4, and 0.5 mg/mL, respectively. The corresponding doses were 0.3, 4, and 5 mg/kg, respectively. The preauricular region was then incised to expose and remove the ELG. The incision site was sutured with silk surgical thread, and povidone-iodine was applied to prevent infection. ELG excision was performed unilaterally, while a sham operation was conducted on the contralateral side. On postoperative days 10, 11, 12, and 13, stPEG/Res was instilled into the eyes three times daily at intervals of 4–12 h. For administration, stPEG/Res was dissolved in a 5% glucose solution to yield a Res concentration of 0.1% (w/v). The vehicle control (stPEG) was administered as a 1.0% (w/v) stPEG solution.

2.7 Tear Volume Measurement

Tear secretion in each eye was measured using phenol red–impregnated cotton threads (Zone-Quick; Ayumi Pharmaceutical Corporation, Tokyo, Japan) [19]. Mice were manually restrained without anesthesia, and the threads were gently placed at the lateral canthus for 20 s. The length of the moistened portion was then measured immediately.

2.8 Histological Analysis Using Hematoxylin-Eosin (H&E) Staining

H&E staining was performed to examine histological changes in ocular tissues. Two weeks after surgery, mice were anesthetized using the protocol described in Section 2.6 and euthanized by decapitation. Whole eyes were collected immediately. The isolated eyes were fixed in 4% paraformaldehyde for 1 h, transferred to 15% sucrose, and kept overnight at 4 °C for cryoprotection. After freezing on dry ice, the specimens were stored at −80 °C before cryosectioning. Sections were cut at a thickness of 12 µm with a cryostat (Leica Biosystems, Wetzlar, Germany) and placed on CREST-coated glass slides (Matsunami Glass Ind., Ltd., Osaka, Japan). H&E staining was carried out using Mayer's hemalum solution and 0.5% aqueous Eosin Y solution following standard histological procedures.

2.9 Immunohistochemical Staining

Double immunofluorescence staining for Iba1 and iNOS was conducted to assess macrophage-associated inflammatory responses. Two weeks after surgery, sections of whole eyes were prepared and mounted on slides as described for H&E staining (Section 2.8). Before antibody labeling, the sections were treated for 1 h at room temperature with a blocking/permeabilization buffer composed of PBS, 0.1% Triton X-100, and 5% normal goat serum. The sections were subsequently reacted overnight at 4 °C with rabbit anti-Iba1 antibody (1:500) and purified mouse anti-iNOS/NOS type II antibody (1:50). After rinsing with PBS, the sections were treated for 1 h at room temperature with cross-adsorbed goat anti-rabbit IgG (H+L) labeled with Alexa Fluor 488 and cross-adsorbed goat anti-mouse IgG

