Cytostatic and Cytotoxic Effects of 5-Fluorouracil on Human Corneal Epithelial Cells and Keratocytes

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Purpose: To investigate the effects of various 5-fluorouracil (5-FU) concentrations, exposure times, and application techniques on in vitro–cultured human corneal cells.

Methods: Human corneal epithelial cell (HCEC) and human corneal keratocyte (HCK) cultures were exposed to different 5-FU concentrations (0.025%–1%) and incubation durations (5 minutes to 2 hours). The cytostatic effect was evaluated as the percentage of inhibition of migration relative to the control. The evaluation of cytotoxic effect included both phase contrast microscopic observations and viability measures performed using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] colorimetric assay. The results are expressed as ratio of optical density (OD) reduction 24 hours after exposure.

Results: The cytostatic effect was time and dose dependent. The 50% inhibiting dose was 0.55% after 1 hour of incubation for HCECs and was 0.5% after 2 hours of incubation for HCKs. A 100% inhibitory effect was never observed at any concentration or incubation duration. No cytotoxic changes were observed using an 5-FU concentration of <1%; 1% 5-FU showed time-dependent cytotoxic changes in HCEC cultures only. MTT analysis showed no OD reduction at 5-FU concentrations of <1%, whereas 1% 5-FU showed OD reduction <50% at any tested exposure time. HCECs showed higher reduction in OD than HCKs.

Conclusions: 5-FU formulations topically used in clinical practice showed limited toxicity in normal cultured corneal epithelial cells and keratocytes.

Key Words: corneal epithelial cells, keratocytes, cell cultures, 5-fluorouracil

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The cell-cycle phase–specific antimetabolite 5-fluorouracil (5-FU) is one of the different antimetabolite drugs that have been used in the treatment of ocular surface neoplasia and to modulate ocular wound healing after glaucoma, pterygium, or refractive surgery.[1]

5-FU is a pyrimidine analog that interferes with nucleic acid synthesis, altering cellular DNA and RNA.[2] Some studies have demonstrated that 5-FU inhibits subconjunctival fibroblast proliferation, justifying its use in topical scleral applications or subconjunctival injections in filtering glaucoma surgery to modulate the postsurgical healing process.[3] Topical 5-FU eye drops were also successfully used in the treatment of ocular surface neoplasia because of its efficacy in the treatment of epithelial tumours.[4–9]

Limited toxicity on normal corneal structures was reported.[10] Side effects seem more relevant toward the corneal epithelium, which represents an innocent bystander.[11,12] Local side effects include punctate keratopathy, filamentary keratopathy, epithelial defects, and whorl-like keratopathy, reported after subconjunctival injections in filtering glaucoma surgery. These side effects were time limited, and reversible after stopping subconjunctival injections. Moreover, they were more common in patients with underlying ocular surface disorders.[2] A controlled slow release of 5-FU has been an attractive topic for the development of new ocular delivery techniques, to further reduce the possible adverse effects of 5-FU treatments in ophthalmology. However, in vivo studies on animal models are still ongoing, and further evaluations are needed to assess the biocompatibility, drug release kinetics, and adverse effects of these new systems.[13–15] The aim of this study was to investigate the effects of different concentrations and exposure times of 5-FU on cultured human corneal cells to better understand the local toxicity of topical 5-FU administration, currently used in clinical ophthalmologic practice. For this purpose, both cultured human corneal epithelial cells (HCECs) and human corneal keratocytes (HCKs) were employed.

MATERIALS AND METHODS

Cellular Cultures

Human donor corneas employed in this study (kindly supplied by Veneto Eye Bank Foundation, Mestre, Italy) were previously processed to be used in corneal transplantation. The remaining corneal rings were used to obtain both epithelial cell and keratocyte cultures.

Culture of Corneal Epithelial Cells

Corneal rings were washed under sterile conditions with balanced salt solution (BSS; BSS Plus; Alcon, Fort Worth, TX), and then mechanical removal of the epithelium was performed.

