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Abstract

Aims: Using an established three-dimensional (3D) toxicological model based on reconstituted human corneal epithelium (HCE), this study investigated the tolerability of four topical intraocular pressure (IOP)-lowering agents: the commercial solutions of benzalkonium chloride (BAC)-containing 0.005% latanoprost, 0.004% travoprost, 0.03% bimatoprost containing 0.02%, 0.015%, and 0.005% BAC, respectively and the preservative-free (PF) tafluprost. Solutions of 0.01% and 0.02% BAC alone were also evaluated for comparison.

Methods: The 3D-HCEs were treated with either solutions for 24 hours followed or not by a 24-hour recovery period. We used a modified MTT procedure to assess cell viability in the HCE. Frozen sections of HCE were analyzed using fluorescence microscopy for the evaluation of apoptosis (TUNEL), inflammation (ICAM-1), and proliferation (Ki67). Corneal epithelial tight junctions (occludin and ZO-1) were also assessed by en-face confocal microscopy in response to the different eye drops.

Results: The MTT test revealed that the cytotoxicity of antiglaucoma eye drops was primarily related to the concentration of their common BAC preservative (0.02%BAC-latanoprost > 0.015%BAC-travoprost > 0.005%BAC-bimatoprost). PF-tafluprost did not induce obvious cytotoxicity and showed the least expression of inflammatory or apoptotic markers and revealed preservation of membrane immunostaining of tight junction proteins in comparison with BAC-containing solutions.

Conclusion: The toxicological model of 3D reconstructed corneal epithelia model confirmed the ocular surface cytotoxicity of BAC-containing antiglaucomatous solutions. Compared to

the formulations containing the toxic preservative BAC, PF-tafluprost was well tolerated without inducing significant corneal epithelium deterioration.

Introduction

Tafluprost is a newly synthesized PGF2alpha-agonist without any toxic preservative [1].It demonstrated no cytotoxicity in human conjunctival epithelial cell lines [2] and was well tolerated following short- and repeated instillations in rabbits[3]. The problems raised by potentially toxic glaucoma treatments should really require more attention to be paid due to the increased number of patients in the world, life-span duration of treatments that require not only efficacy, but also safety, good tolerability, and optimal compliance for patients.

Supplied by SkinEthic® Laboratories, the reconstructed three-dimensional (3D) model of human corneal cells (HCE) is an appropriate alternative toxicological method to the classical Draize test in rabbits.[4] It was proven to have the same characteristic as the corneal epithelium of the human eye in morphology and thickness, and is suitable for investigating the undesired effects of ophthalmic drugs.[5] The objective of this study was to investigate in this 3D-HCE system the tissue changes after the treatment of the currently available commercial prostaglandin (PG)-derived eye drops, namely the commercial solutions of 0.005% latanoprost, 0.004% travoprost, 0.03% bimatoprost (containing 0.020%, 0.015%, and 0.005% of benzalkonium chloride (BAC), respectively), and preservative-free (PF) tafluprost. We analyzed a panel of five selected biomarkers in order to assess the phenomena of apoptosis, inflammation, proliferation and tight junction impairment after a contact with the different antiglaucoma eye drops, which would contribute to a better understanding of human corneal cell reactions following topical antiglaucoma treatments.

Materials and methods

Tissue model and antiglaucoma solution treatments

The 3D-HCE model was supplied by SkinEthic® Laboratories (Nice, France) and consists of immortalized HCE cells grown vertically on a 0.5cm^2 insert permeable polycarbonate filter. All the experimentations were conducted as previously published [5]: 30µl of sterile phosphate-buffered saline (PBS) used as negative control solution, BAC solutions at 0.020% and 0.010% used as positive controls and the commercial solutions of 0.020% BAC-containing latanoprost (Xalatan®; Pfizer, NY, USA), 0.015% BAC-containing travoprost (Travatan®, Alcon, TX, USA), 0.005% BAC-containing bimatoprost (Lumigan®, Allergan, CA, USA) or PF-tafluprost (Taflotan®, Santen Oy, Tampere, Finland) were applied on the apical surface of 3D-HCE for 24 hours (h) and 24 h followed by an additional 24h-recovery period (24h+24h-recovery). The recovery period (24 hours) was chosen in order to observe if toxic effects on HCE were reversible or not.

Six series of 3D-HCE were used for each solution: two series for cell viability MTT testing, two series for immunohistological analyses on cryosections, and two series for immunofluorescent labeling on the most superficial layers of 3D-HCE by en-face confocal microscopic analyses.

Modified MTT test

The experiments were conducted in triplicate. The 3D-HCEs were transferred to 24-well plates containing 300µl of the MTT solution at 0.5g/ml in culture medium with the application onto the apical surface. After the 3h-incubation at 37°C, the 3D-HCEs were incubated with 750 µl isopropanol. After the agitation for 2h, the results were analyzed by the optical density (OD, absorbance) at 570 nm versus OD690 nm, and they were expressed as a

percentage of cell viability compared to the negative control, PBS. Analyses were performed using Safire technology (Tecan, Lyon, France).

Immunofluorescence analyses

After incubations with different solutions, the 3D-HCE samples were transferred into Petri dishes containing PBS to be separated into two pieces using a surgical scalpel. One piece of tissue was embedded in OCT® embedding medium (Tissue-Tek, Miles Inc., Elkhart, IN, USA), and frozen at –80°C for future 10µm vertical cryosections. The other piece was fixed in 4% paraformaldehyde (PFA) for 20 min before immunofluorescent labeling of the tight junction proteins occludin and ZO-1.

1/ Detection of apoptosis (TUNEL assay), inflammation (CD54) and proliferation (Ki67) on 3D-HCE cryosections

- **Apoptosis, TUNEL assay**: A terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) kit containing TUNEL enzyme and TUNEL label (Roche Diagnostics, Meylan, France) was used to detect apoptosis in the tissue layers. The nuclei were labeled with DAPI and the cryosections were mounted in an anti-fade medium (Vectashield; Vector Laboratories, Burlingame, CA, USA).

- **CD54 (ICAM-1) and Ki67 immunostainings**: The samples were firstly fixed with 4% PFA for 10 min and then permeabilized with 0.01%-diluted Triton X100® (Sigma Chemical Company, Saint Louis, MO, USA) for 5 min. The following primary antibodies were incubated: the mouse anti-human ICAM-1 (IgG1, BD Biosciences, Pharmingen, San Diego, CA, USA; 1:100), the mouse anti-human Ki67 (Immunotech, Marseilles, France; 1:25), and negative isotypic control mouse IgG1 (BD Biosciences). Alexa 488 conjugated-goat anti-mouse IgG at a 1:500 dilution (Invitrogen-Molecular Probes, Eugene, OR, USA) was used as second antibody. The nuclei were labeled with propidium iodide (PI) and cryosections were

mounted. Samples were analyzed under a laser confocal microscope equipped with digital camera (E800, PCM 2000, Nikon, Champigny-sur-Marne, France). We counted the immunopositive cells under x 20 objective of the microscope in three different areas, and the results were calculated as the average of counts, and finally presented as cells/field (x20 objective) after each treatment.