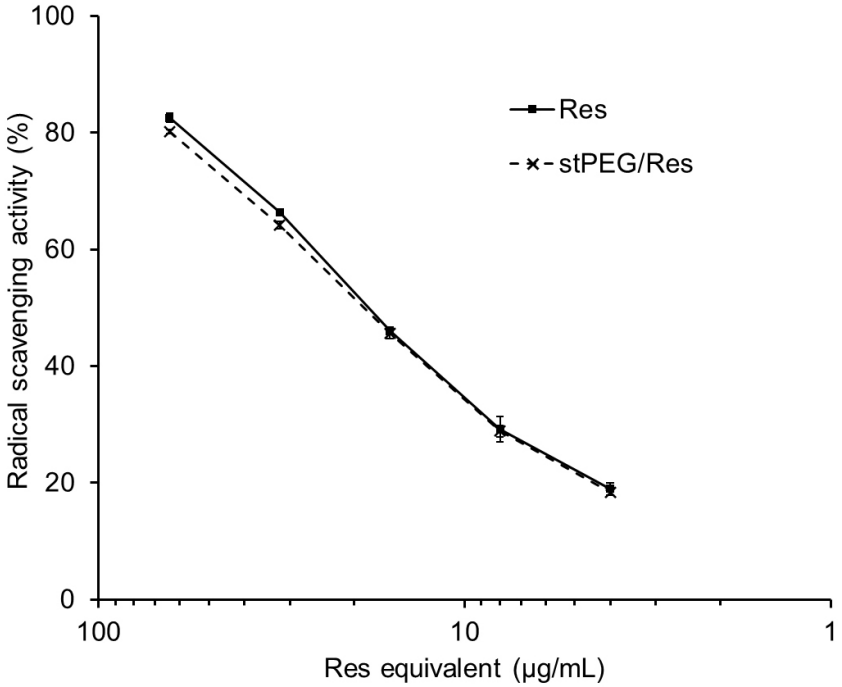

Fig. 1. Antioxidant activity of stPEG/Res evaluated using the DPPH radical-scavenging assay. The antioxidant activity of Res and the stPEG/Res micellar formulation was evaluated using the DPPH radical-scavenging assay. Radical-scavenging activity was determined after incubation with each sample at the indicated concentrations. Data are presented as mean ± SD (n = 3). The n = 3 refers to three independent experiments, not technical triplicates. Statistical comparisons between Res and stPEG/Res at each Res-equivalent concentration were performed using Student's *t*-test. stPEG, polyethylene glycol monostearate; Res, trans-resveratrol; DPPH, 2,2-diphenyl-1-picrylhydrazyl; SD, standard deviation.

(H+L) labeled with Alexa Fluor 594, both diluted 1:250. Fluorescence images were captured with an FV3000 confocal laser-scanning microscope (Olympus, Tokyo, Japan).

2.10 TUNEL Assay

To detect apoptosis, TUNEL assay was performed. Two weeks after surgery, sections of whole eyes were prepared and mounted on slides as described for H&E staining (Section 2.8). The sections were treated with proteinase K (10 μg/mL; Thermo Fisher Scientific, Waltham, MA, USA) for 5 min at room temperature to digest cellular proteins and expose DNA. After permeabilization, the labeling reaction was performed for 90 min at 37 °C using a reaction mixture containing the TdT enzyme (1:10). Fluorescence images were captured with the same confocal microscope described in Section 2.9.

2.11 Statistical Analysis

All results are presented as mean ± standard deviation (SD). Student's *t*-test was used for two-group comparisons, and one-way analysis of variance followed by Tukey's honestly significant difference test was used for multiple-group comparisons. Statistical significance was set at $p < 0.05$. Statistical analyses were conducted using R software, version 4.3.0 (R Foundation for Statistical Computing, Vienna, Austria). For the animal experiments, no formal a priori sample size calculation was performed. The sample size was selected based on experimental feasibility and the minimum number of animals considered necessary to evaluate tear secretion, histological changes, inflammatory responses, and apoptosis in this exploratory study.

3. Results and Discussion

3.1 Evaluation of In Vitro Antioxidant Activity and Toxicity of stPEG/Res Micelles

The ability of Res to scavenge free radicals is considered one of the main mechanisms underlying its antioxidant effects [7,20]. Therefore, the *in vitro* antioxidant activity of stPEG/Res was evaluated by measuring its radical-scavenging capacity using the DPPH assay (Fig. 1). The Res-equivalent concentrations required to achieve 50% DPPH radical-scavenging were 18.3 μg/mL for Res and 18.8 μg/mL for stPEG/Res. In addition, no significant difference in radical-scavenging activity was observed between free Res and stPEG/Res at each Res-equivalent concentration tested, indicating that stPEG/Res retained antioxidant activity comparable to that of free Res. Because the DPPH assay was conducted in ethanol, the micellar structure was likely disrupted during the assay. Thus, the DPPH results demonstrate that neither the presence of stPEG nor the micelle formulation process affected the antioxidant function of Res. Therefore, these findings indicate preser-

Table 1. Previously characterized physicochemical properties of the stPEG/Res formulation.