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Attention was paid to avoid any contamination from the underlying stroma or conjunctival cells. Epithelial cells were collected from the corneal limbus, where corneal epithelial stem cells are located.[16–19] Epithelium was seeded in 4-well plates (Nunc, Denmark) filled with Ham nutrient mixture F12 medium (Sigma Chemicals, St Louis, MO), supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 μL/mL penicillin, 100 μg/mL streptomycin (L-glutamine, penicillin, streptomycin; Sigma Chemicals), 2 mg/mL glucose, and incubated in a humidified incubator at 37°C, 5% CO_2, and 95% air. Subsequently, the cultures were fed twice weekly with fresh culture medium and monitored regularly by phase contrast microscopy until confluence was reached. After reaching confluence, the cells were washed with 0.5 mL of BSS and then enzymatically removed by incubation in the humidified incubator at 37°C, 5% CO_2, and 95% air with 0.2 mL of 0.25% trypsin per well. The reaction was stopped after 20 minutes by the addition of 0.4 mL of cold medium in each well. Then, the epithelial cell suspension was centrifuged at 100 g for 10 minutes, and the cells were resuspended in fresh culture medium after the removal of supernatant, seeded in 6-well plates of 3.5-mm diameter (Corning, New York), and incubated until confluence in the humidified incubator at 37°C, 5% CO_2, and 95% air with twice weekly feeding. The purity of the cell cultures was assessed on the basis of both the typical morphology of human corneal epithelial cells and their reactivity with antibodies to cytokeratins by immunofluorescence analysis. Epithelial cells from the third passage were used for the experiments.

Corneal Keratocyte Culture

Human corneas were washed under sterile conditions, and mechanical removal of both epithelium and endothelium was performed. The remaining stroma was cut into small pieces (1–2 mm large), and an average of 3 pieces per well were placed in 6-well plates of 3.5-mm diameter and left to adherence for 15 minutes in dry air. Then, 2 mL of fresh medium was added in each well and incubated in the humidified incubator at 37°C, 5% CO_2, and 95% air. Once a cellular monolayer was obtained, stromal fragments were removed and the culture was left to grow to confluence with twice weekly feeding and regular monitoring by phase contrast microscopy. Keratocytes at confluence were washed with 0.5 mL BSS and then enzymatically removed by incubation in the humidified incubator at 37°C, 5% CO_2, and 95% air with 0.2 mL of 0.25% trypsin per well. The reaction was stopped after 20 minutes by the addition of 0.4 mL of cold medium in each well. The purity of the cell cultures was assessed on the basis of both the typical morphology of human corneal keratocytes and their reactivity with antibodies to vimentin by immunofluorescence analysis. Keratocytes from the third to sixth passages were used for the experiments. Each assay was repeated 4 times.

Phase Contrast Microscopy

Regular monitoring (every 2 days) of the wells under phase contrast microscopy (Mod CK 2; Olympus, Japan) was performed. Wells showing signs of cellular distress and no confluence (cellular swelling, loss of intercellular contacts, necrosis) were excluded from further analysis.

Drug Preparation and Incubation Protocol

5-FU (Roche, Nutley, NJ) for intravenous administration (50 mg/mL) was diluted with fresh medium to obtain the 5 following concentrations: 0.025%, 0.05%, 0.1%, 0.5%, and 1%. Dilutions were performed under sterile conditions in a laminar flow hood, avoiding any contact with skin. Cell cultures were exposed to these different drug concentrations for the following incubation durations: 5 minutes, 10 minutes, 30 minutes, 1 hour, and 2 hours.