2/ Confocal immunofluorescence on entire epithelia for tight junction staining

The rabbit anti-human occludin (IgG1, Dako, Glostrup, Denmark; 1:100 dilution) and rabbit anti-human ZO-1 (IgG1, Santa Cruz Biotechnology, Santa Cruz, CA) were used for tight junction staining. Alexa 488-conjugated goat anti-mouse was used as second antibody, and finally nuclei were labeled with DAPI. Samples were then analyzed under a laser confocal microscope (E800, PCM 2000, Nikon) for detecting occludin and ZO-1 expressions.

Quantification and Statistical Analysis

TUNEL-, ICAM-1-, and Ki67-positive cells were quantified manually, using a microscopic grid on images under ×400 magnification. The results were expressed as mean cell number per field (cells/field). Standard deviations were indicated.

Control and treatment groups for MTT data and immunopositive cell counts were compared using two-way analysis of variance (ANOVA) followed by the Fisher's adjustment (Statview V; SAS Institute Inc., Cary, NC, USA).

Results

Cell viability: MTT test

PBS that was used as negative control did not affect the cell viability neither at 24h nor 24h+24h-recovery (Fig. 1). PF-tafluprost showed the same level of cell viability as PBS at

24h (90.2%) and at 24h+24h-recovery (100%) without any statistically significant differences compared to PBS. The preservative solutions of BAC at 0.010% and 0.020% induced a cell viability decrease at 24h (65.9% and 42.5% respectively, $P<0.01$ compared to PBS) and at 24h+24h-recovery (62.9% and 43.4% respectively, $P<0.01$ compared to PBS).

The preservative-containing antiglaucoma solutions induced a BAC-concentration-dependent decrease of the cell viability: at 24h, 85.5% for 0.005%BAC-bimatoprost, 75.5% for 0.015%BAC-travoprost ($P<0.01$ compared to PBS group), and 40.1% for 0.020%BAC-latanoprost ($P<0.05$ compared to PF-tafluprost, 0.005%BAC-bimatoprost and 0.015%BAC-travoprost groups, $P<0.01$ compared to PBS and $P<0.05$ compared to 0.010%BAC group), at 24h+24h-recovery, 83.0% for 0.005%BAC-bimatoprost, 65.5% for 0.015%BAC-travoprost ($P<0.01$ compared to PBS group, $P<0.02$ compared to PF-tafluprost group), and 53.2% for 0.020%BAC-latanoprost ($P<0.05$ compared to PF-tafluprost and 0.005%BAC-bimatoprost).

Immunofluorescence analyses and quantification of apoptosis (TUNEL)

Few apoptotic cells were observed after PBS (Fig. 2A) or PF-tafluprost (Fig. 2B) incubations (Fig. 2H): 17.0 cells/field for PBS and 13.3 cells/field for PF-tafluprost at 24h; 14.5 cells/field for PBS and 15.4 cells/field for PF-tafluprost at 24h+24h-recovery. In accordance with our previous study, BAC at 0.010% and 0.020% significantly increased the number of TUNEL-positive cells at 24 h, and at 24 h+24 h-recovery compared to PBS group ($P<0.02$ for 0.010%BAC, $P<0.01$ for 0.020%BAC).

The number of apoptotic cells increased after the BAC-containing antiglaucoma eye drops in a BAC-concentration dependent manner at 24h. In addition, the corneal epithelial cells could not recover after the recovery period and more cells tended to undergo apoptosis with an increase of TUNEL-positive cells at 24h+24h-recovery. Bimatoprost with 0.005% BAC (Fig. 3C) induced moderate expression of apoptosis (Fig. 2H, 23.4 cells/field at 24h and 24.8 cells/field at 24h+24h-recovery), and 0.015%BAC-travoprost (Fig. 2E) with 37.0 cells/field at

24h, and 42.6 cells/field at 24h+24h-recovery (P<0.03 compared to PF-tafluprost and 0.005%BAC-bimatoprost groups at 24h and 24h+24h-recovery). The highest levels of apoptotic cells were found homogenously disseminated after the application of 0.020%BAC-latanoprost (Fig. 2F) and of 0.020% BAC (Fig. 2G): 43.4 cells/field and 44.3 cells/field respectively at 24h; 68.3cells/field and 61.3 cells/field respectively at 24h+24h-recovery. These TUNEL-positive cells were observed especially in the apical cell layers, but also in the middle epithelial layers. Latanoprost with 0.020%BAC induced significantly higher amount of apoptotic cells than did PF-tafluprost, 0.005%BAC-bimatoprost at 24h (P<0.003 for both) and 24h+24h-recovery (P<0.003 for both), as well as 0.010%BAC and 0.015%BAC-travoprost at 24h+24h-recovery (P<0.003).

Immunofluorescence analyses and quantification of the inflammation marker: ICAM-1 (CD54)

PBS-treated 3D-HCE cultures (Fig. 3A, 3H) were found to express CD54 at levels of approximately 113.2cells/field at 24h, and 74.0 cells/field at 24h+24h-recovery. PF-tafluprost induced 121.5cells/field at 24h and 79.0 cells/field at 24h+24h-recovery without any statistically significant differences with PBS-treated samples. BAC 0.005%-bimatoprost induced an increase of CD54-positive cells: 155.5 cells/field at 24h and 134.0 cells/field at 24h+24h-recovery (P<0.001 compared to PBS, P<0.002 compared to 0.02%BAC-latanoprost, P<0.008 compared to 0.015%BAC-travoprost at 24h and 24h+24-recovery). This level of increased CD54 activation was also found with 0.01%BAC: 158.8 cells/field at 24h, and 128.5 cells/field at 24h+24h-recovery (P<0.001 compared to PBS, P<0.002 compared to 0.02%BAC-latanoprost, P<0.008 compared to 0.015%BAC-travoprost at 24h and 24+24-recovery). For 0.005%BAC-bimatoprost and 0.010%BAC, the CD54 positive cells were located throughout all the epithelial layers (Figs. 3C and 3D). However, with more higher concentrations of BAC, this number decreased, probably due to the erosion of corneal

epithelium with toxic solutions: 74.0 cells/field after 0.015%BAC-travoprost treatment, 73.3 cells/field after 0.020%BAC-latanoprost treatment, and only 64.3 cells/field after 0.020% BAC treatment at 24h (for the three groups: $P<0.001$ compared to PBS group, $P<0.005$ compared to 0.010% BAC).

Immunofluorescence analyses of cell proliferation: Ki67

After PBS treatment (Fig. 4A), some proliferating cells were observed, not restricted to the basal layer at 24h or 24h+24h-recovery. PF-tafluprost induced similar aspect as did PBS-treated samples. However, more numerous proliferating cells, with a greater number located in the basal layer were found after 0.005%BAC-bimatoprost (Fig. 4C), 0.01%BAC (Fig. 4D), 0.015%BAC-travoprost (Fig. 4E), 0.02%BAC-latanoprost (Fig. 4F) and 0.02% BAC (Fig. 4G). At 24h+24h-recovery, we could still find some proliferative cells after the treatments with PBS or PF-tafluprost. In this time, following treatment with the solutions containing BAC (0.010%BAC, 0.020%BAC, and the three BAC containing antiglaucoma solutions), no or very rare proliferative cells were observed, again most likely due to the deep impairment of corneal cells after toxic challenge.