Formulation	Res content (% w/w)	Diameter (nm)	Zeta potential (mV)	Saturation solubility of Res (mg/mL)
stPEG/Res	8.6 ± 0.6	11.8 ± 0.2	−17.6 ± 4.6	12.30 ± 0.35

Data are summarized from our previous study [11], in which the same stPEG/Res formulation was characterized. Values are presented as mean ± SD (n = 3). The n = 3 refers to three independent formulation preparations.

vation of the intrinsic antioxidant activity of Res after formulation, but they should not be interpreted as evidence of sustained release from intact micelles or enhanced bioavailability. The stPEG/Res formulation used in this study was prepared based on our previously developed micellar formulation [11], and its previously characterized physicochemical properties are summarized in Table 1 (Ref. [11]). The preservation of antioxidant activity observed here is consistent with previous reports showing that stPEG micelles can solubilize lipophilic antioxidants without diminishing their radical-scavenging properties [12,13].

Next, the cytotoxicity of stPEG/Res toward corneal epithelial cells was assessed *in vitro*. The HCE-T cell line is a reliable model for predicting *in vivo* corneal toxicity of ophthalmic drugs [21,22]. As shown in Fig. 2, although cell viability decreased at higher Res-equivalent concentrations, the cytotoxicity of stPEG/Res at each tested Res-equivalent concentration was comparable to that of free Res, and no significant difference was observed between the two groups. These results suggest that micellization with stPEG did not increase the cytotoxicity of Res in HCE-T cells. Collectively, these *in vitro* findings indicate that micellization with stPEG did not markedly compromise the antioxidant activity or cytotoxicity profile of Res.

3.2 Evaluation of In Vivo Efficacy of stPEG/Res Micelles Using the DED Mouse Model

To evaluate the *in vivo* efficacy of stPEG/Res, an ELG-excision mouse model of DED was employed. This model recapitulates key pathological features of DED, including reduced tear secretion, corneal epithelial damage, and ocular surface inflammation [18]. In this lacrimal gland–resected model, tear volume was measured using phenol red–impregnated cotton threads. The sham-operated group exhibited a tear volume of 2.8 ± 0.6 mm, whereas no measurable tear volume was detected in either the vehicle or stPEG/Res-treated groups (Fig. 3). These findings confirm that the ELG-excision model successfully reproduces dry eye conditions and that stPEG/Res does not stimulate tear secretion. Current therapeutic eye drops for dry eye primarily improve ocular surface health through tear supplementation and/or anti-inflammatory mechanisms [4]. Therefore, the efficacy of stPEG/Res was further evaluated in terms of its protective effects against DED-induced corneal epithelial damage.

First, histological evaluation was conducted using H&E staining (Fig. 4). In the vehicle-treated eyes, corneal ulceration and infiltration of inflammatory cells were observed (arrows). In contrast, these pathological changes were not observed in the stPEG/Res-treated group. These results suggest that stPEG/Res reduced the histological signs of DED, including corneal epithelial damage and inflammatory cell infiltration. Although the cornea is an avascular tissue, infiltration of inflammatory cells is frequently observed. This phenomenon is primarily attributed to leukocyte extravasation from the limbal and conjunctival vasculature, followed by directed migration into the corneal stroma in response to chemokine gradients [23,24].

Next, immunohistochemical staining for Iba1 (a macrophage/microglial marker) and iNOS (a proinflammatory enzyme) was performed (Fig. 5). The results showed that iNOS-positive inflammatory macrophages were evident in the vehicle-treated group (arrows), whereas inflammation surrounding macrophages was markedly reduced in the stPEG/Res-treated corneas, consistent with the H&E staining results. These findings suggest that stPEG/Res effectively suppresses corneal inflammation in DED. Free Res has previously been reported to inhibit iNOS expression in inflammatory models [25]; our results indicate that stPEG-mediated delivery achieves comparable anti-inflammatory effects in the cornea, as evidenced by the reduced infiltration of inflammatory cells into the corneal tissue.

