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HEALTH POTENTIAL OF ALOE VERA AND POLYDEOXYRIBONUCLEOTIDE (PDRN) AGAINST OXIDATIVE STRESS INDUCED CORNEAL DAMAGE: AN IN VITRO MODEL OF FUCHS ENDOTHELIAL CORNEAL DYSTROPHY

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CONTENTS

1. Introduction

Fuchs endothelial corneal dystrophy (FECD) is a bilateral hereditary and slowly progressive disease, which affects the endothelial layer of the cornea of both eyes. The prevalence of FECD is approximately four percent of the adult population. Early manifestation can be observed in patient from 30 to 40 years old, but Fuchs usually become symptomatic over 40 years of age or later. Women are more commonly affected than men are. In addition, family history increases the chance of developing the disease. Symptoms are usually represented by blurred and cloudy vision, especially in the morning that improves during the day, photophobia and feeling of foreign body inside the eye. Fuchs dystrophy can be classified in four stages, which vary from early signs of guttae formation to end-stage subepithelial scarring. Diagnosis is confirmed through ophthalmological examination, corneal pachymetry ad in-vivo confocal. It is characterized by the gradual deterioration of corneal endothelial cells (CECs) developing a characteristic feature (cornea guttate) and is the most common cause of corneal transplantation worldwide [1, 2]. The deterioration of corneal endothelial cells and the consequent guttae formation usually start in the centre of the cornea and slowly involves the periphery as well. The longer the guttae spreads, the more CECs are destroyed and the density of endothelial cells decreases, as these two parameters are inversely proportional.

In this stage, the changes in Fuchs endothelial cells include a modification of the cell size called “polymegethism” and a modification of the cell outline called “pleomorphism”. The corneal endothelium (CE) is a monolayer formed by the CECs situated on the corneal surface that has different functions including: maintaining corneal deturgescence thanks to his barrier function and

regulating corneal hydration, nutrition and transparency. As the CE does not divide in vivo, loss of endothelial cells seen in FECD is permanent. CECs apoptosis caused by oxidative stress may play a pivotal role in the pathogenesis of FECD [3, 4]. Previous studies have already demonstrated a higher level of reactive oxygen species (ROS) in the cornea of FECD patients when compared with the healthy one [5]. The imbalance between oxidant and antioxidant factors in CECs is responsible for the development of endothelial oxidative DNA damage. It is caused by a decreased expression of Nrf2 transcription factor and its antioxidant targets like superoxide dismutases, glutathione S-transferases and peroxiredoxin, which are involved in the scavenging of ROS. The aberrant Nrf2 expression influences the antioxidant system in FECD corneal endothelium and induces free radicals and other reactive species to accumulate, leading to an alteration of tissue homeostasis and activating the p53-dependent apoptotic pathway [6,7].

Reactive oxygen species could be produced after photochemical reactions caused by the exposure to UV light or ionizing radiation. During physiological conditions, there is a cellular equilibrium between ROS production and degradation and low levels of ROS can be found [8]. The imbalance between ROS production and the antioxidant scavenging systems (AOX) causes Oxidative stress (OS). OS is related to several disorders, including Parkinson's and Alzheimer's diseases, cancer, atherosclerosis, diabetes and rheumatoid arthritis [9–11]. Furthermore, OS is also responsible for different ocular pathologies, such as ocular surface disorders, different syndromes of the eye anterior and posterior segment and retinal diseases. The cornea is a transparent and avascular tissue in the anterior segment of the eye and is one of the most densely innervated tissues in the body with refractive and barrier functions [12]. Due to its external localization, it is directly exposed to different

factors, such as air pollution, cigarette smoke and UV radiations, which can induce oxidative damage, and different ocular pathologies such as FECD [13].

Corneal tissue has developed physiological antioxidant systems, which contain free radical scavengers, including superoxide dismutase, glutathione peroxidase and catalase [14]. The imbalance between prooxidant and antioxidant is primarily attributed to the down-regulation of the antioxidant enzymes principally: Lactate dehydrogenase, catalase and glutathione peroxidase. This imbalance leads to structural and functional changes in the corneal tissue. As a consequence of the increased oxidative stress there is a reduction of the number of corneal fibroblasts and corneal endothelial cells in corneas, due to the triggering of the apoptotic process [15–17]. Oxidative damage in the endothelium is caused by an imbalance between oxidant and antioxidant components that leads to ROS production, the dysregulation of pro- and antiapoptotic factors (Bax and Bcl-2), and the triggering of the apoptotic process mediated by Caspase 3 and 8, with consequent CEC loss in the corneas [18–20]. In addition, the increased expression of the transcription factor nuclear factor kappa-B (NF-kB), the subsequent release of the pro-inflammatory cytokine interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) activity have been linked to low CEC density and the development of symptomatic lateonset FECD [21]. TNF-α triggers programmed cell death and prompts the stimulation of the pro-inflammatory cytokine interleukin 1 beta (IL-1β), leading to chronic inflammation and the consequent injury of corneal endothelial tissue [22].

Both elevated ROS levels and oxidative stress play a key role in the development of many corneal diseases, including Fuchs endothelial corneal dystrophy, keratoconus, granular corneal dystrophy type 2 and bullous keratopathy [23,24].

Despite the increased attention to the management of FECD, nowadays the nonsurgical therapy for FECD is palliative and limited to the topical application of hypertonic 5% sodium chloride eye drops. For this reason, finding new treatment approaches for the management of corneal diseases is of great interest. In this context we decided to investigated the effect of two different compound (Aloe Vera extract and Polydeoxyribonucleotide) with anti-oxidant properties for the FECD treatment.

Thus, previous papers have investigated the possibility of using phytotherapic treatments as new strategies for ROS related diseases [25,26]. Health plants are currently used in a number of consumer products due to their medicinal or aesthetic properties. Among them, the Aloe vera extracts are known for their curative properties, and are used both internally and externally on humans in the form of alternative medications, as well as in the home for first aid. Aloe vera has demonstrated wound-healing properties and showed immunomodulatory, antioxidant and anti-inflammatory effects [27]. Indeed, different studies showed that Aloe vera treatment induced inhibition of the inflammatory reactions, reducing the expression of proinflammatory cytokines, like interleukine-6 (IL-6), IL-1β and tumor necrosis factor-α (TNF-α) [23]. Moreover, Aloe vera extracts revealed a modulating effect on the inflammatory cytokine network and antioxidant function including ROS scavenging in eye tissue [24]. Furthermore, previous work established that the use of Nrf2 agonists with antioxidant activity caused cytoprotective effects, a significant decrease in ROS production and ameliorated oxidative stress-levels in FECD corneas, confirming the idea that the implementation of antioxidant system may play a role in the treatment of corneal ROS related diseases [28]. Morover, adenosine receptors have been recognized as a promising target in the management of ROS-related disorders, eye diseases and impaired healing conditions [29–33].

In particular, adenosine A_{2A} receptor (A_{2Ar}) activation showed the ability to modulate the inflammatory response and the apoptotic process, and to improve tissue repair and the healing process [34,35]. Polydeoxyribonucleotide (PDRN) is a biologic drug isolated from the gonads of trout, the antioxidant and anti-inflammatory effects of which have been demonstrated mainly through A_{2Ar} modulation [36,37].

Finally, Jurkunas et al. demonstrated that immortalized normal corneal endothelial cells stimulated with H2O2 may display morphological changes and cellular apoptosis similar to those observed in FECD [5]. For this reason, the goal of this work was to assess the effects of PDRN and Aloe Vera in an H_2O_2-induced in vitro FECD model.

2. Materials and Methods for Aloe Vera set of experiment

2.1. Cell Culture

Epithelial Adenovirus 12-SV40 hybrid transformed HCE cells (ATCC® CRL-11135™) were obtained from LGC Standards S.r.l Milan, Italy. Cells were cultured in a medium prepared with DMEM-F12 basic media supplemented with 10% heat-inactivated FBS, recombinant human epidermal growth factor (5 ng/mL), 5 μg/mL insulin plus 1% antibiotics (penicillin/streptomycin), and incubated at 37 °C with 5% of CO_2. The culture's medium was replaced with a time interval of 2 days and the cells were re-plated.