En-face confocal microscopic analysis of tight junction: occludin and ZO-1

The 3D-HCEs disclosed a thin occludin immunostaining in the most superficial cells, forming a persistent green ring around, leaving a diffuse cytoplasmic staining, after treatments with PBS (Fig. 5A) or PF-tafluprost (Fig. 5B). We could still find a slight occludin expression in the apical cells with 0.005%BAC-bimatoprost (Fig. 5C). This kind of occludin expression clearly disappeared after the treatments with 0.010%BAC (Fig. 5D), 0.015%BAC-travoprost (Fig. 5E), 0.020%BAC-latanoprost (Fig. 5F) and 0.020% BAC (Fig. 5G) at 24h, leaving only dense green patches. After the 24h recovery period, no significant change in occludin protein distribution was found compared to treatment without the recovery period. The same

tendency was found using ZO-1: a thin green ring was found just after the treatment with PBS (Fig. 6H) or PF-tafluprost (Fig. 6I).

Discussion

The toxicological model of 3D-reconstructed cornea epithelial model confirmed the cytotoxicity of BAC-containing solutions with a better approach of what exists in vivo or in vitro. Using a selection of pertinent biomarkers on this 3D-HCE, we could detect and analyze the presence of cell apoptosis, activation/inflammation, proliferation/turnover and cellular tight junctions after application of different test solutions. This model, while respecting the ethical guidelines of animal experimentation, specially the 3R rule (refining, reducing and replacing the use of animals),[6] contributes to a better visualization of the human epithelial structure than the traditional monolayer cell cultures and represents a highly valuable tool between animal and cellular models for toxicity detection. The fluorescence techniques conjugated with confocal microscopy on 3D-reconstructed corneal epithelia were proven to be suitable for the investigation of toxicological markers and gave relevant results compared with the known human data.[5] As on monolayer cell cultures, the 24 hours recovery experiments on 3D-HCE model allow the apoptotic process to further develop and to induce the inhibition of proliferation. The expression of the proliferation marker, KI-67 nuclear antigen decreased after these 24 hours recovery while the apoptosis- and inflammation-related markers increased. These results confirmed that BAC, depending on the time and concentration used, acts as a pro-inflammatory and pro-apoptotic agent able to irreversibly impair the normal epithelium turn-over even after BAC withdrawal. Several in vitro studies have already pointed out these deleterious effects of BAC and BAC-containing antiglaucoma drugs. MTT assay is a simple quantitative method usually used to assess the cell viability in these 3D-HCE models.[7] It seems more reliable and robust than immunofluorescence and

confocal microscopy that are known to provide visual and convincing results for the analysis of cell damage; however, these techniques are deemed to give adjunct results for the purpose of comparison of tested solutions. But, even if based on a smaller number of cells and a quantitative reading that depends on the experimenter, we proved in this study, that these techniques also allow quantification and provide a good understanding of all toxic phenomena and their impact on various biological processes. Consistent with similar analyses in monolayer cell cultures and in rabbit model, in the present study using this new 3D model, we demonstrated that PF-tafluprost presented almost the same aspects as did the negative control with persistent epithelial cell tight junctions and appropriate proliferative level, with no induction of cellular apoptosis or inflammation when compared to the formulations containing toxic preservative BAC. BAC-induced ocular toxicity has been well-documented in cell culture, animal models and in patients.[8] The reconstituted 3D-HCE model, resembling the corneal epithelium of the human eye in morphology and thickness,[9] was already used as a new alternative to the classic Draize eye test for the assessment of the eye irritation potential of chemicals and cosmetic products.[4,10] It confirmed the cytotoxicity of BAC, and this model was sensitive enough to distinguish between different concentrations of BAC, from 0.001% to 0.5%.[5] The versatility and sensitivity of the 3D-HCE models were also investigated and this model was proposed to replace some uses of animal testing in pre-clinical studies. In addition, the occludin gene expression was proposed as an early in vitro sign for mild eye irritation assessment.[11]

Even though multiple studies consistently showed BAC toxicity and potential advantages of BAC-free compounds,[8] there still exist some debate on its cytotoxicity in ocular tissues and potential harmfulness for glaucoma patients. In contrast with our results and all literature data on BAC,[8,12-19] one recent study did not find any loss of viability when testing BAC alone or BAC-containing latanoprost after a short contact duration from 10 to 60 minutes by using the

same model of 3D-corneal epithelium as used in this study.[20] These conflicting results were principally due to the differences in experimental conditions, such as the choice of model or appropriateness of duration of contact. In patients, the age, the quality of tear film, the individual sensitivity of the ocular surface, a preexisting sensitivity or allergy, and the overall number of BAC-containing eye drops and duration of administration could all be considered to influence the impact of toxic preservative on the ocular tissues and may explain wide individual variations. In experimental conditions, the contact duration of BAC is essential to consider and may explain some apparently conflicting results: Khoh-Reiter and Jessen stated in their study that BAC is in contact with the eye structures only for few seconds after instillation. Another study proved that a rapid dilution in the tear film reduced the concentration of BAC to near zero in minutes.[21] However, Champeau et al. detected the presence of BAC in the conjunctival epithelium nine days after a single administration, which suggests prolonged impregnation of BAC in ocular tissue instead of simple dilution in tear film.[22] In rabbits, radiolabelled BAC was found in the palpebral and bulbar conjunctiva and all corneal layers, and single-drop administration resulted in high tissue levels in the anterior ocular tissues retaining for up to 120 hours.[23]

In our model of 3D-culture of corneal epithelium, a 24-hour incubation was therefore considered to better mimic the accumulation of BAC over the long term and allowed to better investigate the cell alterations in the deeper layers of the corneal epithelium. As BAC is usually considered a potent and useful enhancer of drug penetration into deep ocular structures, all experimental models should be considered in light of a significant contact time, depending not on tear concentrations of BAC but instead on much more prolonged contact times, as most likely occurs with a compound that impairs the tear film, disrupts cell membranes, increases corneal epithelium permeability, and is repeatedly used over extremely long periods of time.[8] Complementarily, the 24h recovery appeared justified as for the

monolayer cultures for evaluating late effects, either in the way of possible compensation and recovery or as an amplification of toxic reactions.

In conclusion, our results using the new 3D-HCE model for evaluating BAC-containing prostaglandin analogs and PF-tafluprost were in accordance with previously published works investigating BAC toxicity. The present study provides a valuable method to evaluate the impacts of eye drops in vitro, being more accurate than single monolayers and easier to perform than animal models. The development of 3D-HCEs thus enriches the existing models to study the bioactivity of eye drops in the cornea by investigating and quantifying a set of cellular functions like cellular viability, inflammation, proliferation, apoptosis or cell junctions.