To further investigate the protective effects of stPEG/Res on the corneal epithelium, apoptotic cell death—a hallmark of epithelial damage associated with DED—was evaluated. Apoptosis plays a crucial role in the pathogenesis of DED, as excessive oxidative stress and inflammation can cause epithelial cell loss and disruption of the ocular surface [26]. Therefore, TUNEL staining was performed to visualize apoptotic cells in the different treatment groups. Interestingly, TUNEL staining revealed distinct patterns of apoptosis in the corneal tissue of stPEG/Res-treated DED mice compared with vehicle-treated mice (Fig. 6). In the vehicle group (stPEG alone), apoptotic signals were primarily localized in the corneal stroma (arrows), consistent with tissue damage induced by DED [18]. DAPI staining also confirmed ulceration (arrows), in agreement with the H&E staining results (Fig. 4). In contrast, the stPEG/Res-treated group exhibited apoptotic cells predominantly in the outermost epithelial layer of the cornea, with minimal or no staining observed in the stroma. No apoptotic signals were detected in either layer of the cornea in sham-operated mice. Because the vehicle consisted of stPEG alone and did not induce superficial epithelial apoptosis, it is unlikely that the micelle carrier itself

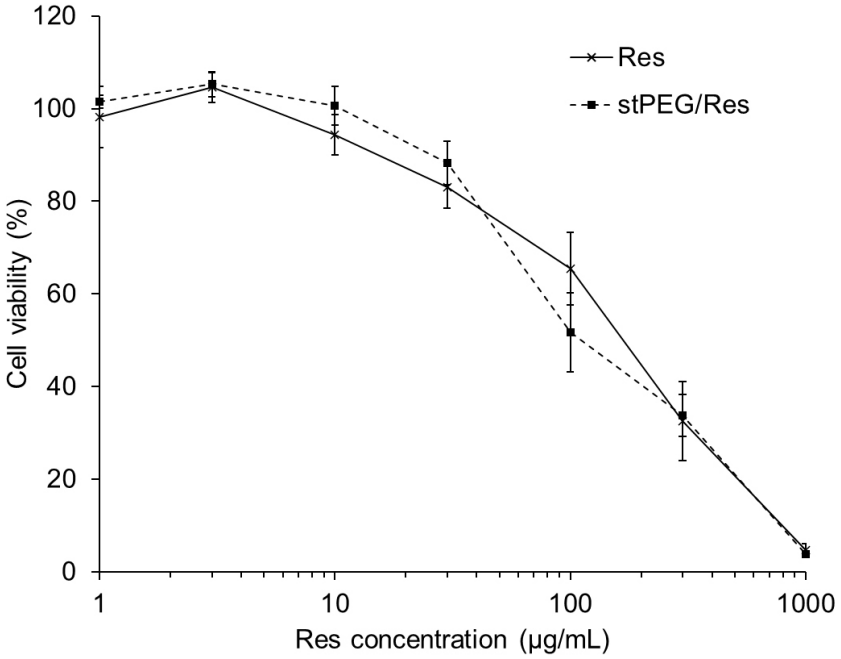

Fig. 2. ***In vitro*** **cytotoxicity of stPEG/Res in HCE-T cells**. The cytotoxicity of Res and stPEG/Res was assessed in human corneal epithelial (HCE-T) cells using the resazurin reduction assay. Cells were treated with the indicated concentrations of each sample for 24 h, followed by incubation with a resazurin-containing solution. Cell viability is expressed as a percentage relative to untreated control cells. Data are shown as mean ± SD (n = 3). Statistical comparisons between Res and stPEG/Res at each Res-equivalent concentration were performed using Student's *t*-test.