2.2. Treatments of Cells

The IHCE cells were cultured in 6-well plates at a density of 3 x 106 cells/well, and were challenged with 200 μM H_2O_2 (Sigma Aldrich, Milan, Italy) for 2 h to establish an oxidative stress model. HCE cells were preincubated with aloe extract 100 μg/mL 2 h before H_2O_2 stimulus. HCE cells treated with 1% DMSO were used as control. The oxidative stress model, the doses and the incubation time of Aloe vera extract were chosen according to previously published papers [5,24,38,39].

2.3. MTT Assay

Cell viability was assessed using the MTT assay. HCE cells were treated with H_2O_2 (500 μM), H_2O_2 + aloe extract (100 μg/mL), when reaching confluence. In particular, CTRL, H_2O_2, H_2O_2 + aloe extract, were examined in a 96-well plate with a density of 8×10^4 cells/well for 24 h in order to evaluate the cytotoxic effect as previously described [40–42].

2.4. Ros Measurament

To evaluate the effect of aloe extract on OS, the production of Total Reactive Oxygen Species (ROS) in HCE cells was measured using an assay kit (Thermo Fisher, Carlsbad, CA, USA). Briefly, after the treatments the HCE cells were cleaned with PBS once, and then were incubated at 37 °C for 30 min with 2′,7′-dichlorofluorescein diacetate. DCFHDA fluorescence distribution of 1×10^4 cells was detected using a flow cytometry at two different wavelength 488 nm for excitation and 525 nm for emission [13].

2.5. Malondialdehyde Assay

The effects of aloe extract as antioxidants against lipid peroxidation in HCE cells were examined using as marker the levels of malondialdehyde (MDA) as previously described in details [43–45].

2.6. Real Time Quantitative PCR Amplification (RT-qPCR)

The m-RNA expression of SOD2, Catalase, Nrf2, Il-1β, TNF-α IL-6, COX-2, Bcl-2, Bax, Caspase-3 and Caspase-8 was evaluated as previously described [46–48]. Primers used to identify both targets and reference genes are catalogued in Table 1.

2.7. Measurements of Cytokines

Nrf2, IL-1β, TNF-α, IL-6, PGE2 and Caspase-8 levels were measured in the cell culture supernatants, Bax, Bcl-2 and Caspase-3 levels were evaluated in the cell culture extracts using an Enzyme-Linked Immunosorbent Assay (ELISA) kits (Abcam, Cambridge, UK or Thermo Fisher, Waltham, MA, USA), in agreement with the instructions reported by the manufacturer [49–52].

2.8. Catalase and SOD Activity Measurement

The Catalase and SOD activity were evaluated in agreement with the manufacturer's protocol of commercial kits (Thermo Fisher, Waltham, MA, USA). All the samples were measured in duplicate, the results were interpolated with the Catalase or SOD standard curve, and the results were expresses in units/mL.

2.9. Collection of Plant Samples and Extract Preparation

Aloe vera plants (Aloe barbadensis Miller) were subjected to vegetative propagation as previously described [53]. Briefly, young shoots were cut from the mother plant and subsequently were planted in 0.5 kg plastic pots with incorporated commercial ground. Plants received tap water once every two weeks and grown under ambient irradiance of (400–1400 μmol m^{-2} s^{-1}) and a temperature of (25 ± 1° C). After 12 months of growth, the earliest fully developed leaves were used for the extract preparation. Fresh leaves were cleaned with distillated water and cut into fragments of around 20 g each. The extraction was performed by grinding a sample of 20 g with 100 mL of 100% methanol using a high performance grinder, followed by agitation for 4 h at 4 °C. After, the extract was evaporated in a bath at 60 °C and then lyophilized for 24h. The obtained powder was weighed and stored until the use.

2.10. Phytochemical Analysis of Aloe vera Extract

In Aloe vera plants, several phytochemical constituents are present such as: anthraquinones, fatty acids, alkaloids, carbohydrates, enzymes, vitamins, mineral and other miscellaneous compounds [25].

A phytochemical screening was performed the Aloe vera methanolic extract was subjected to for the presence or absence of various phytochemical according to standard protocols [54,55].

2.11. Statistical Analysis

Data are shown as the mean ± SD and the values reported are the result of at least five experiments performed in duplicate (two wells for each treatment). To ensure reproducibility, all assays were replicated three times. The various groups were compared and evaluated using one-way ANOVA with Tukey post-test for comparison between the different groups. A p value <0.05 was considered significant. Graphs were prepared using GraphPad Prism (version 8.0 for macOS, San Diego, CA, USA).

3. Materials and Methods for PDRN set of experiment

3.1. Cell Cultures

Human Corneal Endothelial Cells (IHCE) were purchased by Creative Biolabs neuroS London, UK. The IHCE cells were cultured in PriNeu I medium enriched with 10% Fetal Bovine Serum (FBS), recombinant human epidermal growth factor (5 ng/mL), a 5 g/mL insulin plus 1% antibiotic mixture (penicillin/streptomycin), and G418 antibiotic in a humidified incubator at 37° C and with a percentage of 5% CO_2.

3.2. Cell Treatments

The IHCE cells were cultured in 6-well plates at a density of 3 x 10^6 cells/well, and were challenged with 200 µM H_2O_2 (Sigma Aldrich, Milan, Italy) for 2 h to establish an oxidative stress model. After the H_2O_2 stimulus, the cells were treated with PDRN (100 µg/mL) (Placentex Integro, Mastelli Srl, Sanremo, Italy), CGS21680 (1µM A_{2Ar} agonist) (Tocris Bioscience, Bristol, UK) and PDRN (100 µg/mL) in combination with ZM241385 (1 µM A_{2Ar} antagonist) (Tocris Bioscience, Bristol, UK) for 24 h. The induction of the oxidative stress model, the doses and the experimental time were chosen according to previously published papers [5,31,33].

3.3. FDA/PI Staining

The cells were plated at a density of 5 x 10^5 cells/well in a 24-well plate, and were incubated with H_2O_2 (200 µM) for 2 h; then, the cells were treated with PDRN (100 µg/mL), CGS21680 (1 µM) and PDRN + ZM241385 (1 µM) for 24 h. At the end of the treatment period, the cells were washed in sterile PBS and stained with the FDA/PI staining solution, with the addition of 3.2 µL FDA and 20

µL PI at the concentrations of 5mg/mL and 2 mg/mL, respectively, in 2 mL culture medium without FBS, for each well, for 5 min at room temperature in the dark. The viable cells were observed with a fluorescence microscope. ImageJ software for Windows was used to calculate the number of positive cells (Softonic, Barcelona, Spain).

3.4. ROS Measurement

In order to evaluate the effects of PDRN on oxidative stress, the production of total Reactive Oxygen Species (ROS) in the IHCE cells was measured using an assay kit (Thermo Fisher, Carlsbad, CA, USA), as previously described in detail [56].

3.5. Malondialdehyde Assay

The antioxidant effect of PDRN in IHCE cells was evaluated by measuring the malondialdehyde (MDA) levels, as previously described in detail [56].

3.6. Real-Time Quantitative PCR Amplification (RT-qPCR)

Il-1 β, TNF-α, IL-6, IL-10, Bcl-2, Bax, Caspase-3 and Caspase-8 m-RNA expression was assessed as previously described. The primers used to identify both the targets and reference genes are listed in Table 2 [33].

3.7. Measurements of the Cytokines

The IL-1β, TNF- α, IL-6 and IL-10 levels were measured in the cell culture supernatants, using Enzyme-Linked Immunosorbent Assay (ELISA) kits (Abcam, Cambridge, UK), in agreement with the instructions given by the manufacturer [57-59].

3.8. Western Blot Analysis

After 24 h of treatment, the IHCE cells were collected and protein extraction was performed to evaluate pNF-kB, Bax, Bcl-2, Caspase-3 and Caspase-8 expression by Western Blot analysis, as previously described in detail [60].