Figures and legends

Fig. 1: Cell viability MTT test

Cell viability of PBS-, PF-tafluprost-, 0.005%BAC-bimatoprost-, 0.010%BAC-, 0.015%BAC-travoprost-, 0.020%BAC-latanoprost- and 0.020% BAC-treated 3D-HCE after 24h-incubation without or with a 24-h recovery period. Antiglaucoma treatments induced a BAC-dose dependent decrease in cell viability as assessed by the MTT test. PF-tafluprost presented the higher level of viability than all BAC-containing eye drops.

∗ P <0.01 compared to PBS at the same time point;
# P <0.03 compared to 0.010% BAC at the same time point;
$ P <0.002 or P <0.03 ($$) compared to 0.020% BAC at the same time point;
♦ P <0.05 compared to 0.020%BAC-latanoprost at the same time point;
● P <0.02 compared to 0.015%BAC-travoprost at the same time point.

Fig. 2: Apoptosis analysis

Localization of TUNEL positive cells (green) on 3D-HCE samples after PBS (A), PF-tafluprost (B), 0.005%BAC-bimatoprost (C), 0.010%BAC (D), 0.015%BAC-travoprost (E), 0.020%BAC-latanoprost (F) and 0.020% BAC (G) incubation after 24h without (left column) or with the 24h-recovery period (right column). Quantification of TUNEL-positive cells (H) was performed after 24 h of treatment with all solutions followed or not by a 24h-recovery period.

At 24h or 24h+24h-recovery, no or very rare apoptotic cells were observed after PBS (A) or PF-tafluprost (B) treatments. Bimatoprost with 0.005% BAC (C) induced moderate expression of apoptosis, and 0.010%BAC (D), 0.015%BAC-travoprost (E), 0.020%BAC-latanoprost (F) 0.020% BAC (G) all induced a great number of TUNEL-positive cells principally in the apical cell layers, and also in the middle epithelial layers. The nuclei were stained with DAPI (blue).

The quantification of TUNEL-positive cells shows the increase of apoptotic cell number in a BAC dose-dependent manner. BAC at 0.010% and 0.020% significantly increased the number of TUNEL-positive cells at 24h, and at 24h+24h-recovery compared to control. The antiglaucoma eye drops travoprost and latanoprost, which contain higher concentrations of BAC, showed much higher expression of apoptotic cells than did PF-tafluprost or control.

* P<0.02 for 0.010% BAC and P <0.01 for other solutions compared to PBS at the same time point;
# P <0.005 compared to 0.010% BAC at the same time point;
$ P <0.001 compared to 0.020% BAC at the same time point;
♦ P <0.003 compared to 0.020% BAC-latanoprost at the same time point;
● P <0.03 compared to 0.015% BAC-travoprost at the same time point

Fig. 3: Inflammation analysis

Immunolocalization of ICAM-1 (CD54) positive cells (green) on 3D-HCE samples after PBS (A), PF-tafluprost (B), 0.005%BAC-bimatoprost (C), 0.010%BAC (D), 0.015%BAC-travoprost (E), 0.02%BAC-latanoprost (F) or 0.020% BAC (G) treatments after 24 h of treatment without or with the 24-h post-incubation period. Quantification of ICAM-1 (H) was performed after 24 h of treatment with all solutions followed or not by a 24-h post-incubation period.

ICAM-1-expressing cells were already found in the PBS (A) and PF-tafluprost (B) groups with the same level of expression after 24h or 24h+24h. The increase of ICAM-1 expressions was observed after the treatments with 0.005%BAC-bimatoprost (C) and 0.010%BAC (D) with and without the 24-h post-incubation period. However, few remaining ICAM-1 positive cells were observed after treatments with 0.015%BAC-travoprost (E), 0.020%BAC-latanoprost (F) and 0.020% BAC (G) on the 3D-HCE samples due to the erosion. The nuclei

were stained with propidium iodide (PI, red). The quantification of TUNEL-positive cells confirmed the observations.

* P <0.001 compared to PBS at the same time point;
# P<0.005 compared to 0.010% BAC at the same time point;
$ P <0.0001 compared to 0.020% BAC at the same time point;
♦ P <0.002 compared to 0.020%BAC-latanoprost at the same time point;
● P <0.008 compared to 0.015%BAC-travoprost at the same time point.

Fig. 4: Proliferation analysis

Immunolocalization of Ki-67 positive cells (green) on 3D-HCE samples after PBS (A), PF-tafluprost (B), 0.005%BAC-bimatoprost (C), 0.01%BAC (D), 0.015%BAC-travoprost (E), 0.020%BAC-latanoprost (F) or 0.020% BAC (G) treatments after 24h of treatment without (left column) or with the 24h recovery period (right column).

Ki67 immunostaining showed an epithelium with proliferating cells not restricted to the basal layer after PBS treatment (A) at 24h. The same aspect was found after the treatment of PF-tafluprost (B). Numerous proliferating cells, with a great number located in all the layers were found after the treatments of 0.005%BAC-bimatoprost (C), 0.010%BAC (D), 0.015%BAC-travoprost (E), 0.020%BAC-latanoprost (F) and 0.020% BAC (G) at 24h. At 24h+24h-recovery, we observed the persistence of some proliferative cells after the treatments of PBS or PF-tafluprost. However, at this time, there were no more proliferative cells after the treatments with all BAC-containing solutions.

Fig. 5: Tight junction: occludin and ZO-1

Immunofluorescence analysis of occludin (green: A to G) and ZO-1 (green: H to N) expressions using en-face confocal microscopy after treatment with PBS (A), PF-tafluprost (B), 0.005%BAC-bimatoprost (C), 0.010%BAC (D), 0.015%BAC-travoprost (E),

0.020%BAC-latanoprost (F) and 0.020% BAC (G) treatments after 24 h (left column) or 24h+24h-recovery (right column). The nuclei were stained with DAPI (blue).

After the treatment with PBS or PF-tafluprost, the 3D-HCE disclosed a thin occludin/ZO-1 immunostaining in the most superficial cells of samples by forming a green ring around, leaving a diffuse cytoplasmic staining. We could still find a slight tight junction expression in the apical cells with 0.005%BAC-bimatoprost (C). The expressions disappeared completely after the treatments with 0.010%BAC (D), 0.015%BAC-travoprost (E), 0.020%BAC-latanoprost (F) and 0.020% BAC (G) at 24h or 24h+24h-recovery, leaving dense green patches. Note that the nuclei became smaller after the treatments of high dose BAC-containing solutions.