Cytostatic Effect Evaluation

To evaluate the cytostatic effects of 5-FU, we considered the migratory capacity of cells. In confluent cell cultures, a well-defined wound was made with a 35-mm blade. The blade was handheld with the aid of a dermatome and a downward pressure was applied, ensuring the formation of an evenly marked edge and a sharp linear mark on the plastic. A cotton swab was used to scrape off the cells from one side of the blade. A pipette was used to aspirate the medium and the debris, and then the wounded monolayer was washed twice with BSS and examined under phase contrast microscopy to verify that each culture well contained a cell-rich area, a marked edge designating the wound line, and a cell-free area. Then, scheduled incubations were performed, with one control well incubated with medium only in each plate. At the end of each incubation period, the supernatant was immediately and completely drained and the cellular monolayer was washed twice with BSS. Two milliliters of fresh medium was added in each well, and the plates were left in the incubator for 48 hours. Cultures were washed with BSS, fixed, and stained with 0.075% toluidine blue in ethanol, pH 3.5, for 5 minutes at room temperature. Cultures were then washed with absolute ethanol[20] and left to dry air at room temperature. Stained cells were examined under a phase contrast microscope (Olympus) equipped with an ocular grid to quantify cell migration. At a ×400 magnification, the total number of cells 250 μm beyond the wound was calculated in at least 3 different fields. The results are expressed as the percentage of migration inhibition relative to the control: % inhibition = 100 – (A/B × 100), where A is the number of migrated cells after 5-FU exposure and B is the number of migrated cells after medium incubation.

Cytotoxic Effect Evaluation

We choose cell viability as a parameter to evaluate the cytotoxic effects of 5-FU. Qualitative evaluation was performed on confluent cultures in 24-well plates (Corning) that underwent the scheduled drug incubations. Cultures were then observed every 2 days for 30 days using a phase contrast microscope with twice a week feeding with fresh medium. Any morphological modifications such as vacuoles, swelling, loss of intercellular contacts, and apoptosis were documented.

A quantitative assay was performed on confluent cultures in 96-well plates (Nunc), exposed using the same drug incubation protocol. The rapid colorimetric Mossmann test—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test[21]—was performed at 24 hours after incubation, with the following protocol: culture medium drying,

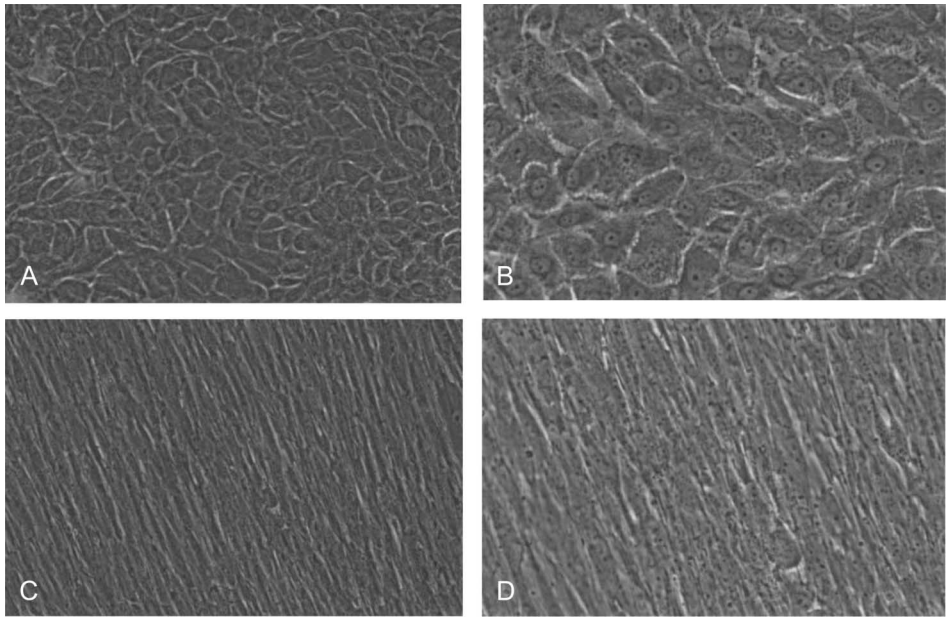

FIGURE 1. Phase contrast micrographs showing primary corneal cultures: confluent epithelial cell monolayers, (A) ×20 and (B) ×40, and confluent corneal keratocyte monolayers, (C) ×20 and (D) ×40. Morphological difference between the 2 corneal cell populations is evident: epithelial cells show their characteristic mosaic pattern, whereas keratocytes show the typical parallel elongated aspect.

addition of 100 μM MTT solution 5 mg/mL; (Sigma Chemicals) in RPMI 1640-SR-medium (Sigma Chemicals) in each well, 4 hours of incubation at 37°C, drying, addition of 100 μL of extraction solution (0.04 N HCl) in isopropyl alcohol, 5-minute incubation at room temperature, vigorous shacking, and spectrophotometric lecture (ELISA reader) at 590 nm with 630 nm as reference. Values are reported as the percentage of reduction of optical density (OD) relative to the control: % OD reduction = 100 × (OD control – OD test)/OD control, where OD control is the optical density of the control well and OD test is the optical density of the 5-FU-exposed well. OD variations result from the mitochondrial dehydrogenase action in only viable cells that transform tetrazolium, allowing a color change when isopropyl alcohol is added.

