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Tamarind seed polysaccharide attenuates oxidative stress in corneal epithelial cells exposed to UV and blue light

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Abstract

The ocular surface is constantly exposed to environmental radiation. While the damaging effects of ultraviolet (UV) radiation are well established, increasing exposure to artificial blue light has raised concerns regarding photo-induced oxidative stress at the corneal level. Preventive strategies to protect corneal epithelial cells from light-induced damage are therefore of growing interest.

The protective effects of a tamarind seed polysaccharide (TSP)-based ophthalmic formulation were evaluated in rabbit corneal epithelial (RCE) cells exposed to blue light (405 nm) or UV radiation (365 nm). Cell viability, intracellular and mitochondrial oxidative stress, and cell morphology were assessed using complementary biochemical and high-content imaging approaches.

Blue-light exposure moderately increased oxidative stress without affecting cell viability, whereas UV irradiation induced significant cytotoxicity and ROS accumulation. TSP pre-treatment significantly reduced cytosolic and mitochondrial ROS in a dose-dependent manner under both irradiation conditions and completely prevented UV-induced loss of viability. The fluorescence image analysis confirmed increased mitochondrial superoxide after irradiation, which was significantly attenuated by TSP, with a significant irradiation–treatment interaction. High-content imaging demonstrated preservation of mitochondrial organization and epithelial morphology in TSP-treated cells while no intrinsic cytotoxic or pro-oxidant effects were observed in non-irradiated samples.

TSP exerts cytoprotective effects by attenuating oxidative stress and preserving mitochondrial integrity in corneal epithelial cells exposed to blue and UV light. These results support its potential use for protecting the ocular surface against environmental and digital light exposure.

Introduction

The eye is continuously exposed to environmental radiation, with solar light representing the primary natural source capable of inducing ocular damage. Solar radiation spans ultraviolet (UV), visible, and infrared wavelengths; within the visible spectrum, blue light (380–500 nm) is of particular concern, especially at shorter wavelengths around 400–450 nm, which are characterized by higher photon energy. While the harmful effects of UV radiation on ocular tissues are well established [1], increasing attention has been directed toward blue light due to its growing relevance in modern lifestyles [2-4].

In recent years, exposure to artificial sources of blue light has dramatically increased because of the widespread use of digital devices such as smartphones, tablets, computers, and LED-based displays [5]. This phenomenon is particularly relevant in pediatric populations, where screen use begins at increasingly early ages and often involves prolonged daily exposure [6,7]. International health organizations [8] and ophthalmological societies [9] have therefore recommended limiting screen time in young children to reduce potential risks to visual development and ocular health. Although epidemiological studies primarily associate prolonged screen exposure with digital eye strain, dry eye symptoms, and visual discomfort, growing experimental evidence suggests that chronic blue-light exposure may also contribute to oxidative stress at the ocular surface.

The pediatric eye may be especially susceptible to light-induced damage. Compared with adults, children exhibit higher transparency of ocular media, allowing greater transmission of short-wavelength light to anterior ocular tissues [10]. Moreover, antioxidant defense systems are still developing during childhood, potentially increasing susceptibility to oxidative injury. These factors

raise concerns that prolonged exposure to blue light from digital screens during early life could represent an underestimated risk factor for cumulative corneal stress.

Both UV radiation and blue light are known to induce oxidative stress through the generation of reactive oxygen species (ROS). Excessive ROS disrupt cellular redox homeostasis and lead to oxidative damage of lipids, proteins, and nucleic acids, ultimately impairing cell function and viability. At the corneal level, oxidative stress has been implicated in epithelial barrier dysfunction, inflammation, tear film instability, and altered wound-healing responses. While UV radiation induces direct photochemical damage, blue light primarily exerts its effects through mitochondrial dysfunction and photosensitizer-mediated ROS generation, resulting in sub-lethal but biologically relevant oxidative stress [11].

In response to growing concerns regarding light-induced ocular damage, several ophthalmic formulations have been developed to protect the ocular surface from UV and blue-light exposure. These products typically combine antioxidants, osmoprotectants, and lubricating agents to stabilize the tear film and mitigate oxidative stress. Commercial examples include formulations containing riboflavin as a natural UV absorber, vitamin E derivatives as free radical scavengers, ectoin as a cellular osmoprotectant, and hyaluronic acid to improve hydration and surface protection. Although these products may partially attenuate incident radiation or reduce oxidative stress at the tear film level, their mechanisms of action are largely limited to surface protection, and relatively little attention has

been devoted to compounds capable of modulating intracellular and mitochondrial oxidative pathways directly involved in photo-induced cellular damage.

Tamarind seed polysaccharide (TSP®; Farmigea SpA, Pisa, Italy) is a high-molecular-weight, neutral branched polysaccharide widely used in ophthalmic formulations for the management of dry eye disease due to its mucoadhesive, lubricating, and tear film–stabilizing properties. Beyond its mechanical and rheological functions, previous studies have demonstrated that TSP can preserve cell viability and reduce oxidative damage in corneal-derived cells exposed to UV radiation [12]. However, its potential role in protecting corneal epithelial cells from blue-light-induced oxidative stress, as well as its effects on mitochondrial redox balance, remains poorly explored.