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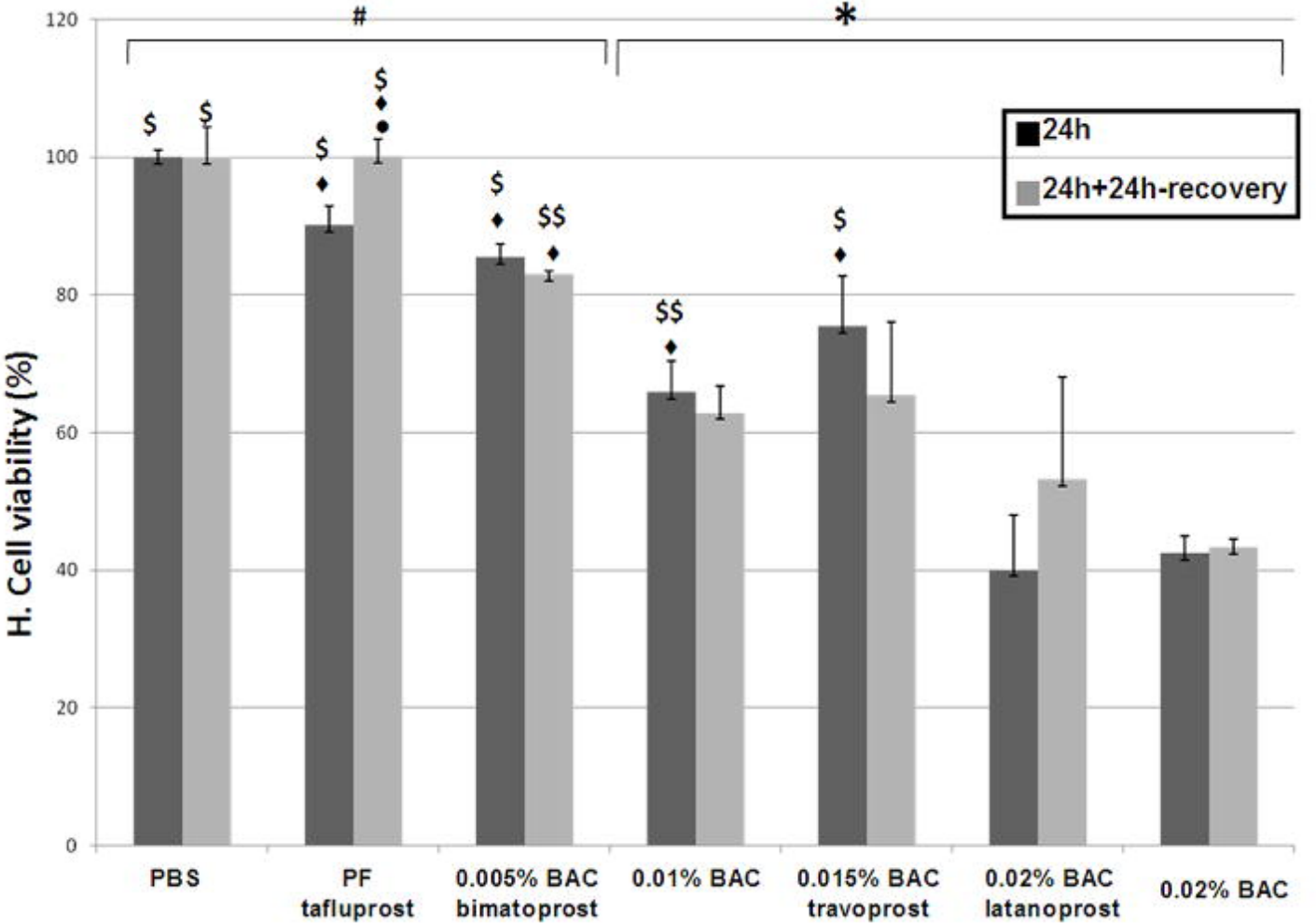