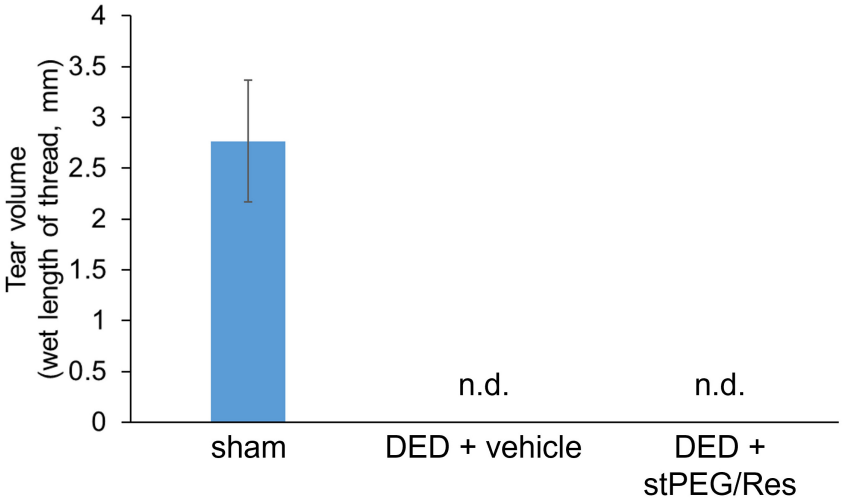

Fig. 3. Tear volume measurement in a lacrimal gland–excised mouse model of dry eye disease. Tear secretion was measured using phenol red–impregnated cotton threads in sham-operated mice, vehicle-treated DED mice, and stPEG/Res-treated DED mice. Tear volume was quantified as the length of the moistened thread after 20 s. Data are expressed as mean ± SD (n = 6). DED, dry eye disease; n.d., not detected.

was responsible for this phenomenon. Rather, the epithelial apoptosis observed in the stPEG/Res group may reflect a pharmacological effect of Res. Res has been reported to induce apoptosis selectively in damaged cells [27], suggesting a potential mechanism for removing dysfunctional epithelial cells. Alternatively, this epithelial apoptosis may represent a regulated tissue remodeling process that promotes epithelial regeneration and barrier restoration under oxidative stress conditions. These findings warrant further investigation into the temporal dynamics of epithelial turnover and repair following stPEG/Res treatment. Together with the H&E and Iba1/iNOS staining results, the

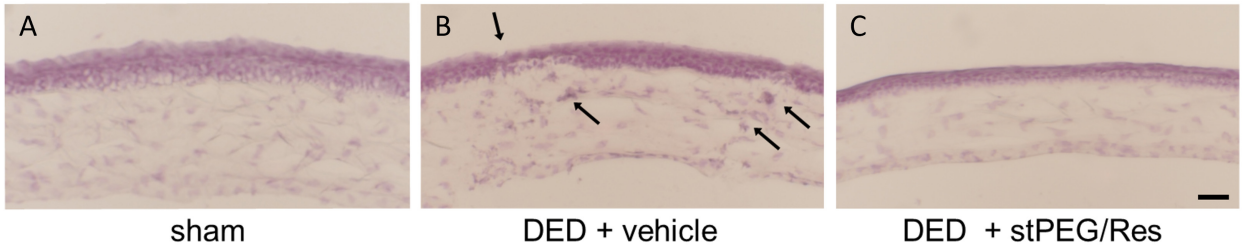

Fig. 4. Histological evaluation of corneal tissue using H&E staining. Representative H&E–stained corneal sections are shown for (A) sham-operated mice, (B) vehicle-treated DED mice, and (C) stPEG/Res-treated DED mice. Arrows indicate corneal ulceration and inflammatory cell infiltration. Images are representative of four independent mice per group. Scale bar = 50 μm. H&E, Hematoxylin-Eosin.