3.9. Statistical Analysis

The data presented are the results of at least five experiments, and are expressed as the mean ± SD. In order to guarantee repeatability, all of the assays were carried out in duplicate. The differences between the groups were evaluated and analysed using one-way ANOVA with the Tukey post-test. A p value of less than 0.05 was considered significant. SPSS Statistics for Windows v22.0 was used for the statistical analysis (SPSS, Inc, Chicago, IL, USA), and GraphPad Prism was used to create the graphs (Version 8.0 for macOS, San Diego, CA, USA).

4. Results for ALOE VERA set of experiment

4.1. Phytochemical Screening of Aloe vera Extract

A wide range of various phytochemicals; alkaloids, flavonoids, glycosides, phenolic compounds, tannins and saponin, steroids and terpenoids, glycosides were tested with their appropriate protocols and reagents. The Aloe vera extract showed presence of most of the phytochemicals tested, the characterization is depicted in Table 3.

4.2. Effects of Aloe Extract on Cell Vitality and Oxidative Stress

The viability of HCE cells was drastically reduced after exposure to 500 μM H_2O_2 for 24 h, as compared with the control group ($p < 0.0001$ versus control group; Figure 1A). Pre-treatment for 2h with aloe extract at 100 μg/mL significantly increased the cell viability of HCE cells incubated with H2O2 ($p < 0.0001$ vs. H_2O_2 group; Figure 1A). Furthermore, the incubation of aloe extract did not affect cell viability, thereby showing that this natural extract does not have a cytotoxic effect. To examine the ROS level in this oxidative stress setting and the effects of aloe extract, we measured the ROS production. An exposure to H_2O_2 at a concentration of 500 μM for 24 h resulted in high levels of ROS compared to the control group ($p < 0.0001$ vs. control group; Figure 1B). Whereas, aloe extract at a concentration of 100 μg/mL significantly suppressed the production of ROS ($p < 0.0001$ vs. H_2O_2 group; Figure 1B), indicating that aloe extract suppressed ROS production under the oxidative stress induced by H_2O_2. An important aspect of damage caused by ROS, especially by H_2O_2, is the oxidation of lipids, such as MDA. We observed a significant increase in MDA generation by

H_2O_2 stimulus ($p < 0.0001$ vs. control group; Figure 1C). Moreover, aloe extract at 100 μg/mL significantly inhibited MDA levels compared to the H_2O_2 group ($p < 0.0001$ vs. H_2O_2 group; Figure 1C).

4.3.Effects of Aloe Extract on mRNA Expression and Activity of Antioxidant Markers

To analyze the effects of aloe extract during oxidative stress, we measured the gene expression and mature protein levels of Nrf2, chief regulator of the antioxidant system, and the enzyme activities and gene expression of SOD2 (one of the major antioxidant defence systems against free radicals) and catalase (one of the most important antioxidant enzymes) involved in antioxidant defences. Exposure of HCE cells to H_2O_2 for 24h significantly decreased mRNA expression of Nrf2, SOD2 and Catalase compared to the control group ($p < 0.0001$ vs. control group; Figure 2). While, 2h of pre-treatment with aloe extract significantly upregulated the gene expression of Nrf2, SOD2 and Catalase when compared to cell cultures challenged with H_2O_2 alone ($p < 0.0001$ vs. H_2O_2 group; Figure 2). Moreover, Nrf2 mature protein levels were significantly reduced in the H_2O_2 group as compared to the control group ($p < 0.0001$ vs. control group; Figure 2). Meanwhile, 2h pre-treatment with aloe extract significantly increased Nrf2 levels in the cells after exposure to H_2O_2 stimulus ($p < 0.0001$ vs. H_2O_2 group; Figure 2). In addition, it was revealed that catalase and SOD activity was decreased in HCE cells after exposure to H_2O_2 at a concentration of 500 μM for 24 h ($p < 0.0001$ versus control group) while 2h pre-treatment with aloe extract at 100 μg/mL significantly upregulated the catalase and SOD activity in HCE cells after exposure to H_2O_2 ($p < 0.0001$ vs. H_2O_2 group; Figure 2).

4.4 Effects of Aloe Extracts on Inflammatory Markers

As estimated, H_2O_2 induced a significant upregulation of mRNA expression of proinflammatory enzyme COX-2, a common feature of inflammation caused by oxidative stress, and a marked expression of proinflammatory cytokines IL-1β, IL-6 and TNF-α compared to control group ($p < 0.0001$ vs. control group; Figure 3). Pre-treatment with aloe extract in H_2O_2 stimulated HCE cells suppressed the increased mRNA for the inflammatory enzyme COX-2 and caused a marked reduction in the expression of the message of the inflammatory cytokine TNF-α, IL-6 and IL-1β ($p < 0.0001$ vs. H_2O_2 group; Figure 3). To confirm the anti-inflammatory effect of the aloe extract we measured the mature protein levels in the supernatants of HCE cells stimulated with H_2O_2. TNF-α, IL-6 and IL-1β levels were markedly increased ($p < 0.0001$ vs. control group; Figure 3). By contrast aloe extract pre-treatment blunted the increase of TNF-α, IL-6 and IL-1β in the HCE cells stimulated with H_2O_2 ($p < 0.0001$ vs. H_2O_2 group; Figure 3). PGE2 levels metabolite of COX-2 was also markedly released in the supernatants of HCE cells upon H_2O_2 stimulation ($p < 0.0001$ vs. control group; Figure 3). Pre-treatment with aloe extract resulted in a powerful reduction of PGE2 levels ($p < 0.0001$ vs. H_2O_2 group; Figure 3).

4.5. Effects of Aloe Extract on Apoptosis

To examine the role of aloe extract in regulating apoptosis in HCE cells stimulated with H_2O_2, the mRNA expression of Bcl-2, Bax, Caspase-3 and Caspase-8 was evaluated. HCE cells challenged

with H_2O_2 for 24 h showed a marked reduction of Bcl-2 expression with a concomitant up-regulation in the mRNA expression of Bax, Caspase-3 and Caspase-8 compared to control cells ($p < 0.0001$ vs. control group; Figure 4). Conversely, when cells were pretreated with Aloe extract for 2 h, mRNA expression of Bcl-2, Bax, Caspase-3 and Caspase-8 were reversed ($p < 0.0001$ vs. H_2O_2 group; Figure 4). To better evaluate the antiapoptotic effect of aloe extract we measured the protein levels of Bcl-2, Bax, Caspase-3 and Caspase-8 in HCE cells challenged with H_2O_2. After HCE cells were challenged with H_2O_2 for 24 h Bcl-2 protein expression levels were significantly decreased with a contemporaneous increase in protein expression of Bax, Caspase-3 and Caspase-8 compared to control cells ($p < 0.0001$ vs. control group; Figure 4). By contrast, when cells were pretreated with aloe extract for 2 h, a significant growth in Bcl-2 protein expression with a simultaneous reduction of Bax, Caspase-3 and Caspase-8 protein levels were observed ($p < 0.0001$ vs. H_2O_2 group; Figure 4).

5. Results for PDRN set of experiment

5.1. Effects of PDRN on Cell Viability

The control cells showed 100% viability, whereas cell exposure to H_2O_2 200 µM for 2 h significantly reduced IHCE viability compared to the controls ($p < 0.0001$ vs. the control group; Figure 5). The IHCE cells' viability following H_2O_2 challenge and treatment with PDRN for 24 h was extensively increased ($p < 0.0001$ vs. H_2O_2 group; Figure 1). Furthermore, the treatment with the specific A_{2Ar} agonist CGS21680 significantly augmented IHCE cells' cell viability, thus confirming A_{2Ar}'s involvement in the promotion of cell viability. The co-incubation with the A2Ar antagonist ZM241385 abrogated the effects of PDRN, thus pointing out PDRN's mechanism of action in this cell type and in an oxidative stress condition ($p < 0.0001$ vs. the PDRN group; Figure 5).