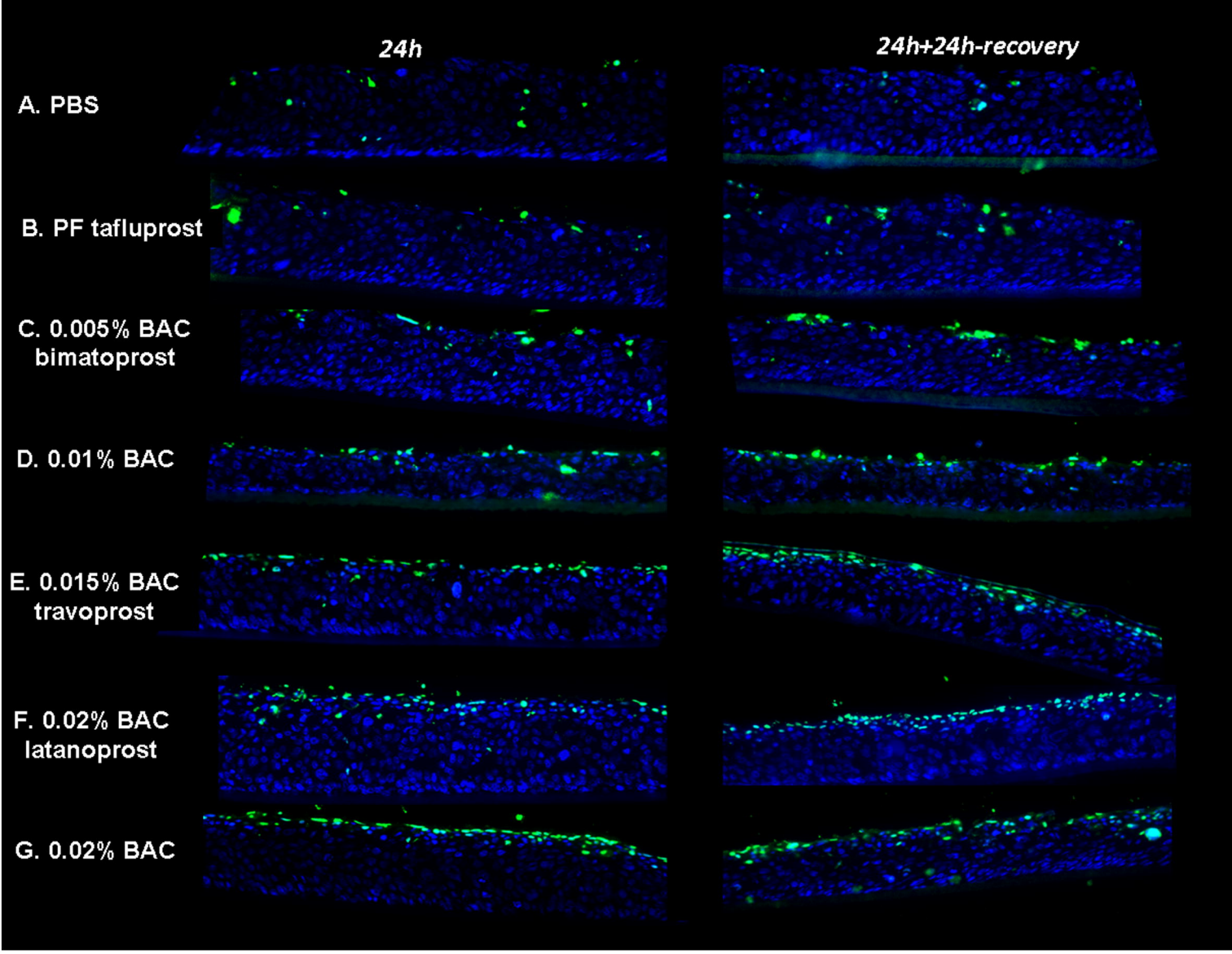
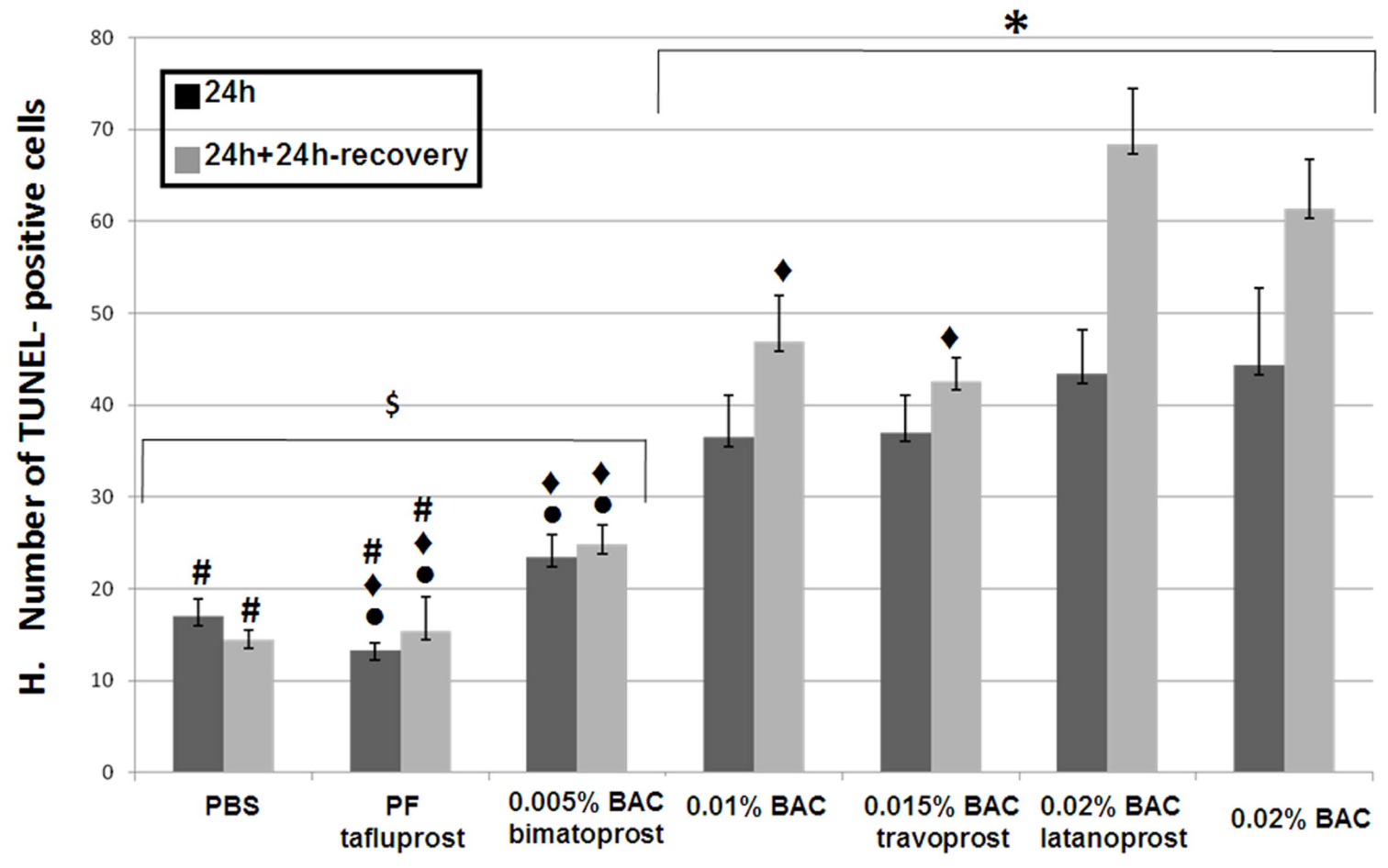
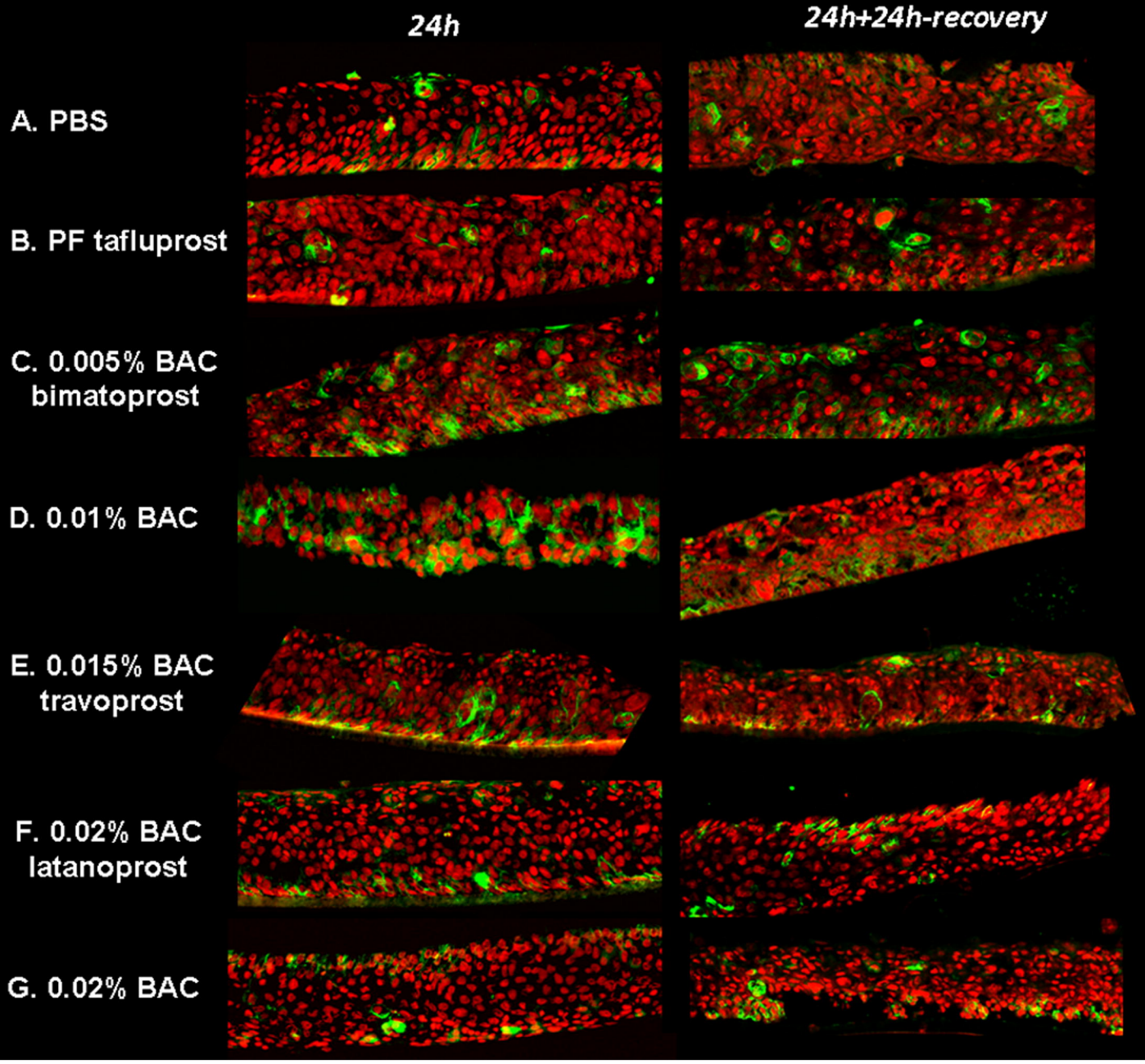