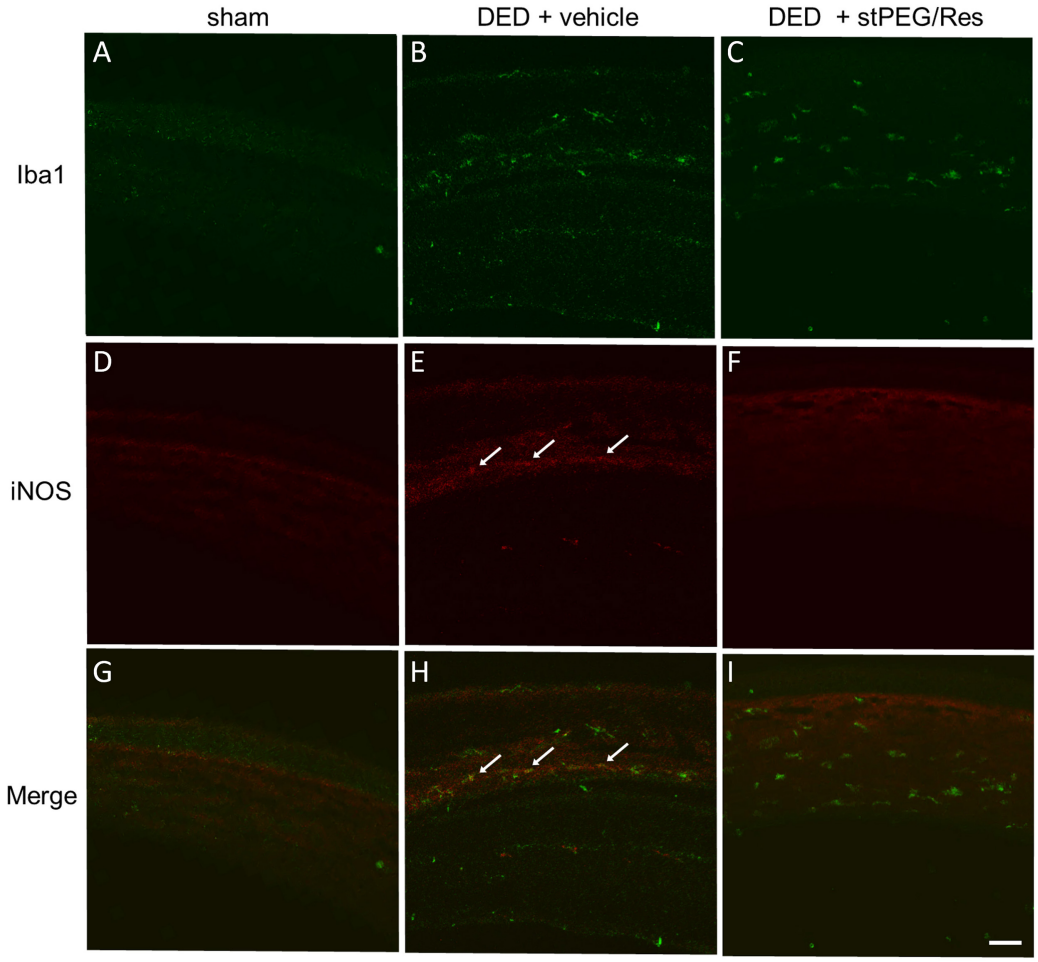

Fig. 5. Immunohistochemical analysis of corneal inflammation markers. Representative immunofluorescence images of corneal sections stained for Iba1 and iNOS are shown. Panels (A–C), (D–F), and (G–I) show Iba1 staining, iNOS staining, and merged images, respectively. In each row, the left panels (A,D,G), middle panels (B,E,H), and right panels (C,F,I) correspond to sham-operated mice, vehicle-treated DED mice, and stPEG/Res-treated DED mice, respectively. Iba1-positive cells are shown in green, and iNOS-positive signals are shown in red. Arrows indicate representative iNOS-positive inflammatory cells. Images are representative of four independent mice per group. Scale bar = 50 μm.

TUNEL findings support the possibility that stPEG/Res reduces corneal tissue damage, inflammatory responses, and apoptosis-related changes in the DED model. However, because these effects were evaluated using a limited number of histological and immunohistochemical markers, further molecular analyses using additional inflammatory and apoptotic markers will be required to clarify the detailed mechanisms of stPEG/Res action in DED.