Statistical Analyses

Statistical analyses (analysis of variance with Bonferroni test) were performed using SAS version 8.2 statistical package (SAS Institute, Cary, NC). A P value of <0.05 was considered statistically significant.

RESULTS

Phase Contrast Microscopy

Both HCECs and HCKs produced confluent cellular monolayers (Fig. 1). HCECs reached confluence after 21 ± 3 days and HCKs after 14 ± 2 days. Some wells showed signs of cellular distress, whereas others contained mixed epithelial

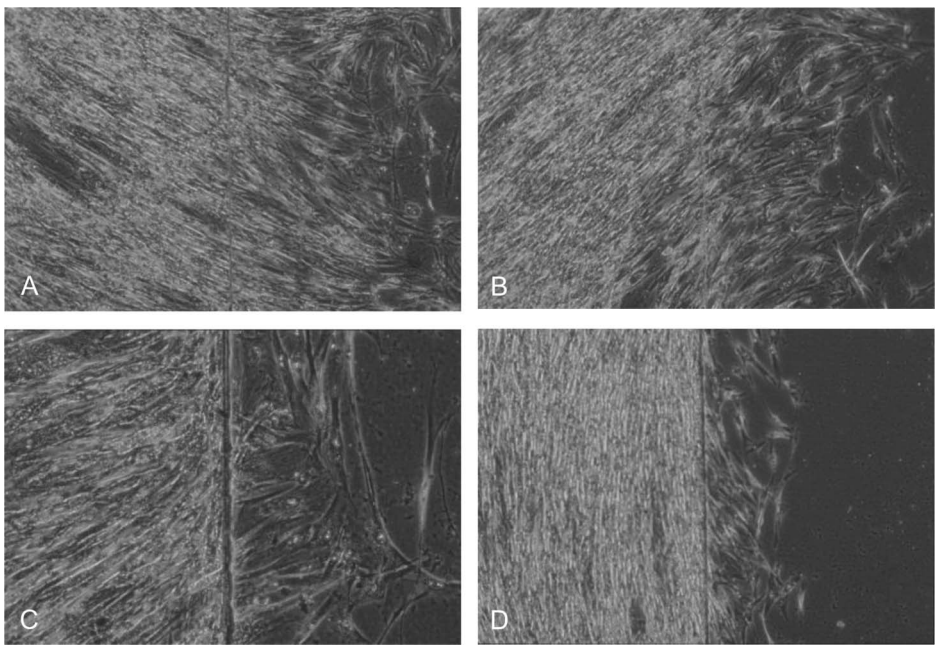

FIGURE 2. Phase contrast micrographs showing cytostatic effects on keratocyte cultures at different concentrations of 5-FU: (A) control well; (B) 0.5% 5-FU, 1-hour incubation; (C) 1% 5-FU, 1-hour incubation (C); and 1% 5-FU, 2-hour incubation.

and keratocyte cellular populations. Both were excluded from further analysis.

Cytostatic Effect

The cytostatic effect was evaluated by quantifying cellular inhibition of migration/proliferation on corneal HCECs and HCKs (Fig. 2). At incubation durations ranging from 5 to 30 minutes, any tested concentration significantly inhibited epithelial cell migration (Table 1). After 1 hour of incubation, only 1% 5-FU was a significant HCEC migration inhibitor, whereas after 2 hours of incubation, a similar effect was obtained with ≥0.5% 5-FU (Fig. 3). The 50% inhibiting dose (ID_{50}) of exposed epithelial cells after 1 hour of incubation was 0.55%, and after 2 hours, it was 0.35%. A 100% inhibitory effect was never observed at any concentration or time duration.