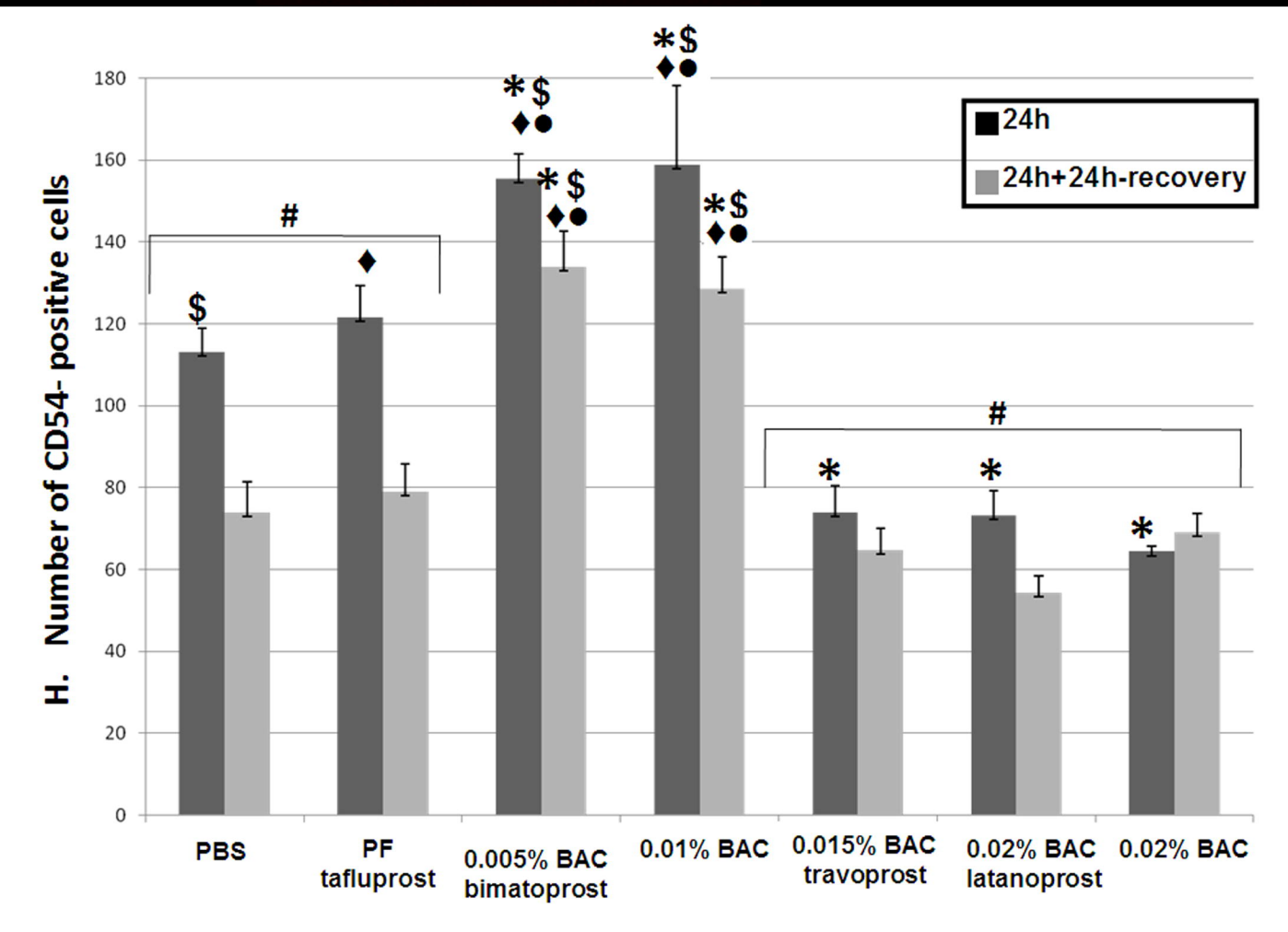
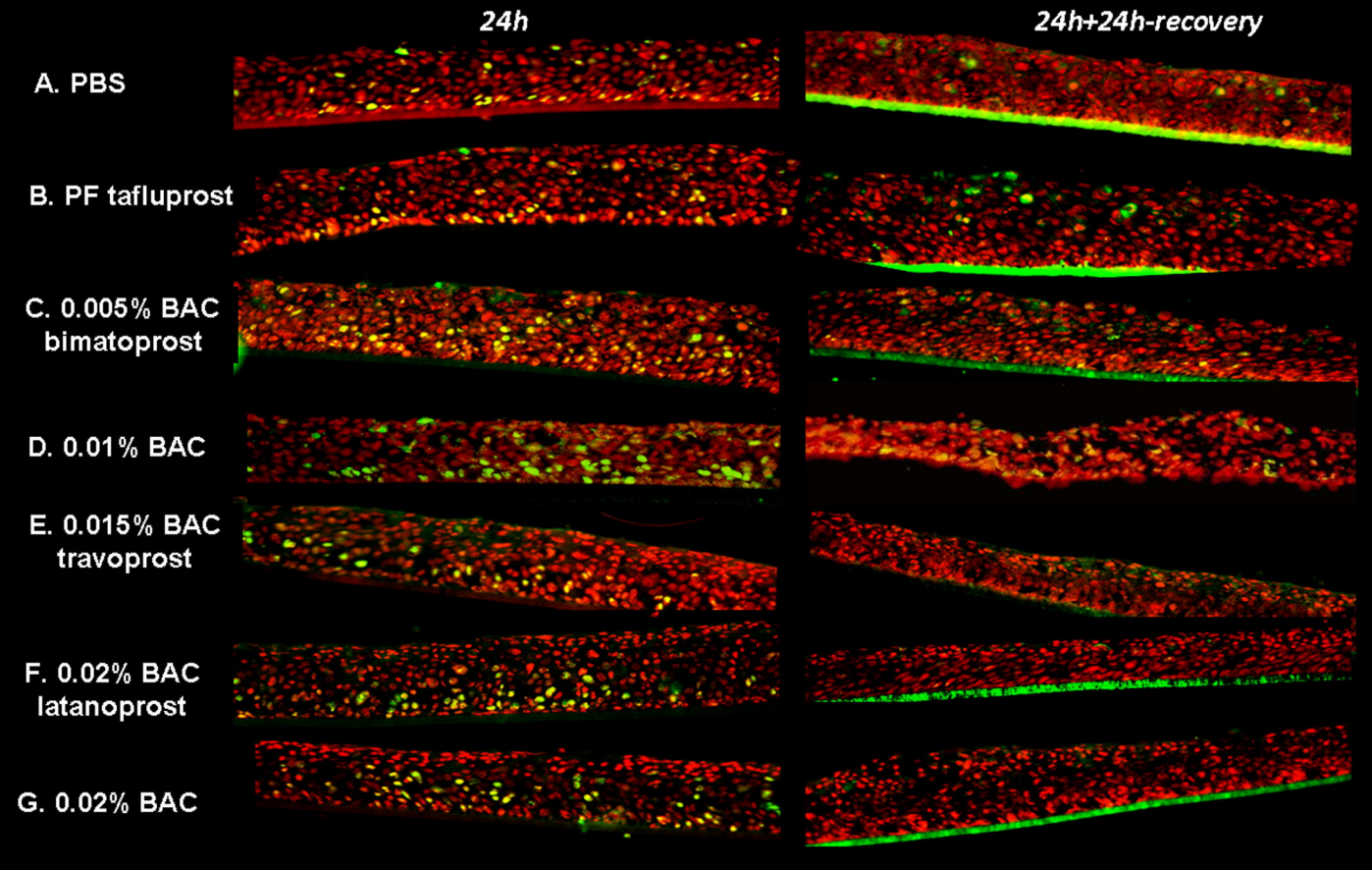