In this context, the present study investigates the protective effects of a TSP-based ophthalmic formulation against corneal damage induced by UV and blue light irradiation. By integrating cell viability assays, cytosolic and mitochondrial oxidative stress measurements, and high-content imaging analysis, this work aims to provide mechanistic insight into the antioxidant and cytoprotective properties of TSP, with relevance to the increasing exposure of the ocular surface to artificial light sources in both adult and pediatric populations.

Material and Methods

Materials

For this study, a rabbit corneal epithelial cell line (RCE n. 95081046) obtained from the ECACC (Salisbury, UK) was used.

The growth medium had the following composition: Dulbecco's modified Eagle's medium with Ham's nutrient mixture F12 (1:1) (DMEM/F12) with addition of L-

glutamine (2 mM), penicillin (100 IU/mL), streptomycin (0.1 mg/mL), amphotericin B (0.25 µg/mL), fetal bovine serum heat-inactivated (15% v/v), insulin (5 µg/mL), and epidermal growth factor (10 ng/mL) (Merck Life Sciences, Milan, Italy). Cells were grown at 37°C in a humidified atmosphere with 5% CO2 and used at passages 20 to 25. Other materials: TSP 1% and TSP 0.5% eyedrops (Farmigea SpA, Pisa, Italy); cell proliferation reagent WST-1 (Roche Diagnostic, Milan, Italy); 2,7-dichlorofluorescein diacetate (DCF-DA; Merck Life Sciences, Milan, Italy); MitoSOX™ Red assay (Life Technologies, Milan, Italy).

Irradiation Conditions

The test was performed on RCE cells seeded in 96-well plates at a concentration of $3x10^5$ cells/mL. After 24 hours and once 70% confluence was reached, the plates were irradiated either with a lamp emitting radiation in the blue light range (405 nm) or with a UV-emitting LED system (365 nm). The irradiation conditions, kept constant during the treatment period and determined in a previous study [13] were the following:

- Distance between the lamp and the plate containing the cells: 12.5 cm for blue light; 16.5 cm for UV light. This distance allowed for the modulation of the irradiation power.
- Duration of irradiation: 30 minutes, as a longer exposure of the cells outside the incubator could have induced additional damage beyond that caused by the irradiation.
- Incubation following irradiation (recovery period): 24 hours.

These irradiation conditions provide a controlled high-energy photon exposure capable of inducing dose-dependent ROS generation and redox-associated biochemical changes while preserving experimental conditions compatible with RCE cell viability.

The irradiation power was measured using an HD2302.0 photoradiometer (Delta OHM) and resulted 0.82 J/cm^2 for both sources. The control cells, which were not subjected to irradiation, were protected from light by covering the corresponding wells with a dark

plastic sheet. Control cells were placed in the outer columns of the plate, ensuring that they were farthest from the lamp light beam, to prevent radiation from reaching the cells and compromising their viability.

TSP Treatments

The evaluation of the potential protective activity of TSP against light-induced damage in RCE cells was performed using commercial TSP eye drops (1.0% and 0.5%), diluted 1:1 with cell growth medium before use to obtain final concentrations of 0.5% and 0.25%, respectively. Cells were treated before irradiation and maintained in the corresponding formulation until cell viability or ROS determination.

Cell Viability

RCE cell viability was quantified using WST-1 cell proliferation reagent. After the 24 h recovery period, the reagent medium containing the formulation was removed and the cells were washed three times with PBS. Then, 100 µL of a fresh growth medium and 10 µL of the reagent WST-1 were added to each well. The cells were incubated for 2 h at 37 °C, then the microplates were thoroughly shaken for 30 s and the absorbance was measured at 450 nm using a microplate reader (Asys UVM 340, Biochrom Ltd., UK). The background absorbance was measured from the wells containing only the dye solution in culture medium, and the results were expressed as the percentage absorbance of the treated *vs.* control (non-irradiated and not TSP-treated, Ctr dark) wells.

Oxidative Stress Assessment

Quantification of reactive oxygen species (ROS) generated from RCE cells following exposure of cells to light was performed utilizing DCF-DA (2,7-dichlorofluorescein diacetate) reagent. After the 24 h recovery period, cells were washed three times with PBS, 100 µL/well of a 10 µM DCF-DA solution was applied and the cells were incubated for 45 min before fluorescence was monitored at λem = 480 nm and λex =

585 nm using a multimode microplate reader (Varioskan™ L; Thermo Scientific™). Background absorbance was measured on wells containing only the dye solution, and the results were expressed as a percentage of the fluorescence emitted from treated *vs.* control (non-irradiated and not TSP-treated, Ctr dark) wells.