A significant migratory inhibition was reached in keratocytes after 2 hours of incubation with 0.5% 5-FU (Table 2). Durations of <2 hours did not result in any significant level of inhibition. A 100% inhibition was never observed for any time duration or concentration (Fig. 4). ID_{50} was 0.5% after 2 hours of incubation.

Cytotoxic Effect

The cytotoxic effect was evaluated both qualitatively and quantitatively. Qualitative evaluation was performed on HCECs by phase contrast microscopy examination up to 30 days after drug exposure. After exposure with 5-FU

TABLE 1. Cytostatic Effect of 5-FU on HCECs and Statistical Analysis

5-FU Concentration (%)	5 min	10 min
0.025	0	0
0.05	0	8.6 ± 2.7
0.10	0	15.2 ± 5
0.50	0	31.9 ± 7.2
1	0	32 ± 6.8
Analysis of variance		$F = 23.25$, $P < 0.0001$

Bonferroni analysis

5-FU (%)	0.025					0.05				
	5 min	10 min	30 min	1 h	2 h	5 min	10 min	30 min	1 h	2 h
0.05		ns	ns	ns	ns					
0.10		*	*	ns	ns		ns	ns	ns	ns
0.50		*	*	*	*		*	*	*	*
1.00		*	*	*	*		*	*	*	*

5-FU Concentration (%)	30 min	1 h	2 h
0.025	0	4.1 ± 3	7.5 ± 3.5
0.05	13.9 ± 3	17.9 ± 8.2	20 ± 7
0.10	18.2 ± 6.2	26.7 ± 9.8	30 ± 10.2
0.50	32 ± 6.9	47.1 ± 8.3	57.6 ± 11.1
1	40.8 ± 9.7	70 ± 10.5	85 ± 12.2
Analysis of variance	$F = 20.07$, $P < 0.0001$	$F = 28.50$, $P < 0.0001$	$F = 33.36$, $P < 0.0001$

Bonferroni analysis

5-FU (%)	0.10					0.50				
	5 min	10 min	30 min	1 h	2 h	5 min	10 min	30 min	1 h	2 h
0.05										
0.10										
0.50		*	ns	ns	*					
1.00		*	*	*	*		ns	ns	ns	*

5-FU migration inhibition ratio (mean ± SD) at different concentrations and exposure times. The cytostatic effect is time and dose dependent. Each assay was repeated 4 times. Bonferroni test, level of significance: $P < 0.05$; $^*P < 0.05$
ns, not significant.

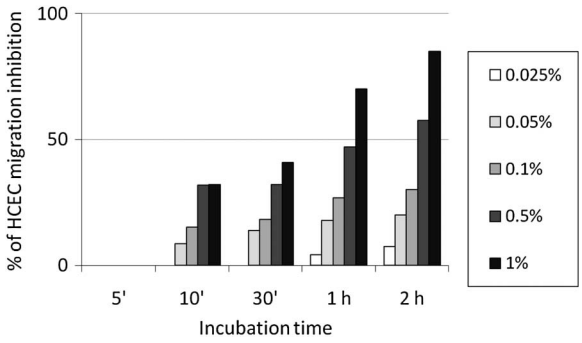

FIGURE 3. Effect of 5-FU on HCEC migration inhibition and ID_{50} at different drug concentrations and exposure times with SD error bars.

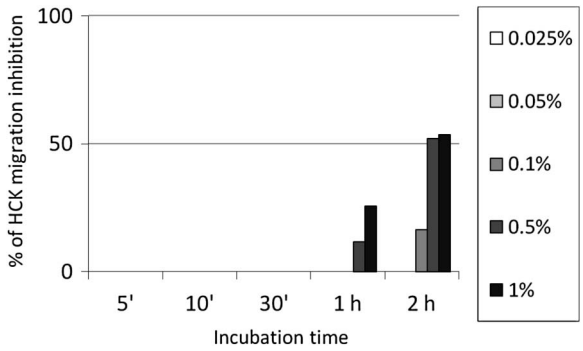

FIGURE 4. Effect of 5-FU on HCK migration inhibition and ID_{50} at different drug concentrations and exposure times with SD error bars.

concentrations of <1%, no morphological change was observed. At 5-FU concentrations of ≥1%, cellular morphological changes directly related to duration of exposure were observed. Vacuolization started at 21 ± 2 days in cells exposed for 30 minutes, at 14 ± 2 days after 1-hour exposure, and at 10 ± 1 days after 2-hour exposure. Rounding of cells and loss of intercellular contacts appeared at 24 ± 2 days after 1-hour exposure and at 22 ± 2 days after 2-hour exposure (Fig. 5).