5.2. The Effects of PDRN on Oxidative Stress

A significant increase in ROS levels was observed in IHCE cells as a consequence of H_2O_2 stimulation ($p < 0.0001$ vs. the control group; Figure 6A). PDRN and CGS treatment significantly reduced ROS levels compared to untreated IHCE cells, confirming that A_{2Ar} stimulation is involved in the antioxidant effects of PDRN ($p < 0.0001$ vs. the H_2O_2 group; Figure 6A). The co-incubation with ZM241385 blunted the antioxidant effects of PDRN, confirming that PDRN acts through A_{2Ar} modulation (Figure 6A). In order to better characterize the antioxidant effects of PDRN, the malondialdeyide (MDA) levels were measured in the IHCE cells. Low levels of MDA were detected in the control cells, whereas H_2O_2 challenge considerably increased the MDA levels ($p < 0.0001$ vs.

the control group; Figure 6B). Both PDRN and CGS21680 decreased MDA production ($p< 0.0001$ vs. H_2O_2 group; Figure 6B), while co-incubation with ZM241385 counteracted the beneficial effects of PDRN (Figure 6B).

5.3. The Effects of PDRN on Inflammatory Markers

The gene expression of TNF- α, IL-1β, IL- 6 and IL-10 was studied in order to evaluate the anti-inflammatory effects of PDRN in this experimental model. H_2O_2 exposure caused a significant increase in TNF- α, IL-1 β and IL- 6 gene expression, as well as a significant decrease of IL-10 expression compared to untreated cells ($p < 0.0001$ vs. the control group; Figure 7). PDRN treatment blunted TNF- α, IL-1 β and IL-6 expression, and upregulated IL-10 mRNA expression compared to the H_2O_2 group ($p < 0.0001$ vs. the H_2O_2 group; Figure 7). CGS21680, a selective A_{2Ar} agonist, caused similar effects, indicating that A_{2Ar} is involved in inflammatory cascade modulation ($p < 0.0001$ vs. the H_2O_2 group; Figure 7). The co-incubation with the A_{2Ar} antagonist ZM241385 abrogated the effects of PDRN, thus corroborating the involvement of A_{2Ar} in PDRN's mechanism of action (Figure 7).

In addition, the expression of mature proteins was evaluated in order to confirm PDRN's anti-inflammatory effects. TNF- α, IL-1 β, p-NF-kB and IL-6 protein expression were markedly increased in H2O2-stimulated cells compared to the untreated cells ($p < 0.0001$ vs. the control group; Figure 4). PDRN treatment dampened TNF- α, IL-1β , p-NF-kB and IL-6 increased expression following H_2O_2 incubation ($p < 0.0001$ vs. the H_2O_2 group; Figure 8). Additionally, IL-10 protein expression

was markedly reduced following H_2O_2 incubation compared to the untreated cells ($p < 0.0001$ vs. the control group; Figure 8). PDRN and CGS21680 treatments showed a significant increase of IL-10 protein levels, confirming that PDRN anti-inflammatory effects were related to A_{2Ar} activation ($p < 0.0001$ vs. the H_2O_2 group; Figure 8). The co-incubation with the A_{2Ar} antagonist ZM241385 reverted the effects of PDRN, indicating that A_{2Ar} activation is involved in PDRN's mechanism of action (Figure 8).

5.4. Effects of PDRN on Apoptosis

The gene expressions of Bcl-2, Bax, Caspase-3 and Caspase-8 were studied in order to evaluate the apoptosis process. IHCE cells challenged with H_2O_2 for 2 h exhibited a significant down-regulation of Bcl-2 mRNA levels with a simultaneous increase of Bax, Caspase-3 and Caspase-8 compared to the untreated cells ($p < 0.0001$ vs. the control group; Figure 9). On the other hand, PDRN treatment for 24 h completely inverted the gene expression of Bcl-2, Bax, Caspase-3 and Caspase-8 ($p < 0.0001$ vs. the H_2O_2 group; Figure 9). CGS21680, a selective A_{2Ar} agonist, showed similar effects, thus confirming the role of A_{2Ar} activation in the modulation of the apoptotic process ($p < 0.0001$ vs. H_2O_2 group; Figure 9).

The A_{2Ar} antagonist ZM241385 counteracted the effects of PDRN, highlighting the role of A_{2Ar} in PDRN's mode of action (Figure 9). In order to confirm the antiapoptotic effect of PDRN, we also evaluated the mature protein. A significant decrease of the Bcl-2 protein levels together with a concurrent increase of Bax, Caspase-3 and Caspase-8 were detected following H_2O_2 challenge

compared to the untreated cells ($p < 0.0001$ vs. the control group; Figure 10). PDRN or CGS21680 treatment for 24 h caused a significant increase of Bcl-2 protein expression with a concomitant decrease of Bax, Caspase-3 and Caspase-8 protein levels ($p < 0.0001$ vs. the H_2O_2 group; Figure 10). ZM241385 blocked the effects of PDRN, again confirming the role of A_{2Ar} activation in apoptosis modulation (Figure 10).

6. Discussion

Fuchs is a corneal endothelium degenerative condition characterized by the accumulation of focal guttae, contributing to oedema of the cornea and vision loss. The corneal endothelium is a single layer that serves as a barrier, maintains a particular level of corneal hydration and preserves corneal stromal clarity through precise spatial collagen fiber arrangement. End-stage FECD corneal endothelial cells are reduced in number and appear attenuated, inducing progressive stromal swelling and resulting in blurred vision. These pathological conditions are related to CECs apoptosis caused by an exaggerated ROS production that lead to oxidative stress. ROS can be divided into two forms: radical and nonradical species. Hydrogen peroxide (H_2O_2), superoxide anion (O2−), ozone (O3), and nitric oxide (NO) belong to the first category. Oxidative stress may have distinct effects, and the cellular response depends on its behaviour. The signalling role of ROS has also been shown to be important for the integrity of living organisms and their aging process. In addition, ROS may be involved in various damage mechanisms, such as membrane lipid peroxidation, protein structure damage and also in different ocular pathologies such as ocular surface disorders.

In this scenario, evaluating the efficacy of natural extracts could be of interest. Indeed, medicinal plants and their derivatives have shown several beneficial effects, including the reduction of reactive oxygen species (ROS) (antioxidant activity), the prevention of cell apoptosis, and the modulation of pro-inflammatory factors. In particular, among medicinal plants, Aloe vera (A. barbadensis Miller) has a number of pharmacological properties, such as antioxidant, immunomodulatory, bactericidal, antiviral, antifungal and anti-inflammatory due to the presence of a variety of chemicals like flavonoids, anthraquinones, enzymes, vitamins, and phenolic acids. In addition, in previous studies,

Aloe vera extract showed the ability to speed up re-epithelialization and minimize fibrosis in superficial corneal lesions [24,53].

In light of these preceding observations, in the present study, we evaluated the efficacy of an extract from Aloe vera in an “in vitro” model of FECD, induced by H_2O_2 stimulus. Previous papers have investigated the possibility that hydrogen peroxide, its products, and/or other oxidant species may be in part responsible for the functional and structural alterations of corneal endothelial cells during ocular inflammatory disease processes [61]. Moreover, preceding works have demonstrated that the corneal endothelium is susceptible to oxidative stress, which leads to inflammation and apoptosis [5, 62– 64]. Our results have shown that hydrogen peroxide causes the alteration and apoptosis of corneal endothelial cells according to previous published papers [65, 66]. Pre-incubation with aloe

extract significantly reduced oxidative stress markers and upregulated the expression of Nrf2, SOD2 and Catalase when compared to cells cultures challenged with H_2O_2 alone.

These effects may be attributed to Aloin, a substance with many biological activities present in Aloe vera. Indeed, Aloin has been demonstrated to have anti-oxidant effects in two different models of oxidative stress damage in skin fibroblasts and macrophages [39,67].

These results are in accordance with other studies that demonstrated the protective effects of Nrf2 agonists on oxidative stress in corneal endothelium due to the antioxidant activity of these compounds, corroborating the idea that the implementation of antioxidant systems may play a role in the treatment of corneal ROS related diseases [28].