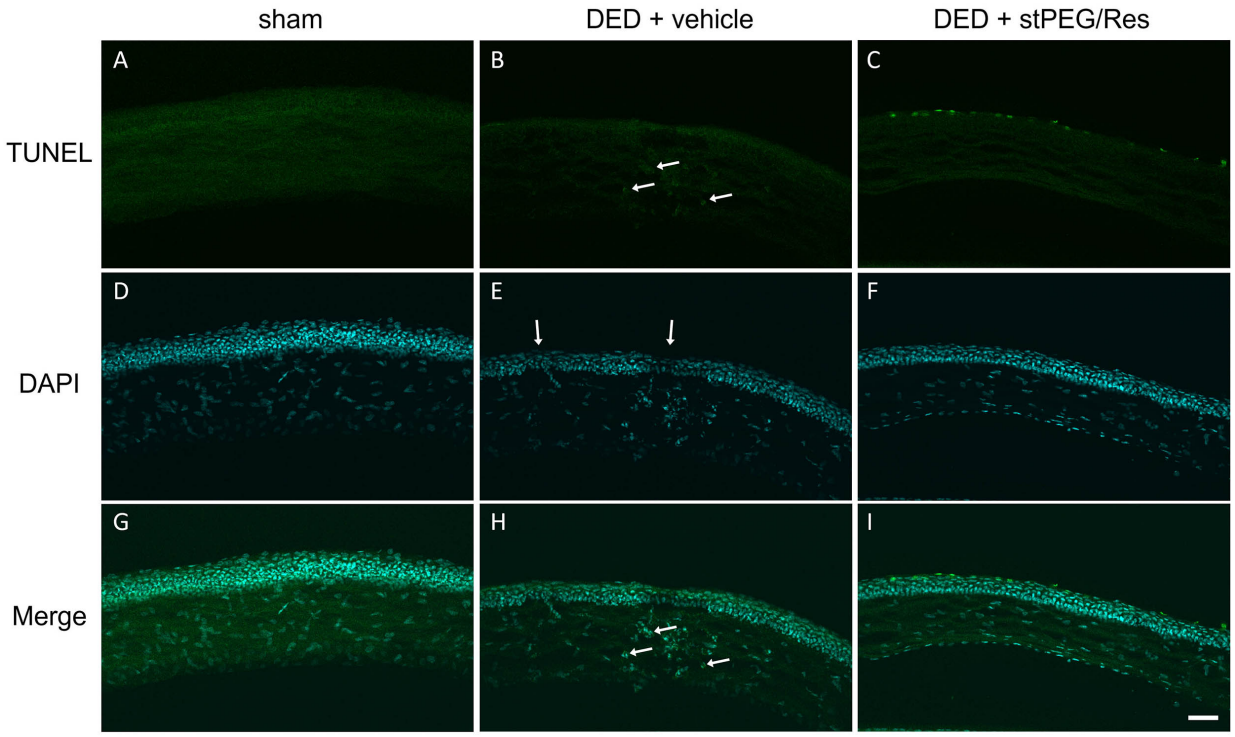

Fig. 6. Detection of apoptotic cells in corneal tissue using the TUNEL assay. Representative fluorescence images of corneal sections stained by the TUNEL assay and counterstained with DAPI are shown. Panels (A–C), (D–F), and (G–I) show TUNEL staining, DAPI staining, and merged images, respectively. In each row, the left panels (A,D,G), middle panels (B,E,H), and right panels (C,F,I) correspond to sham-operated mice, vehicle-treated DED mice, and stPEG/Res-treated DED mice, respectively. TUNEL-positive cells are shown in green, and nuclei counterstained with DAPI are shown in blue. Arrows indicate representative TUNEL-positive cells and corneal epithelial defects. Images are representative of four independent mice per group. Scale bar = 50 µm. TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling; DAPI, 4′,6-diamidino-2-phenylindole.

Given that the concentration of the eye drop formulation in this study was 0.1% (w/v) Res-equivalent, the actual amount reaching the ocular surface was likely diluted by tear fluid and blinking. The cytotoxicity results (Fig. 2) showed that stPEG/Res exhibited concentration-dependent cytotoxicity at higher Res-equivalent concentrations. Therefore, although stPEG/Res showed protective effects in the DED model, if the apoptosis observed in the superficial layer of the corneal epithelium in the stPEG/Res-treated group represents a toxic rather than a therapeutic response, it may be mitigated by adjusting the concentration and/or frequency of administration. Future investigations assessing Res release kinetics under ophthalmic conditions, pharmacokinetics, ocular distribution, and dose-dependent efficacy and safety following topical application will be essential to clarify the relationship between Res release and therapeutic or toxicological responses and to establish an optimal dosing regimen.