#
*
24h
24h+24h-recovery
H. Cell viability (%)
120
100
80
60
40
20
0
PBS
PF tafluprost
0.005% BAC bimatoprost
0.01% BAC
0.015% BAC travoprost
0.02% BAC latanoprost
0.02% BAC

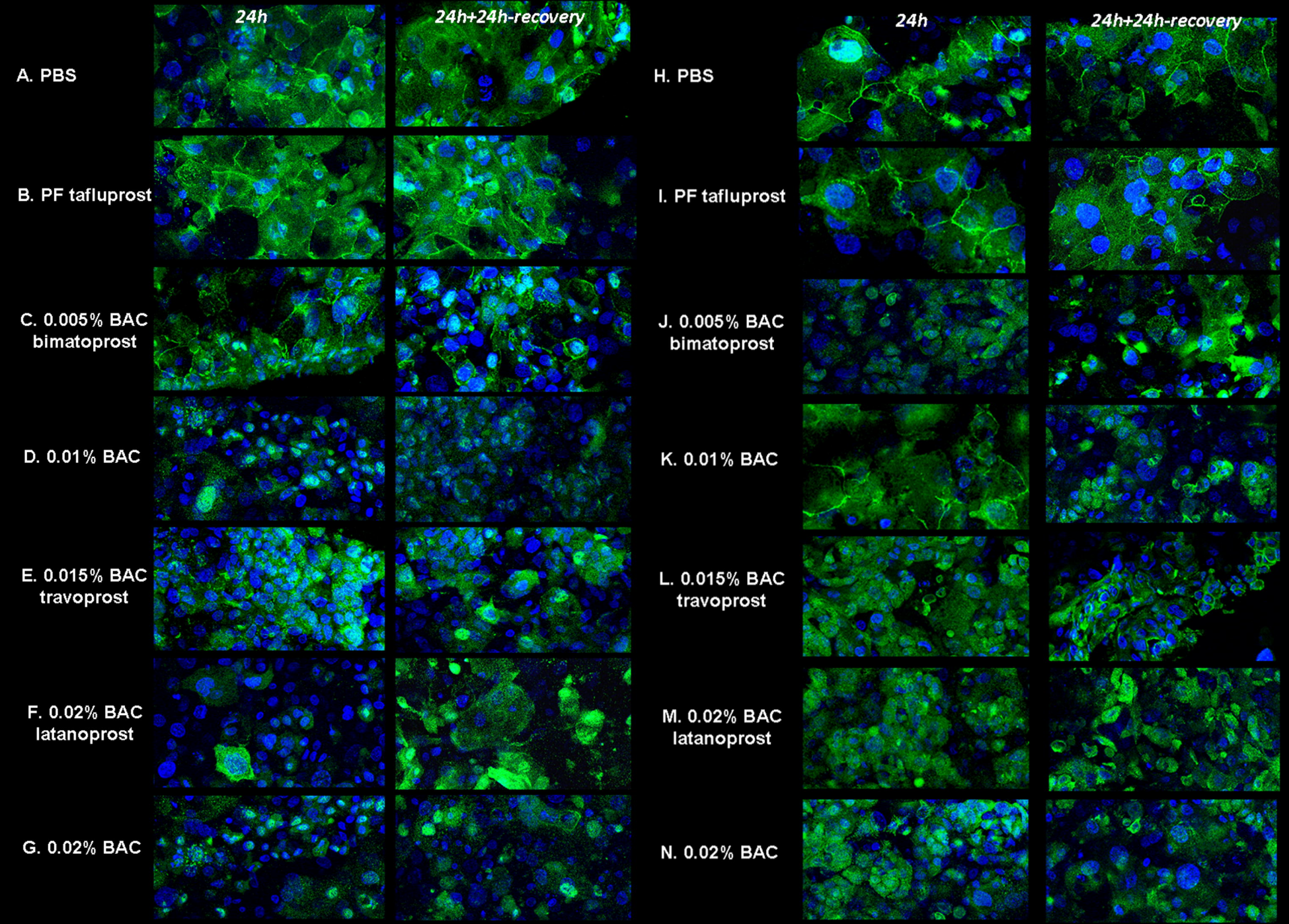

24h
24h+24h-recovery
A. PBS
B. PF tafluprost
C. 0.005% BAC bimatoprost
D. 0.01% BAC
E. 0.015% BAC travoprost
F. 0.02% BAC latanoprost
G. 0.02% BAC


H. Number of TUNEL- positive cells
24h
24h+24h-recovery
*
$
#
♦
●
0
10
20
30
40
50
60
70
80
PBS
PF tafluprost
0.005% BAC bimatoprost
0.01% BAC
0.015% BAC travoprost
0.02% BAC latanoprost
0.02% BAC


24h
24h+24h-recovery
A. PBS
B. PF tafluprost
C. 0.005% BAC bimatoprost
D. 0.01% BAC
E. 0.015% BAC travoprost
F. 0.02% BAC latanoprost
G. 0.02% BAC


H. Number of CD54- positive cells
180
160
140
120
100
80
60
40
20
0
24h
24h+24h-recovery
PBS
PF tafluprost
0.005% BAC bimatoprost
0.01% BAC
0.015% BAC travoprost
0.02% BAC latanoprost
0.02% BAC


24h
24h+24h-recovery
A. PBS
B. PF tafluprost
C. 0.005% BAC bimatoprost
D. 0.01% BAC
E. 0.015% BAC travoprost
F. 0.02% BAC latanoprost
G. 0.02% BAC


24h
24h+24h-recovery
A. PBS
B. PF tafluprost
C. 0.005% BAC bimatoprost
D. 0.01% BAC
E. 0.015% BAC travoprost
F. 0.02% BAC latanoprost
G. 0.02% BAC
24h
24h+24h-recovery
H. PBS
I. PF tafluprost
J. 0.005% BAC bimatoprost
K. 0.01% BAC
L. 0.015% BAC travoprost
M. 0.02% BAC latanoprost
N. 0.02% BAC