Moreover, pre-treatment with aloe extract in H_2O_2 stimulated HCE cells, suppressed the increased mRNA for the COX-2 enzyme and caused a marked reduction in the expression of the message and protein levels of the inflammatory cytokine TNF-α, IL-6 and IL-1β when compared to cell cultures challenged with H_2O_2 alone. These findings may be related to several Aloe compounds such as Aloin and Aloe-emodin that exhibited a great suppression of pro-inflammatory cytokine expression in murine macrophages and in human gingival fibroblast under oxidative stress stimulus [25,39]. Furthermore, our results agree with the findings of other previous studies showing that Aloe vera's anti-inflammatory activity is linked to the downregulations of the arachidonic acid pathway via cyclooxygenase [68]. Moreover, it has been elucidated that the presence of sterols in the Aloe vera extract may reduce the production of the phospholipase A2 enzyme, which is accountable for the release of arachidonic acid precursors in the synthesis of prostaglandins. As a consequence, Aloe vera extract's anti-inflammatory activity is the result of the inhibition of both prostaglandin and leukotriene synthesis [68]. Furthermore, it has been demonstrated that the herb's extracts may inhibit the inflammatory process in different ways by reducing the number of circulating cytokines and also inhibiting the adhesion ability of leukocytes in the injury site. [69]. In addition, previous papers have demonstrated that medical herbal plants have antioxidant effects in human corneal cells and their administration to the eye influences local homeostasis among other effects on the cytokine network [70]. In particular, it has been well-demonstrated that Aloe vera extract is full of various biologically active constituents with different therapeutic properties, such as: Wound-healing properties and immunomodulatory, anti-inflammatory and antioxidant effects [25,71]

Additionally, our results demonstrated that pre-treatment with Aloe vera extract for two hours increased Bcl-2 levels and reduced Bax, Caspase-3 and Caspase-8 mRNA expression and protein levels compared to cell cultures challenged with H_2O_2 alone. These results agree with the findings of previous studies showing that nature-derived antioxidant application had anti-inflammatory and anti-apoptotic activities, and may avoid the aggravation of different illnesses caused by oxidative stress in eye models [72–74].

Moreover, previous papers have already established that the activity of Aloe vera extracts did not have toxic effects even at high concentrations and can be helpful as a complimentary treatment for eye disorders. Aloe vera has well-established pharmacological proprieties that allow the activation and inhibition of different enzymes, modulating the metabolism of cells, the expression of anti-inflammatory, antifungal and antibacterial proprieties [75].

Additionally, in a previous study the healing of corneal epithelial lesions mechanically induced in rabbits after Aloe vera application was observed, without toxic effect [71, 76]. Furthermore, Aloe vera extracts demonstrated a concentration-dependent ROS scavenging action that can be attributed to the phenolic compounds present in the extracts and this is considered its principal beneficial activity [77].

Considering that, the compounds with antioxidant activity showed excellent efficacy for the treatment of corneal pathologies, we decided to evaluate in the same experimental model another compound the Polydeoxyribonucleotide (PDRN) a DNA-derived drug which is extracted from the sperm cells of salmon trout that, in addition to the antioxidant activity, also has anti-inflammatory and a pro-

angiogenetic properties. These therapeutic effects, mediated by the activation of adenosine A_{2A} receptor and salvage pathways, suggest its use in regenerative medicine.

Previous papers have demonstrated that adenosine A_{2Ar} activation prevents ROS generation, also reducing inflammation and the apoptotic process [78, 79]. PDRN, which is recognized as an A_{2Ar} agonist, showed several properties—including antioxidant, anti-apoptotic and anti-inflammatory ones—in several pre-clinical in vivo disease models, such as airway inflammation, cerebral ischemia, interstitial cystitis, acute lung injury, and ischemic colitis [80–84]. Moreover, a previous clinical trial demonstrated that PDRN eye drops stimulated corneal epithelium regeneration, thus supporting the hypothesis that it could be used for the treatment of corneal diseases [32, 35]. Therefore, in the present study, the efficacy of PDRN was evaluated in an in vitro FECD model induced by H_2O_2 stimulation. Previous studies have demonstrated that hydrogen peroxide stimulation may promote the functional and structural changes that occur in corneal endothelial cells in FECD [5, 56]. Furthermore, in this experimental setting, CECs challenged with H_2O_2 showed an increase of oxidative stress, inflammatory and apoptotic processes, in accordance with previous papers [85, 86]. PDRN treatment drastically reduced oxidative stress markers such as ROS content and MDA levels compared to the H_2O_2 group. By contrast, these effects were abolished when the A_{2Ar} antagonist ZM241385 was used together with PDRN, thus demonstrating that its antioxidant effects occurred through adenosine A_{2Ar} stimulation.

Moreover, CECs challenged with H_2O_2 and treated with the specific A_{2Ar} agonist CGS21680 showed a marked reduction of either ROS or MDA levels, thus confirming the involvement of A_{2Ar} in oxidative stress suppression in these experimental conditions. These results are consistent with

previous articles that showed the protective effects of PDRN against ROS-related diseases, such as IBD [30], supporting the idea that the implementation of an antioxidant system through adenosine A_{2Ar} activation may also play a role in the treatment of diseases characterized by oxidative stress, such as FECD.

Moreover, H_2O_2 stimulation activated the transcriptional factor NF-kB, which in turn enhanced the expression of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6, with a concomitant downregulation of the anti-inflammatory cytokine IL-10. PDRN significantly reduced pro-inflammatory cytokine expression and increased IL-10 levels compared to the untreated H_2O_2 group of cells. CECs treated with CGS21680 showed similar results, while ZM241385 co-incubation inhibited the beneficial effects of PDRN, demonstrating again that PDRN activity occurs through A_{2Ar} stimulation. These findings are consistent with earlier data, which have characterized well the anti-inflammatory effects mediated by A_{2Ar} in several preclinical inflammatory models [87,88], and with recent papers that pointed out the inactivation of the NF-kB pathway mediated by PDRN [89,90].

The activation of the oxidative stress mechanism may be also responsible for the induction of cell death processes; in fact, H_2O_2 stimulus induced apoptosis in CECs. PDRN treatment decreased the expression of the proteins involved in apoptosis—such as Bax, Caspase-3 and Caspase-8—whereas it increased Bcl-2 levels compared to untreated H_2O_2-stimulated cells. Furthermore, cells treated with CGS21680 showed a modulation of the apoptotic process; conversely, by adding ZM241385 to PDRN-treated cells, the positive effects observed when the compound was used alone were abolished, thus confirming that PDRN acts through modulation of adenosine A_{2Ar}. As mentioned, these results

are in line with the data obtained from previous studies, and indicate that adenosine A_{2Ar} modulation might prevent the worsening of oxidative stress-related diseases in eye models [91,92].

These preliminary findings are intriguing, and point to new therapeutic alternatives, however, both of these studies are subject to some limitations. In particular, the limitation of the Aloe Vera set of experiment are that we evaluated only the effects of Aloe Vera extract in this “in vitro” model of FECD, additional studies should be performed to compare the efficacy of this extract to other extracts from medical plants with antioxidant activities. Additionally, we did not asses in detail the potential ophthalmological side effects arising from human Aloe Vera use, and such aspects should be carefully evaluated in future studies when considering a longer disease course and human lifespan.

Furthermore, in the PDRN set of experiment a major limitation of the study is that the effects of PDRN were evaluated in an in vitro FECD model; hence, additional in vivo research would be required in order to characterize its efficacy.

7. Conclusion

To date there are no non-surgical treatments for FECD [82], but the present experiment demonstrated the efficacy of this extract from Aloe Vera as treatment for FECD due to its modulating effect on the inflammatory cytokine network and antioxidant function, including ROS scavenging in eye tissue. Since, Aloe Vera extracts are currently on the market for the treatment of other pathologies and severe side effects were not reported, the extract could be readily available for clinical trials in FECD patients. Therefore, these results exalt the potential future application of Aloe extract as a complimentary treatment option for patients with FECD, in a possible formulation of eye drops.