An important feature of this formulation is its amenability to both topical (eye drop) and systemic delivery. In the present study, we investigated topical administration; however, the same stPEG/Res micellar formulation was previously demonstrated to be effective following intravenous administration in an intracerebral hemorrhage model [11]. Given the growing interest in antioxidant-based therapies for various diseases, the dual applicability of stPEG/Res represents a significant advantage. Overall, the stPEG/Res micellar system provides a low-toxicity and efficient platform for delivering Res to ocular tissues. By harnessing the pharmacological benefits of Res while overcoming its poor aqueous solubility, stPEG/Res represents a promising formulation for the treatment of dry eye disease and potentially other inflammatory ocular disorders.

4. Limitations

This study has several limitations. First, neither the release kinetics of Res from stPEG/Res micelles under ophthalmic conditions nor the ocular pharmacokinetics and tissue distribution of Res after topical administration were evaluated in this study. Therefore, although the therapeutic effects observed in the DED model suggest that topically administered stPEG/Res can exert pharmacological effects on the ocular surface, the amount of Res released from the micelles, its residence time on the ocular surface, and its distribution in ocular tissues remain to be clarified. These factors may influence the interpretation of the exposure–

response relationship and the optimization of dosing concentration and administration frequency. Future studies should evaluate Res release from stPEG/Res using appropriate release studies and clarify the ocular pharmacokinetics and tissue distribution of Res after topical administration.

Second, the anti-inflammatory and anti-apoptotic effects of stPEG/Res were evaluated mainly using a limited number of assay systems and markers in this study. Although H&E staining and Iba1/iNOS immunostaining suggested suppression of corneal inflammation, and TUNEL staining suggested reduced apoptosis-related tissue damage, these endpoints were not further validated using additional molecular or biochemical assays, such as quantitative polymerase chain reaction (PCR), western blotting, enzyme-linked immunosorbent assay (ELISA), cytokine measurements, or additional apoptosis markers. Therefore, the present results should be interpreted as histological and immunohistochemical evidence supporting reduced inflammation and apoptosis-related tissue damage, rather than as quantitative evidence obtained from multiple independent assay systems. Additional complementary analyses will be necessary to validate these anti-inflammatory and anti-apoptotic effects and to clarify the underlying mechanisms of stPEG/Res action in DED.

Third, several formulation-development issues must be addressed before further ophthalmic application. The formulation used in this study was prepared for animal experiments, but sterility validation as a final ophthalmic product was not performed. Moreover, although acetone used during preparation was removed by evaporation during stirring followed by lyophilization, the amount of residual acetone in the final formulation was not quantitatively measured. Thus, further development should include validation of an appropriate sterilization process, such as membrane filtration or aseptic preparation, as well as quantitative analysis of residual solvents.

5. Conclusions

In this study, we developed a novel ophthalmic formulation consisting of water-soluble micelles composed of Res and stPEG. The antioxidant activity of Res was preserved after micellization, as demonstrated by the DPPH assay, indicating that stPEG does not interfere with its radical-scavenging function. Furthermore, stPEG/Res exhibited cytotoxicity comparable to that of free Res in HCE-T cells, suggesting that micellization does not compromise the formulation's safety in corneal epithelial cells. In a mouse model of DED induced by ELG excision, topical administration of stPEG/Res reduced corneal epithelial damage, inflammatory cell infiltration, inflammatory responses, and apoptosis. Collectively, these findings suggest that stPEG/Res micelles constitute a promising ophthalmic delivery platform for Res, with potential applicability in the treatment of DED and other ocular surface disorders.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article. Additional raw data are available from the corresponding authors upon reasonable request.

Author Contributions

AM, KB, and MO designed the study. AM, KB, RO, YKam, and YKaw performed the experiments. SS contributed to the design and interpretation of the immunohistochemical and histological analyses. YKan and TT contributed to the design and interpretation of the formulation and physicochemical characterization experiments. AM, KB, and MO analyzed the data. AM and KB drafted the manuscript. KB, SS, YKan, TT, and MO reviewed and edited the manuscript. KB and MO supervised the study and acquired funding. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal experiments were conducted in accordance with the ARRIVE guidelines, Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals used for scientific purposes, and the Fukuyama University guidelines for animal experimentation. The experimental protocols were approved by the Animal Ethics Committee of Fukuyama University (approval number: 2025-A-06, approved on October 24, 2025).

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/Pharmazie53494.

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