Furthermore, mitochondrial superoxide production was measured using the MitoSOX™ Red assay. After the 24 h recovery period, cells were washed three times with PBS, incubated with 1 μM MitoSOX reagent for 30 min at 37 °C in DMEM without phenol red, washed again with PBS, and supplemented with 100 μl of DMEM without phenol red. Images were acquired using Operetta CLS high-content imaging device (PerkinElmer, Hamburg, Germany) and analyzed with Harmony 4.6 software (PerkinElmer, Hamburg, Germany). Imaging was performed at 63× magnification, capturing 48-64 fields per sample in at least three biological and technical replicates. Three channels were collected: nuclei, cytoplasmic MitoSOX fluorescence, and merged composites. Quantitative parameters extracted from Operetta analysis software included MitoSOX cytoplasmic fluorescence intensity (cyt), MitoSOX mitochondrial spot fluorescence intensity (spot). Fluorescence data were normalized to the mean of negative control (Ctr dark; non-irradiated and not TSP-treated).

The protective efficacy (PE %) of the compound against cellular damage due to irradiation was determined by the following equation relating the intensity values:

$$\mathrm{PE\%} = \frac{\text{(light) - (light+compound)}}{\text{(light)} - \text{(Untreated)}} \times 100$$

Representative Operetta images were selected for each condition using identical exposure and scaling settings.

Statistical Analysis

Statistical analyses were performed by GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA).

For each endpoint, descriptive statistics were calculated, unless otherwise stated, data are expressed as mean ± SEM from three independent experiments. One-way analysis of variance (ANOVA) was used to assess overall group differences, followed by Sidak's multiple-comparison post hoc test. Targeted comparisons relevant to oxidative stress and compound protection were additionally evaluated by two-way ANOVA followed by Sidak's multiple-comparison post hoc test. Significance thresholds were set at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$.

Results and Discussion

Cell Viability and ROS Levels

Cell viability assessed by the WST-1 assay indicated that blue-light irradiation, under the experimental conditions applied, did not impair RCE cell survival. One-way ANOVA detected no significant differences among groups (Fig. 1**a**) and irradiated untreated cells (BL-NT) maintained viability values comparable to the physiological control (Ctr dark), reaching 102.95 ± 1.49%. Consistently, pre-treatment with TSP at either 0.25% or 0.50%, in irradiated or dark conditions, did not significantly modify cell viability, confirming the absence of TSP-induced cytotoxicity and supporting its suitability for ophthalmic use.

In contrast, UV irradiation produced a pronounced reduction in metabolic activity. UV-NT cells exhibited significantly reduced viability (84.74 ± 3.14%) compared with all

other experimental groups (Fig. 1**b**). One-way ANOVA confirmed a strong treatment effect, and Sidak's multiple comparisons test revealed that UV-NT differed significantly from every other condition (# in Fig. 1**b**), highlighting the high sensitivity of corneal epithelial cells to UV insult.

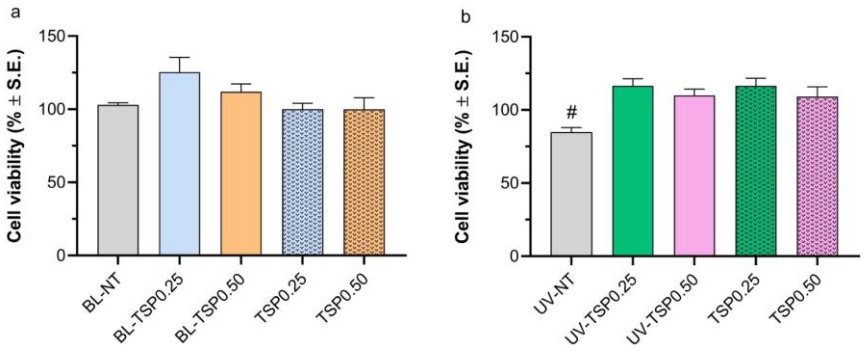

Figure 1. Cell viability in RCE cells irradiated by blue light (a) and UV (b).
BL-NT and UV-NT: irradiated but not TSP-treated cells; BL-TSP0.25 and UV-TSP0.25: cells treated with TSP 0.25% and irradiated; BL-TSP0.5 and UV-TSP0.5: cells treated with TSP 0.50% and irradiated; TSP0.25: cells treated with TSP 0.25% and non-irradiated; TSP0.5: cells treated with TSP 0.50% and non-irradiated. Statistical evaluation by One-way ANOVA multiple comparison using Sidak's test: #statistically different from all the others.

This observation is consistent with the established cytotoxic potential of UV radiation, which induces DNA damage, lipid peroxidation, and mitochondrial dysfunction in corneal epithelial cells. Notably, TSP pre-treatment effectively counteracted UV-mediated damage: both concentrations (0.25% and 0.50%) maintained cell viability at levels indistinguishable from the physiological control (Ctr dark; all not significant), indicating a robust cytoprotective effect in agreement with previous findings in corneal fibroblasts SIRC cells [12].

The complete prevention of UV-induced viability loss indicates that TSP is capable of preserving metabolic competence under severe oxidative challenge, suggesting that its

protective action extends beyond tear film stabilization and involves modulation of intracellular stress pathways.

Blue-light exposure triggered an oxidative response in RCE cells, as evaluated by DCF-DA fluorescence. One-way ANOVA revealed a highly significant treatment effect ($F = 13.42$, $p < 0.0001$; Tab. 1), indicating modulation of intracellular ROS levels across experimental conditions (Fig. 2**a**).