5-FU concentrations of <1% showed no cytotoxic effect on HCKs. No morphological change was observed even at 1% 5-FU, except for vacuolization starting at 25 ± 2 days in 1-hour exposed cells and at 22 ± 3 days in 2-hour exposed cells (Figs. 6, 7).

Quantitative evaluation performed on HCECs using colorimetric assay analysis 24 hours after 5-FU exposure showed no reduction in OD when using 5-FU concentrations of <1%. One percent 5-FU showed 2 ± 1.1 reduction ratio after 5-minute exposure, 7.12 ± 4.3 after 10-minute exposure, 24.4 ± 6.5 after 30-minute exposure, 28.6 ± 7.1 after 1-hour exposure, and 35.1 ± 2.7 after 2-hour exposure (Fig. 8). Similar results were observed for HCKs. No reduction in OD after incubation with 5-FU concentrations of <1% was observed; 1% showed no reduction after 5-minute exposure, and the reduction ratios were 2.24 ± 2.1 after 10-minute exposure, 21.8 ± 6.9 after 30-minute exposure, 26.7 ± 8.0 after 1-hour exposure, and 27.9 ± 4.5 after 2-hour exposure (Fig. 8). Differences in the OD ratio between HCECs and HCKs were statistically significant only after 2 hours of incubation (t = 2.726; P = 0.034).

DISCUSSION

The therapeutic use of 5-FU is a known standard procedure in high-risk glaucoma surgery to reduce postoperative excessive fibrotic reaction.[2,3] Furthermore, 5-FU has been used as an adjuvant or in primary treatment in selected cases of conjunctival epithelial tumors.[4–9] Several ocular drug delivery systems, like poly(lactic acid), poly(lactide-co-glycolide), and chitosan nanoparticles, are under investigation in animal models to enhance absorption, improve bioavailability, reduce side effects, and sustain intraocular delivery of 5-FU.[22] Slow-release 5-FU delivery systems are currently unavailable in clinical practice. One of the major restrictions in the use of 5-FU for topical administration is the relatively limited knowledge of the effects of variable dosages and exposure times on ocular structures. Previous studies on this topic were conducted on animal models and concerned only epithelial or endothelial corneal cells.[23,24] Some authors reported significant side effects on corneal epithelium after 5-FU exposure. These data suggest caution in treating eyes with corneal defects.[11,12] Ando et al[25] observed significant reductions in reepithelization of damaged corneas, but no reduction in the mitotic index of healthy corneas after 5% 5-FU exposure and no effect after 1% 5-FU exposure. Wong et al[26] reported a significant difference in 5-FU inhibition of Tenon capsule fibroblast proliferation in mouse and human cells. An ID_{50} against rabbit fibroblasts was still detectable after 24 hours of incubation with 5-FU by the ^{3}H-thymidine uptake assay, whereas the ID_{50} against human fibroblasts was still detectable after 48 hours of incubation. Khaw et al[27] reported a 50%

TABLE 2. Cytostatic Effect of 5-FU on HCKs

5-FU Concentration (%)	5 min	10 min	30 min	1 h	2 h
0.025	0	0	0	0	0
0.05	0	0	0	0	0
0.10	0	0	0	0	16.4 ± 6.3
0.50	0	0	0	11.6 ± 4.2	52 ± 12.5
1	0	0	0	25.6 ± 9.3	53.5 ± 11.2
Analysis of variance				F = 18.47, P < 0.0001	F = 33.34, P < 0.0001

5-FU migration inhibition ratio (mean ± SD) at different concentrations and exposure times. The cytostatic effect is time and dose dependent. Each assay was repeated 4 times.