Instead, PDRN might be deemed to be a novel treatment for FECD, because this adenosine A_{2Ar} agonist is already on the market for a variety of uses and it could be readily available for a clinical trial in FECD patients. Moreover, previous works demonstrated that the half-life of PDRN is around 12–17 h, indicating that it may be suitable for once-daily administration in ordinary clinical practice [30]. Finally, it should be pointed out that PDRN is already commercially available, it is well tolerated, and it showed a very good safety profile across several clinical trials and in a variety of therapeutic applications [93–95]. These intriguing preclinical findings, in light of PDRN's strong translational potential, should be confirmed in a FECD clinical context.

In conclusion, our findings suggest that the application of Aloe Vera extract or PDRN may protect the cornea from oxidative stress and may provide a scientific basis for the use of this extract in the treatment of corneal inflammation. This effect, in light of its high translational potential, merits confirmation in a clinical setting.

8. Figures

Gene	Gene Access Number	Sequence
β-actin	NG_007992	Fw:5′AGAGCTACGAGCTGCCTGAC3′
		Rw:5′AGCACTGTGTTGGCGTACAG3′
SOD2	NG_008729	Fw:5′GAGAAGTACCAGGAGGCGTTG3′
		Rw:5′GAGCCTTGGACACCAACAGAT3′
Catalase	NG_013339	Fw:5′ACTGAGGTCCACCCTGACTAC3′
		Rw:5′TCGCATTCTTAGGCTTCTCA3′
Nrf2	NC_000002.12	Fw:5′CTCCACAGAAGACCCCAACC3′
		Rw:5′TCTGCAATTCTGAGCAGCCA3′
IL-1β	NG_008851	Fw:5′TGAGCTCGCCAGTGAAATGA3′
		Rw:5′AGATTCGTAGCTGGATGCCG3′
TNF-α	NG_007462	Fw:5′CAGAGGGCCTGTACCTCATC3′
		Rw:5′GGAAGACCCCTCCCAGATAG3′
IL-6	NG_011640	Fw:5′TTCGGTCCAGTTGCCTTCTC3′
		Rw:5′CAGCTCTGGCTTGTTCCTCA3′
COX-2	NG_028206	Fw:5′GTTCCACCCGCAGTACAGAA3′
		Rw:5′AGGGCTTCAGCATAAAGCGT3′
Bcl-2	NG_009361	Fw:5′GCTCTTGAGATCTCCGGTTG3′
		Rw:5′AATGCATAAGGCAACGATCC3′
Bax	NG_012191	Fw:5′TTTGCTTCAGGGTTTCATCC3′
		Rw:5′CAGTTGAAGTTGCCGTCAGA3′
Caspase-3	NC_000004.12	Fw:5′CCTGGTTCATCCAGTCGCTT
		Rw:5′ TCTGTTGCCACCTTTCGGTT
Caspase-8	NC_007117.7	Fw:5′GGTTAGGGGACTCGGAGACT3′
		Rw:5′CAGGCTCAGGAACTTGAGGG3′

Table 1. Primer list

Gene	Sequence
β-actin	Fw:5′AGAGCTACGAGCTGCCTGAC3′
	Rw:5′AGCACTGTGTTGGCGTACAG3′
IL-1β	Fw:5′TGAGCTCGCCAGTGAAATGA3′
	Rw:5′AGATTCGTAGCTGGATGCCG3′
TNF-α	Fw:5′CAGAGGGCCTGTACCTCATC3′
	Rw:5′GGAAGACCCCTCCCAGATAG3′
IL-6	Fw:5′TTCGGTCCAGTTGCCTTCTC3′
	Rw:5′CAGCTCTGGCTTGTTCCTCA3′
IL-10	Fw:5′TGGCGCGGTGGATTCATAC3′
	Rw:5′AGGGGTCTGTTTTGTTGGCA3′
Bcl-2	Fw:5′GCTCTTGAGATCTCCGGTTG3′
	Rw:5′AATGCATAAGGCAACGATCC3′
Bax	Fw:5′TTTGCTTCAGGGTTTCATCC3′
	Rw:5′CAGTTGAAGTTGCCGTCAGA3′
Caspase-3	Fw:5′CCTGGTTCATCCAGTCGCTT
	Rw:5′ TCTGTTGCCACCTTTCGGTT
Caspase-8	Fw:5′GGTTAGGGGACTCGGAGACT3′
	Rw:5′CAGGCTCAGGAACTTGAGGG3′

Table 2. Primer list.

Reducing sugar
Phenolic compounds
Alkaloids
Flavonoids
Steroids and Terpenoids
Tannins
Glycosides
Anthraquinones

Table 3. Qualitative analyses of the phytochemical components of Aloe vera extract.

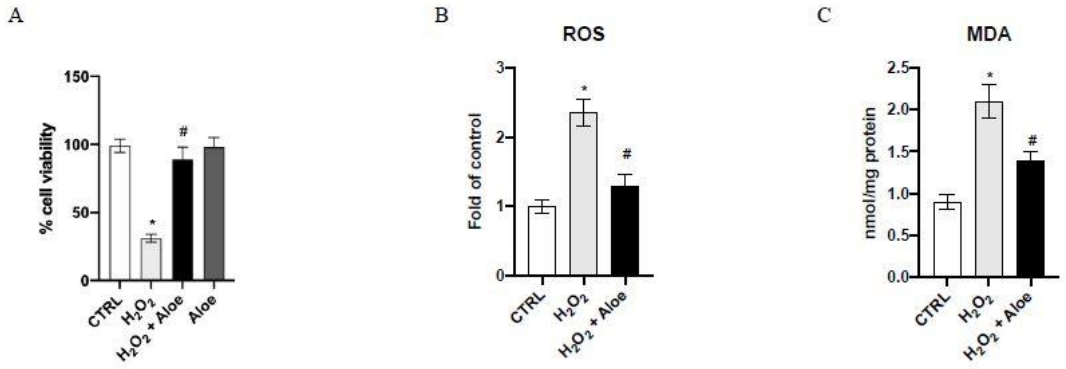

Figure 1. Effects of pre-treatment with aloe extract on cell viability (A), ROS production (B), MDA generation (C). Values are expressed as the means ± SD. * $p < 0.0001$ vs. CTRL; # $p < 0.0001$ vs. H_2O_2.

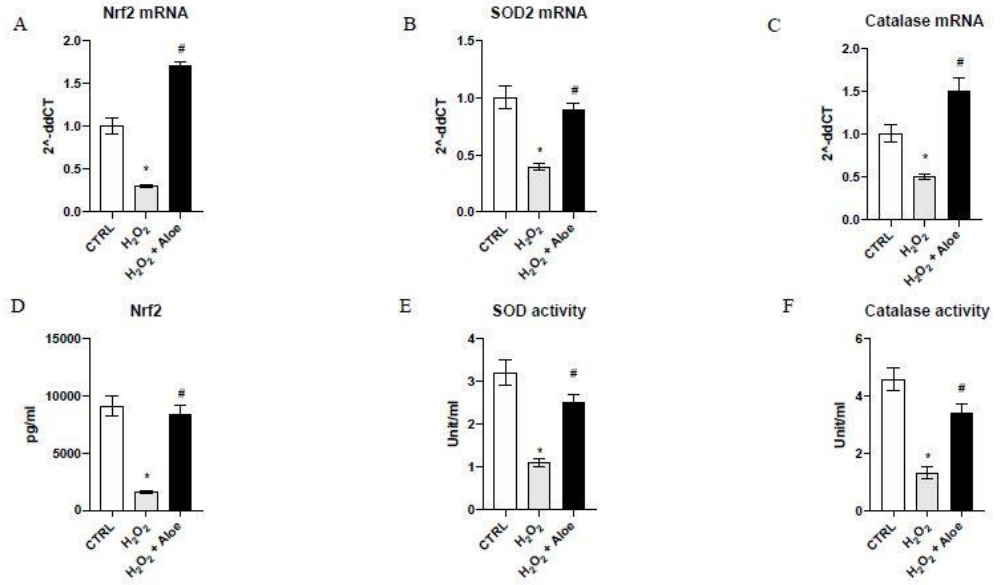

Figure 2. Effects of pre-treatment with aloe extract on Nrf2 (**A**), SOD2 (**B**), Catalase (**C**) mRNA expression. Effects of pretreatment with aloe extract on Nrf2 levels (**D**), SOD activity (**E**), Catalase activity (**F**). Values are expressed as the means ± SD. * $p < 0.0001$ vs. CTRL; # $p < 0.0001$ vs. H_2O_2.