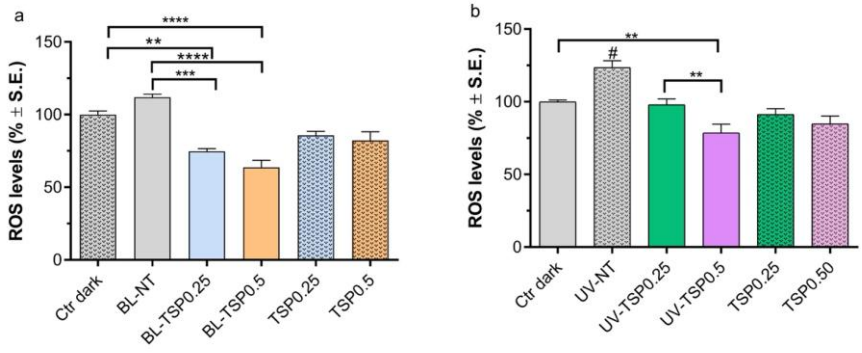

Figure 2. ROS levels in RCE cells irradiated by blue light (a) and UV (b).

BL-NT and UV-NT: irradiated but not TSP-treated cells; BL-TSP0.25 and UV-TSP0.25: cells treated with TSP 0.25% and irradiated; BL-TSP0.5 and UV-TSP0.5: cells treated with TSP 0.50% and irradiated; TSP0.25: cells treated with TSP 0.25% and non-irradiated; TSP0.5: cells treated with TSP 0.50% and non-irradiated; Ctr dark: non-irradiated and not TSP-treated cells. Statistical evaluation by One-way ANOVA multiple comparison using Sidak's test: $^{\#}$statistically different from all the others.

Table 1. Statistical data (one-way ANOVA) of ROS levels evaluated by DCF-DA in RCE cells irradiated by blue light and UV.

Multiple comparisons	Mean differences	Adjusted p-value (Sidak)
	Blue light	
BL-NT vs Ctr dark	12.01±8.14	0.9004
BL-$TSP_{0.25}$ vs BL-NT	−37.29±8.14	0.0002
BL-$TSP_{0.5}$ vs BL-NT	−48.25±7.43	<0.0001
BL-$TSP_{0.25}$ vs Ctr dark	−25.28±6.64	0.0033
BL- $TSP_{0.5}$ vs Ctr dark	−36.24±5.75	<0.0001
$TSP_{0.25}$ vs Ctr dark	−14.22±9.39	0.8819
$TSP_{0.5}$ vs Ctr dark	−17.77±7.93	0.339
	Ultraviolet	
UV-NT vs Ctr dark	23.54	0.0128
UV-TSP0.25 vs UV-NT	−25.64	0.0016
UV- TSP0.5 vs UV-NT	−44.93	<0.0001
UV- TSP0.25 vs Ctr dark	−2.09	>0.9999
UV- TSP0.5 vs Ctr dark	−21.38	0.0075
TSP0.25 vs Ctr dark	−8.726	0.9848
TSP0.5 vs Ctr dark	−15.14	0.5599

Although BL-NT cells exhibited slightly higher ROS values than Ctr dark, this increase did not reach statistical significance (mean difference = 12.01 ± 8.14), supporting the notion that blue light induces a moderate oxidative imbalance rather than severe redox

disruption. These results confirm that blue-light exposure at the applied irradiance primarily triggers sub-lethal cellular stress in corneal epithelial cells without compromising metabolic activity. These results are consistent with previous evidence indicating blue-light-induced mitochondrial ROS generation without overt cytotoxicity [14].

TSP pre-treatment markedly reduced ROS accumulation in irradiated cells. Compared with BL-NT, BL-TSP0.50 and BL-TSP0.25 yielded substantial decreases in ROS levels (mean differences = −48.25 and −37.29, respectively; both $p \leq 0.0002$). In addition, both concentrations resulted in significantly lower ROS levels compared with the physiological control, Ctr dark (mean differences = −36.24, $p < 0.0001$ for BL-TSP0.50 and −25.28, $p = 0.0033$ for BL-TSP0.25), confirming a strong antioxidant activity of TSP under photo-oxidative conditions. In dark conditions, TSP induced only a mild and non-significant reduction in basal ROS levels, suggesting that its antioxidant effect is primarily activated in response to oxidative challenge rather than constitutively altering redox homeostasis.

UV irradiation elicited a markedly stronger oxidative response than blue light with an increase in fluorescence well above baseline (Fig. 2**b**). One-way ANOVA showed a highly significant treatment effect ($F = 9.902$, $p < 0.0001$; Tab. 1). UV-NT cells displayed ROS levels significantly higher than the physiological control (mean difference = 23.54, $p = 0.0128$), confirming the induction of substantial oxidative stress. TSP pre-treatment significantly attenuated UV-induced ROS accumulation in a concentration-dependent manner. UV-TSP0.50 showed the strongest reduction compared with UV-NT (mean difference = −44.93, $p < 0.0001$) also exhibiting ROS levels significantly lower than the physiological baseline (mean difference = −21.38 *vs* Ctr dark, $p = 0.0075$). Similarly, UV-TSP0.25 significantly reduced ROS levels relative

to UV-NT (mean difference = −25.64, p = 0.0016), although values remained above baseline. These findings indicate that TSP is particularly effective under conditions of high oxidative burden, such as UV exposure.