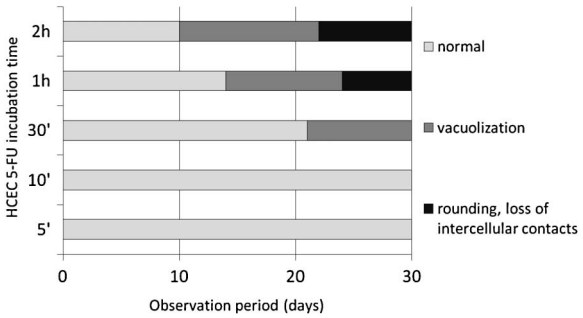

FIGURE 5. Onset of cytotoxic effects of 1% 5-FU on HCECs after several incubation periods during a 30-day observation period.

reduction in proliferation after 5-minute exposures to high 5-FU doses (25,000 μg/mL) in the human Tenon capsule fibroblasts. Huhtala et al[23] studied 5-FU effects on SV40-immortalized HCEC cultures, reporting that the treatment of HCECs with 0.0005 to 5 mg/mL 5-FU for 1 hour had no effect on cell viability. They also found that exposure for 24 hours to a high concentration of 5-FU (5 mg/mL) reduced the cell number to 50% versus controls and resulted in complete cell death after 72 hours. Each one of these studies contributed to the knowledge of 5-FU interactions with superficial ocular structures. All previous studies were directed to one corneal cell type or another. We included both HCEC and HCK cultures and used a wide range of 5-FU concentrations to obtain a more reliable experimental model and to better understand the toxic effects on corneal stromal cells.

To avoid errors, neither adhesion support (such as collagen or 3T3 fibroblast–covered plates) nor growth enhancers were used to obtain confluent cellular cultures. We evaluated a large spectrum of drug concentrations and exposure times to investigate the side effects of the different administration schedules. The reason for choosing 5-FU concentrations of ≤1% derives from our clinical experience in the pharmacological treatment of conjunctival tumours.[7–9] Our data on HCEC cultures showed, at each time interval, no major cytostatic effect for drug concentrations of <0.35%. In the migration inhibition experiments, the ID_{50} values were 0.35%, 0.55%, 1.4%, 1.8%, and 2.75% after 2-hour, 1-hour, 30-minute, 10-minute, and 5-minute exposures, respectively. Microscopic observations of cytotoxic effect showed no evidence of morphological changes at 5-FU concentrations of <1%. At this specific drug dilution, we found the earliest signs of cellular distress after a 30-minute incubation. The appearance of morphological changes was related to the exposure time.

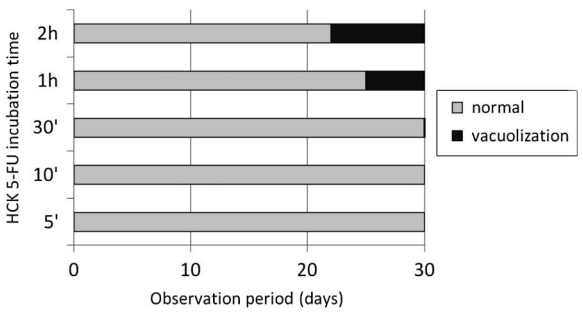

FIGURE 6. Onset of cytotoxic effects of 1% 5-FU on HCKs after several incubation periods during a 30-day observation period.

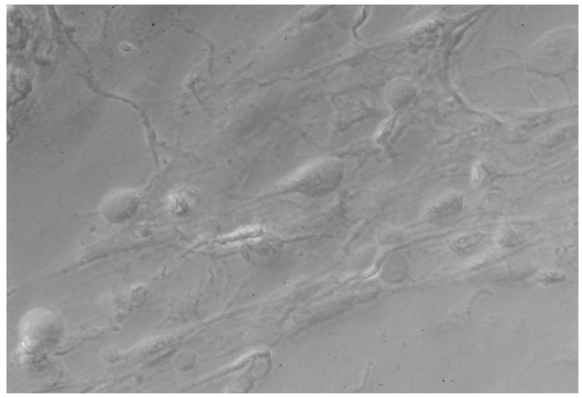

FIGURE 7. Vacuolization of HCKs after 27 days in 1% 5-FU exposed for 2 hours.