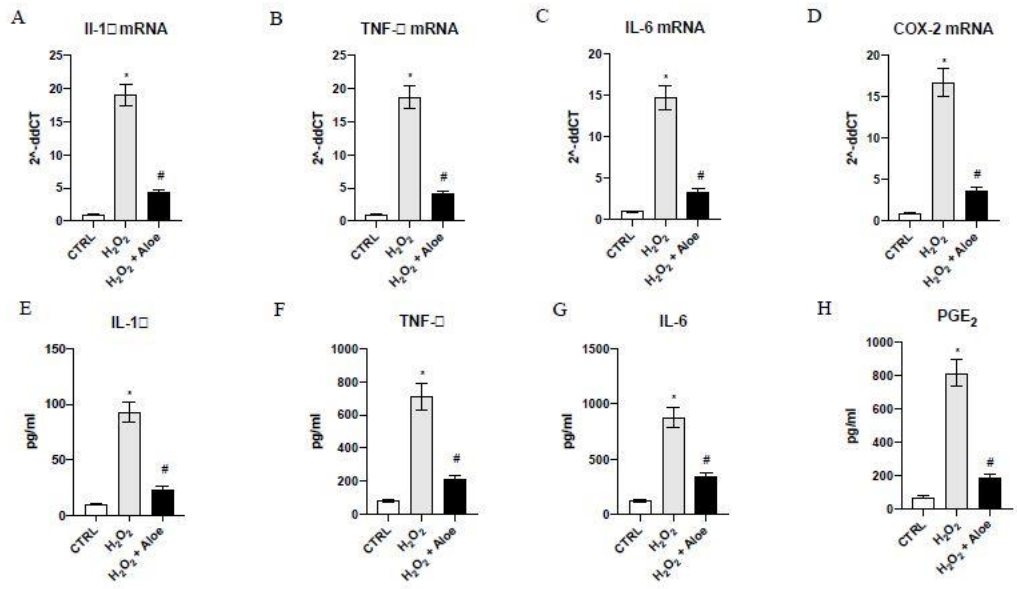

Figure 3. Effects of pre-treatment with aloe extract on IL-1β (A), TNF-α (B), IL-6 (C), COX-2 (D) mRNA expression. Effects of pre-treatment with aloe extract on IL-1β (E), TNF-α (F), IL-6 (G), PGE2 (H) levels. Values are expressed as the means ± SD. * $p < 0.0001$ vs. CTRL; # $p < 0.0001$ vs. H_2O_2.

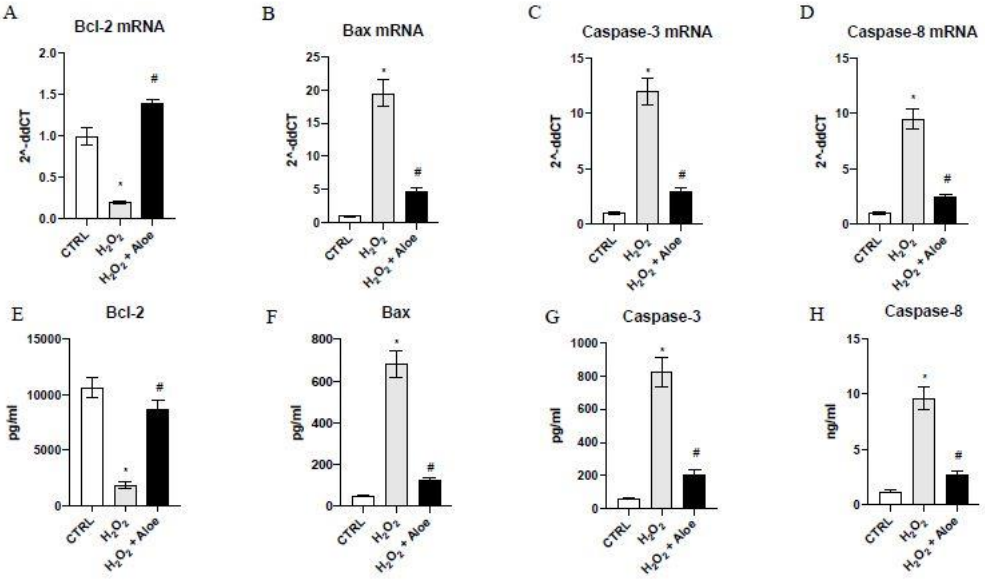

Figure 4. Effects of pre-treatment with aloe extract on Bcl-2 (A), Bax (B), Caspase-3 (C), Caspase-8 (D) mRNA expression. Effects of pre-treatment with aloe extract on Bcl-2 (E), Bax (F), Caspase-3 (G), Caspase-8 (H) levels. Values are expressed as the means ± SD. * $p < 0.0001$ vs. CTRL; # $p < 0.0001$ vs. H_2O_2.

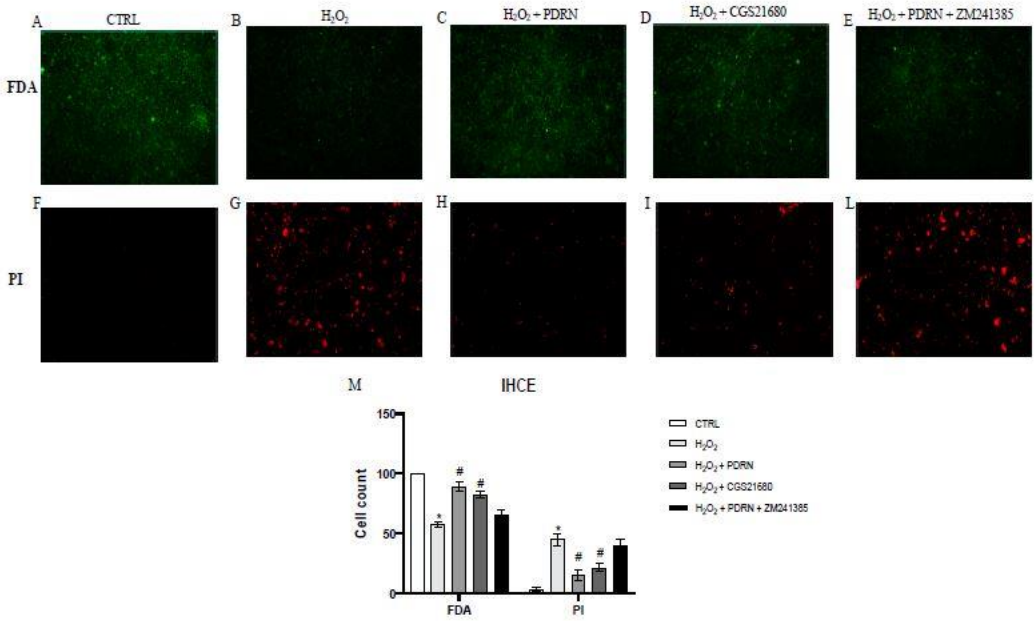

Figure 5. The figure shows the availability of the IHCE cell line treated with PDRN, as evaluated by FDA/PI staining. In panels (A–E) the green colour staining indicates viable IHCE cells; in panels F–L the red staining indicates IHCE cells in apoptosis. Panel (M) shows the IHCE cell count. The data are expressed as means ± SD. * $p < 0.0001$ vs. CTRL; # $p < 0.0001$ vs. H_2O_2.