Blue Light Irradiation: Mitochondrial Superoxide Production

Given the central role of mitochondria in redox signaling and oxidative damage, mitochondrial superoxide production was further investigated using MitoSOX high-content imaging. Blue-light exposure resulted in a marked increase in RCE mitochondrial oxidative stress (Fig. 3).

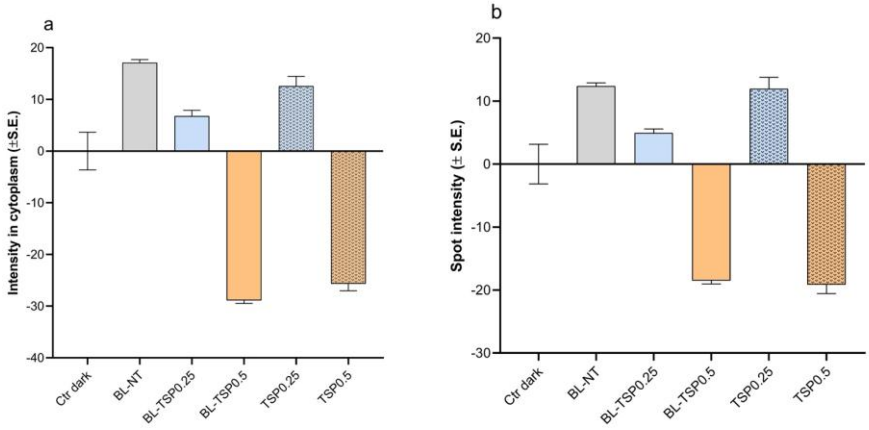

Figure 3. Cytoplasmic (a) and mitochondrial spot (b) MitoSOX intensity in blue light-irradiated RCE cells. Fluorescence data were normalized to the mean of Ctr dark. BL-NT: irradiated but not TSP-treated cells; BL-TSP0.25: cells treated with TSP 0.25% and irradiated; BL-TSP0.5: cells treated with TSP 0.50% and irradiated; TSP0.25: cells treated with TSP0.25% and non-irradiated; TSP0.5: cells treated with TSP 0.50% and non-irradiated; Ctr dark: non-irradiated and not TSP-treated cells.

One-way ANOVA revealed a highly significant overall effect of treatment for both cytoplasmic and mitochondrial spot MitoSOX intensity ($F = 67.67$ and $F = 46.46$, respectively; both $p < 0.0001$), with high coefficients of determination ($R^2 = 0.77$ for cy and $R^2 = 0.70$ for spot analysis). Post-hoc Sidak's multiple comparisons (Tab. 2) confirmed that BL irradiation significantly increased MitoSOX signal compared with

Ctr dark (mean differences = −17.11 ± 3.63 for cyt and −12.38 ± 3.16 for spot intensity; p = 0.0001 and p = 0.0024, respectively), validating the induction of mitochondrial oxidative stress.

Table 2. Statistical data (one-way and two-way ANOVA) of spot and cyt MitoSox fluorescence intensity measured in RCE cells irradiated by blue light.

Multiple comparisons one-way	**Spot intensity**		**Cyt intensity**	
	Mean differences	Adjusted p-value (Sidak)	Mean differences	Adjusted p-value (Sidak)
Ctr dark vs BL-NT	-12.38±3.16	0.0024	-17.11±3.63	0.0001
BL-NT vs BL-$TSP_{0.25}$	7.46±3.25	0.3032	10.35±3.73	0.0947
BL-NT vs BL-$TSP_{0.5}$	30.89±3.25	<0.0001	46.00±3.73	<0.0001
Ctr dark vs BL-$TSP_{0.25}$	−4.92±2.54	0.5757	-6.76±2.92	0.2903
Ctr dark vs BL- $TSP_{0.5}$	18.51±2.54	<0.0001	28.89±2.92	<0.0001
Ctr dark vs $TSP_{0.25}$	−11.93±2.71	0.0004	-12.56±3.11	0.0016
Ctr dark vs $TSP_{0.5}$	19.15±2.71	<0.0001	25.66±3.11	<0.0001
Source of variation two-way	Total variation (%)	p-value	Total variation (%)	p-value
Interaction	6.345	<0.0001	5.880	<0.0001
TSP	67.64	<0.0001	74.57	<0.0001
BL	0.4211	0.2396	0.4362	0.1698

Under blue-light exposure, TSP markedly attenuated the MitoSOX signal in a concentration-dependent manner. In cytoplasmic measurements, TSP0.50 induced a strong reduction in fluorescence compared with irradiated untreated cells (mean difference = 46.00 ± 3.73; $p < 0.0001$), whereas TSP0.25 produced only a non-significant trend toward protection (mean difference = 10.35 ± 3.73; $p = 0.0947$). A similar pattern was observed for mitochondrial spot intensity, with TSP0.50 significantly lowering the MitoSOX signal compared to BL-NT (mean difference = 30.89 ± 3.25; $p < 0.0001$).