In HCK cultures, a significant cytostatic effect was found for incubation durations of ≥2 hours with 0.5% 5-FU. None of the tested drug dilutions resulted in 100% inhibition of migration. The analysis of the cytotoxic effect on HCK cultures revealed no morphological changes in the cultures exposed to 5-FU dilutions of <1%. At higher drug concentrations, changes similar to those of the epithelial cultures were observed, even if later in time and slighter in damage.

The MTT test's viability marker is based on the mitochondrial breathing chain. Our results on quantitative MTT evaluation of drug cytotoxicity did not show any reduction in the OD ratio of ≥50% for 5-FU dilutions of <1%. This may be explained by a sort of cellular stasis determined by the 5-FU active metabolites that are unable to cause cellular death, but only a reduction in the cellular metabolic rate. Our data seem to be confirmed by a toxicity study of 5-FU on corneal bovine endothelial cells in which each drug dilution of <5% did not show significant OD reduction differences.[24] These results suggest the absence of irreversible toxic effects on the examined corneal cells. If we induce a wound in a well plate

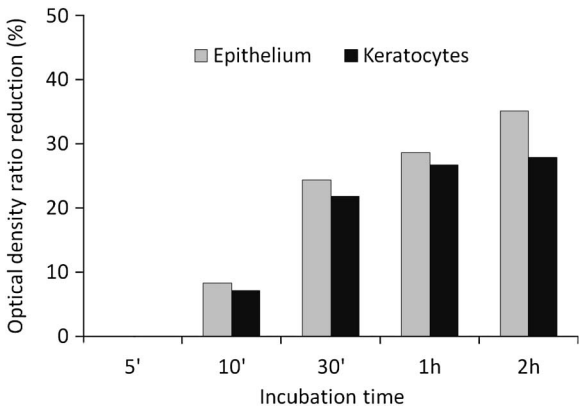

FIGURE 8. OD ratio reduction after 1% 5-FU at different exposure times.

of healthy confluent corneal cells, we mimic the event of a superficial corneal abrasion. The healing process necessarily quickens the cell cycle to restore the integrity of the corneal surface barrier as soon as possible. This active replicative state causes an increase in the mitotic index and in the vulnerability to a cell-cycle–specific drug like 5-FU. This may explain the limited development of cytotoxic effects on confluent cells, and the greater cytostatic effects on HCECs than on HCKs because of the higher mitotic rate of the epithelial cells.

Concerning dilution and the mode of administration used in the treatment of corneoconjunctival tumors with topical 5-FU drops, Parrozzani et al[9] recently demonstrated that 1% 5-FU, as a single agent or in combined therapy, must be considered safe and effective. Human corneal cells were examined in vivo using confocal microscopy. No significant differences were observed between the treated eye and the fellow/control eye in endothelial cell count, pleomorphism and polymegathism, anterior stromal keratocyte density, and beadings and central corneal epithelial thickness with a follow-up of >5 years. Our in vitro data, extending the knowledge on topical 5-FU administration safety profile, complete these in vivo findings.

5-FU is a pyrimidine analog that inhibits cellular proliferation interacting with the S-phase cells. Although this drug has no cytotoxic effects on in vitro cultures, side effects in the cornea and conjunctiva have been reported in vivo.[3,11,12] Reversible side effects of 5-FU as a topical chemotherapeutic agent include conjunctival discomfort and hyperemia, punctate epithelial keratopathy, and corneal epithelial defects.

In conclusion, despite its high efficacy in neoplastic cells, toxic effects of topical 5-FU on normal corneal cells seem to be limited and transient in up to 1% dilution. These events are likely to be more frequent in >2% dilutions. These results underline the cautious use of 5-FU in glaucoma filtering surgery because high drug concentrations are added to corneal tissues distressed by previous antihypertensive topical therapies. However, these results also confirm the wide safety interval of 1% 5-FU employed as a topical agent in conjunctival epithelial tumors.

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