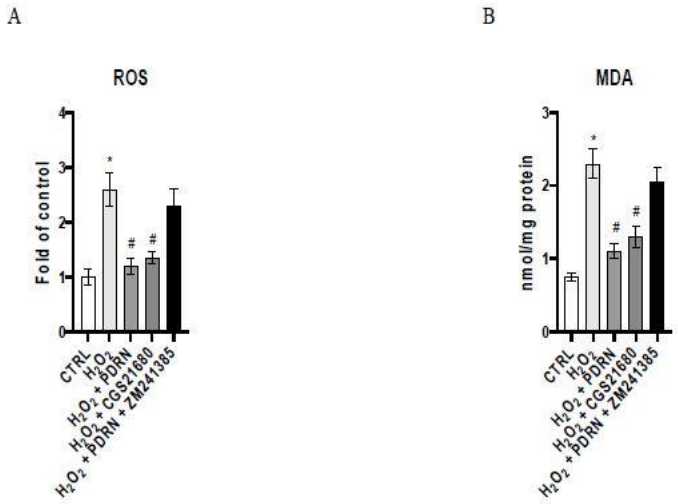

Figure 6. Effects of PDRN treatment on ROS production (A) and MDA generation (B) in IHCE cells.The values are expressed as the means ± SD. * $p < 0.0001$ vs. CTRL; # $p < 0.0001$ vs. H_2O_2.

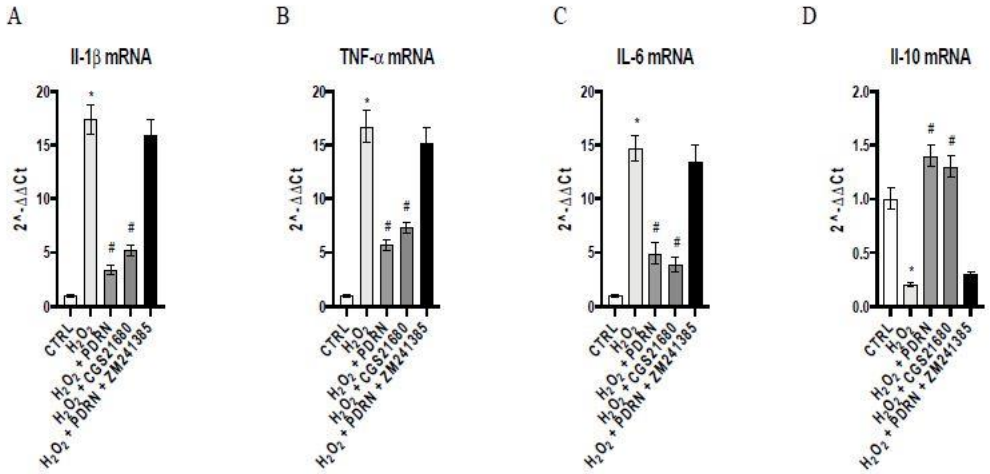

Figure 7. The graphs represent the qPCR results of Il-1β (A), TNF-α (B), Il-6 (C) and Il-10 (D) gene expression in IHCE cells. The values are expressed as the means and SD. * $p < 0.0001$ vs. CTRL; # $p < 0.0001$ vs. H_2O_2.

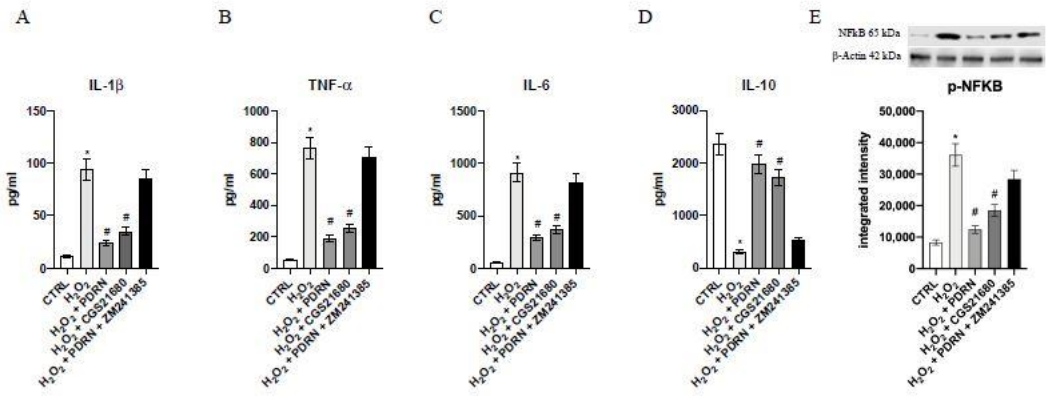

Figure 8. The graphs represent the Il-1β (A), TNF-α (B), Il-6 (C), Il-10 (D) and p-NF-κB (E) protein expression in IHCE cells. The values are expressed as the means and SD. * $p < 0.0001$ vs. CTRL; # $p < 0.0001$ vs. H_2O_2.

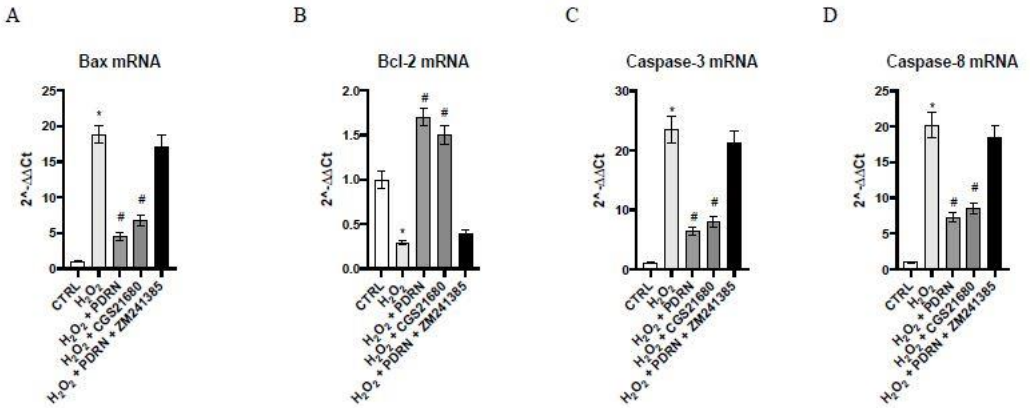

Figure 9. The graphs represent the gene expression results of Bax (A), Bcl-2 (B), Caspase-3 (C) and Caspase-8 (D) in IHCE cells. The values are expressed as the means and SD. * $p < 0.0001$ vs. CTRL; # $p < 0.0001$ vs. H_2O_2.

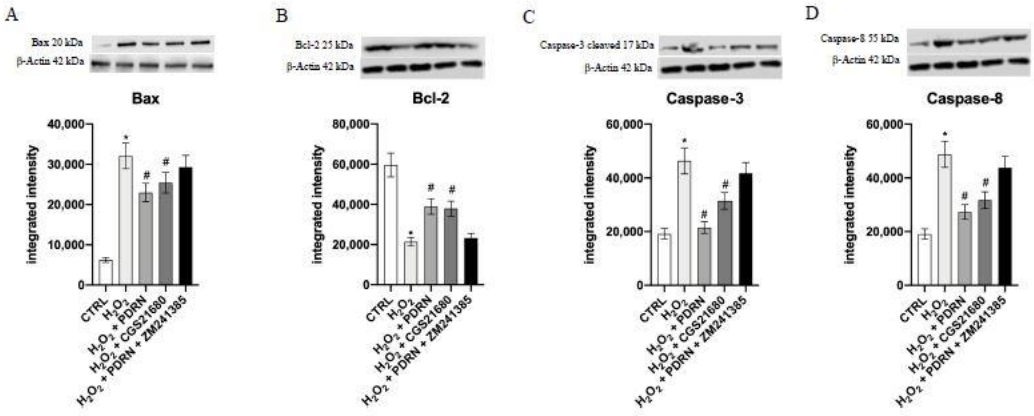

Figure 10. The graphs represent Bax (A), Bcl-2 (B), Caspase-3 (C) and Caspase-8 (D) protein expression in IHCE cells. The values are expressed as the means and SD. * $p < 0.0001$ vs. CTRL; # $p < 0.0001$ vs. H_2O_2.

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