The two-way ANOVA further substantiated these results. For both cyt and spot MitoSOX intensity, the compound factor accounted for most of the total variance (74.57% and 67.64%, respectively; both $p < 0.0001$), whereas the blue light factor alone was not significant ($p > 0.16$). Critically, the interaction term (BL × TSP) was significant ($p < 0.0001$), representing approximately 6% of the total variation, which demonstrates that the compound effect was specifically dependent on the presence of photo-induced oxidative stress. This interaction pattern seems to indicate that TSP does not indiscriminately suppress mitochondrial activity, but rather modulates oxidative pathways selectively under stress conditions, supporting a context-dependent antioxidant mechanism.

Consistent with the statistical analysis, the percentage of protection calculated from normalized means indicated that TSP exerted a strong, dose-dependent effect: protection reached 60.51% and 268.85% for cyt MitoSOX, and 60.26% and 249.51% for spot intensity, for $TSP_{0.25}$ and $TSP_{0.5}$, respectively.

Morphological analysis by high-content imaging provided qualitative confirmation and mechanistic support for the quantitative results. Figure 4 shows the high-content fluorescent imaging where distinct morphological alterations in RCE cells exposed to

BL, with clear differences between Ctr dark, irradiated cells, and cells treated with low or high concentrations of the compound are evinced.

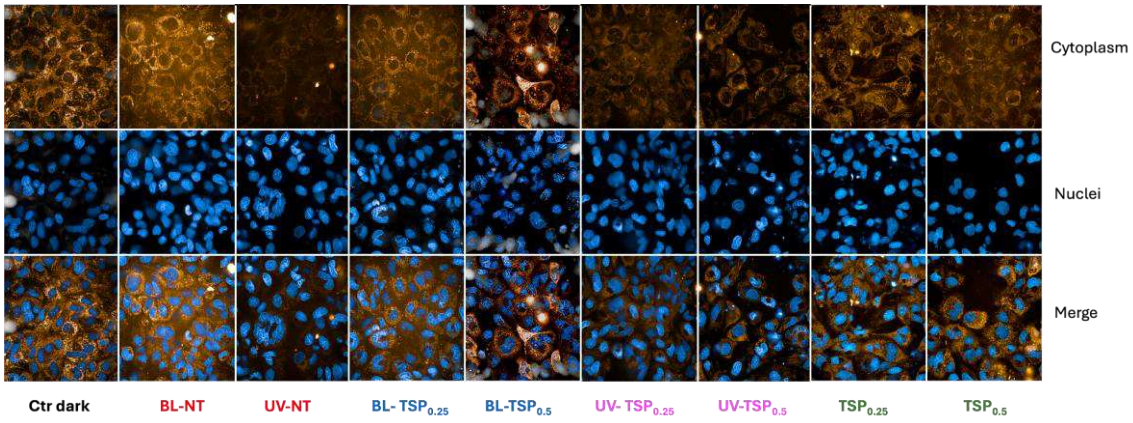

Figure 4. – Representative images of RCE cells acquired by Operetta CLS high-content imaging device (63x) under blue light and UV irradiation. BL-NT and UV-NT: irradiated but not TSP-treated cells; BL-TSP0.25 and UV-TSP0.25: cells treated with TSP 0.25% and irradiated; BL-TSP0.5 and UV-TSP0.5: cells treated with TSP 0.50% and irradiated; TSP0.25: cells treated with TSP 0.25% and non-irradiated; TSP0.5: cells treated with TSP 0.50% and non-irradiated; Ctr dark: non-irradiated and not TSP-treated cells.

In Ctr dark, cells displayed a typical epithelial morphology with high confluence, smooth cell boundaries, and uniformly stained oval nuclei, consistent with the morphology described for healthy corneal epithelial cells in vitro [15]. Chromatin showed a homogeneous distribution, and cytoplasmic staining exhibited a fine mitochondrial reticulum typical of non-stressed epithelial cells [16].

Exposure to blue light alone produced hallmark features of photo-oxidative damage. Nuclei became enlarged, irregularly shaped, and often lobulated, displaying heterogeneous chromatin patterns and bright foci suggestive of condensation or early DNA injury, changes widely reported under phototoxic or oxidative stress [17-19]. The cytoplasmic signal increased becoming granular and heterogeneous, consistent with mitochondrial fragmentation, perinuclear clustering, and loss of reticular organization, a well-recognized responses of mitochondria exposed to oxidative or phototoxic stress.

These abnormalities were accompanied by a reduction in cell density, in line with light-induced cytotoxicity described in corneal epithelial cells [14,16].

Treatment with the low-concentration compound during blue light exposure mitigated several of these effects. Nuclear morphology was less distorted than in irradiated untreated cells, showing more regular contours and a more uniform chromatin distribution. Cytoplasmic staining appeared less granular, with partial restoration of a continuous mitochondrial network. Mitochondrial clustering was attenuated, suggesting improved maintenance of mitochondrial dynamics, possibly involving protection of fission–fusion balance under oxidative stress [20]. Cell density was moderately higher than in BL-NT samples, supporting a partial cytoprotective effect.

Cells treated with the high-concentration compound exhibited the most preserved morphology. Chromatin staining appeared more homogeneous, and nuclei displayed markedly smoother contours compared with both BL-NT and low-concentration conditions. Cytoplasmic mitochondrial signal displayed a more reticulated, network-like appearance, indicative of maintained mitochondrial integrity, rather than the fragmented pattern typically induced by blue-light oxidative injury [17]. Overall cell density was higher, and individual cells appeared larger and with regular shape, consistent with dose-dependent protection typical of antioxidant or mitoprotective compounds.

Importantly, compound treatment in dark conditions did not induce morphological abnormalities. Both concentrations preserved normal nuclear morphology and chromatin organization, and mitochondrial staining remained reticulated without signs of swelling, fragmentation, or clustering. These results indicate that the compound does not induce intrinsic cytotoxicity or nuclear/mitochondrial stress in the absence of irradiation, supporting a conditional protective activity activated primarily under photo-oxidative challenge.

These observations align with known mechanisms of oxidative and phototoxic injury to ocular surface cells and support a protective role of the compound against blue-light-induced cellular damage [21].

UV Irradiation: Mitochondrial Superoxide Production

UV irradiation induced more severe morphological and mitochondrial alterations than blue light, in agreement with the quantitative differences observed in both cytosolic ROS and MitoSOX measurements. As shown in Fig. 5, UV exposure caused a marked increase in mitochondrial superoxide production, with one-way ANOVA revealing a highly significant treatment effect for both cytoplasmic and mitochondrial spot MitoSOX intensity ($F = 37.09$ and $F = 24.02$, respectively; both $p < 0.0001$). Post-hoc Sidak's multiple comparisons (Tab. 3) confirmed that UV-NT cells exhibited significantly higher MitoSOX fluorescence compared with Ctr dark (mean differences = -23.16 ± 4.19 for cyt and -17.57 ± 3.49 for spot intensity; both $p < 0.0001$), quantitatively demonstrating severe mitochondrial oxidative stress induced by UV irradiation.

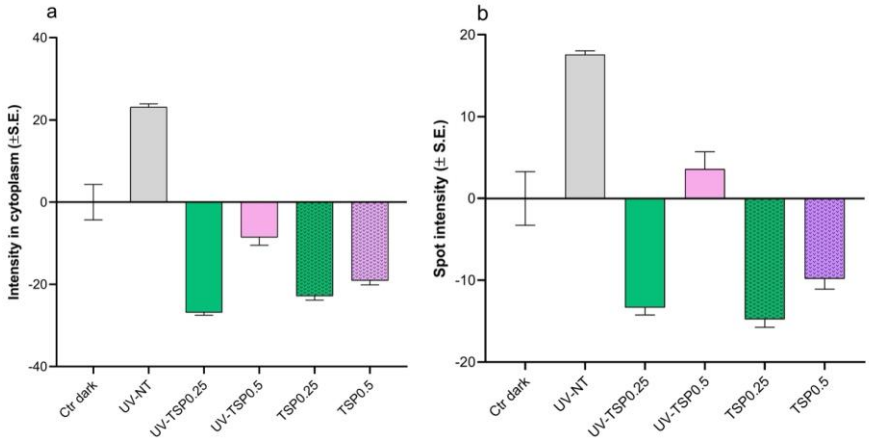

Figure 5. Cytoplasmic (a) and mitochondrial spot (b) MitoSOX fluorescence intensity in UV-irradiated RCE cells. Fluorescence data were normalized to the mean of Ctr dark. UV-NT: irradiated but not TSP-treated cells; UV-TSP0.25: cells treated with TSP 0.25% and irradiated; UV-TSP0.5: cells treated with TSP 0.50% and irradiated; TSP0.25: cells treated with TSP 0.25% and non-irradiated; TSP0.5: cells treated with TSP 0.50% and non-irradiated; Ctr dark: non-irradiated and not TSP-treated cells.

Table 3. Statistical data (one-way and two-way ANOVA) of spot and cyt MitoSox fluorescence intensity measured in RCE cells irradiated by UV.

Multiple comparisons one-way	**Spot intensity**		**Cyt intensity**	
	Mean differences	Adjusted p-value (Sidak)	Mean differences	Adjusted p-value (Sidak)
Ctr dark vs UV-NT	-17.57±3.49	<0.0001	-23.16±4.19	<0.0001
UV-NT vs UV-$TSP_{0.25}$	30.92±3.59	<0.0001	50.03±4.32	<0.0001
UV-NT vs UV-$TSP_{0.5}$	13.98±3.59	0.0027	31.73±4.32	<0.0001
Ctr dark vs UV-$TSP_{0.25}$	13.35±2.81	0.0001	26.87±3.37	<0.0001
Ctr dark vs UV-$TSP_{0.5}$	-3.60±2.81	0.9669	8.57±3.37	0.1735
Ctr dark vs $TSP_{0.25}$	14.79±2.99	<0.0001	22.89±3.59	<0.0001
Ctr dark vs $TSP_{0.5}$	9.83±2.99	0.0211	19.13±3.59	<0.0001
Source of variation two-way	Total variation (%)	p-value	Total variation (%)	p-value
Interaction	7.28	0.0007	8.27	<0.0001
TSP	47.40	<0.0001	60.66	<0.0001
UV	5.03	0.0015	6.80	<0.0001

Pre-treatment with TSP significantly attenuated UV-induced mitochondrial superoxide production, as supported by both numerical and morphological data. In cyt measurements, TSP0.25 and TSP0.50 significantly reduced MitoSOX intensity compared to UV-NT (mean differences = 50.03 ± 4.32 and 31.73 ± 4.32, respectively; both $p < 0.0001$). A comparable pattern was observed for mitochondrial spot intensity, with significant reductions with respect to UV-NT for both concentrations (mean

differences = 30.92 ± 3.59 for TSP0.25, $p < 0.0001$, and 13.98 ± 3.59 for TSP0.50, $p = 0.0027$). These quantitative results are consistent with the images shown in Fig. 4, where TSP pre-treatment markedly reduced diffuse cytoplasmic fluorescence and partially restored a punctate mitochondrial distribution.

Two-way ANOVA further substantiated the interaction between UV irradiation and TSP treatment. For cytoplasmic MitoSOX intensity, the compound factor accounted for 60.66% of the total variance ($p < 0.0001$), whereas UV irradiation alone contributed 6.80% ($p < 0.0001$), and their interaction explained 8.27% of the variance ($p < 0.0001$). Similar results were obtained for mitochondrial spot intensity, where TSP explained 47.40% of total variance ($p < 0.0001$), UV irradiation 5.03% ($p = 0.0015$), and the interaction 7.28% ($p = 0.0007$). These interaction effects indicate that the antioxidant action of TSP is specifically engaged under UV-induced oxidative stress rather than constitutively suppressing mitochondrial activity. Such results reinforce the role of mitochondria as central targets of photo-oxidative injury in corneal epithelial cells and suggest that TSP can preserve mitochondrial integrity under conditions of high oxidative burden.

Under UV irradiation, the compound provided 216.02% protection at low concentration and 137.01% protection at the high concentration for cytoplasmic MitoSOX intensity. For mitochondrial spots, the protection reached 176% and 79.5% for low and high concentrations, respectively.

Morphological analysis provided further support for these quantitative findings: UV-irradiated cells exhibited intense and diffused MitoSOX fluorescence, loss of punctate mitochondrial organization, and pronounced cytoplasmic disarray, all features indicative of mitochondrial dysfunction and excessive ROS accumulation (Fig. 4). TSP pre-treatment improved cellular morphology in parallel with the numerical reduction of

MitoSOX signal. These imaging results are consistent with the statistical analyses showing robust protective efficacy of both doses: at the lower concentration, TSP produced a marked recovery of mitochondrial compartmentalization and reduced cytoplasmic diffusion of fluorescence, whereas the higher concentration also exerted a protective effect, although with a slightly less homogeneous distribution.

This apparent discrepancy is consistent with a saturation or redistribution phenomenon rather than reduced antioxidant efficacy and does not contradict the strong numerical reduction in mitochondrial superoxide levels.

Together, the close correspondence between numerical MitoSOX data, ANOVA outcomes, and imaging analysis demonstrates that TSP preserves mitochondrial integrity and cellular architecture under UV-induced stress. Compared with blue-light exposure, UV irradiation produces quantitatively greater oxidative damage and structural disruption, but in both conditions, TSP effectively limits mitochondrial superoxide generation and stabilizes epithelial morphology, providing a mechanistic basis for its cytoprotective effects.

Overall, the integrated analysis of cell viability, cytosolic ROS, mitochondrial superoxide production, and high-content imaging highlight that TSP confers effective protection against both blue-light- and UV-induced corneal epithelial damage. While blue light induces moderate oxidative stress without affecting viability, UV exposure causes severe oxidative injury and metabolic impairment. In both contexts, TSP reduces cytosolic and mitochondrial ROS, preserves cellular architecture, and, under UV irradiation, fully prevents loss of cell viability, supporting its potential use as a protective agent for the ocular surface.

In the context of increasing exposure to artificial blue light and environmental UV radiation, these data provide a mechanistic rationale for the use of TSP-based

formulations to limit photo-induced oxidative damage and contribute to the maintenance of corneal homeostasis.

Since the formulation does not contain known radiation-shielding excipients the protective activity observed in this study is likely attributable primarily to TS-polysaccharide component. Whether this effect results from a direct interaction with corneal epithelial cells or from indirect modulation of the extracellular microenvironment remains to be clarified. Further studies are warranted to elucidate the relationship between the molecular structure of the high-molecular-weight polysaccharide and its potential interaction with blue-light and UV-induced oxidative processes. In addition, the observed reduction of ROS levels under basal conditions highlights the need to further investigate the antioxidant mechanisms of TS-polysaccharide, including its possible role in enhancing endogenous redox buffering systems [16,20].

Authors' contributions: conception and design of the work: SB and DM; acquisition and analysis: VP, ST and SGB; interpretation of data: SB, DM, and PC; writing-original draft preparation: SB, DM, and VP; writing-review and editing: PC, ST and SGB; project administration: FN.

All authors read and approved the final manuscript and have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Data availability: the datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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