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Effect of different artificial tears against desiccation in cultured human epithelial cells

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: A large number of artificial tears is widely used to treat dry eye symptoms. To test the efficacy of these drugs independent of individual parameters *in vitro* models are required. As described previously, we employed a reproducible *in vitro* cell culture system to evaluate the desiccation protection capability of some artificial tears. In the present paper data is presented of another set of pharmaceutical agents.

Material/Methods: Conjunctival epithelial cell line Chang 1-5c-4 (series 1) and the corneal cell line 2.040 pRSV-T (series 2) were cultured under standard conditions. Confluent cells were wetted for 20 min with artificial tears (Arufil® Uno, Arufil®, Lacrimal®, Lacophthal® sine, Siccaprotect®, Tears Again®, Vidisept® EDO, Vistil®, Wet Comod®) or PBS as a control. After exposure to a constant air flow for 0, 15, 30 and 45 minutes respectively, cells were incubated with the vital dye alamarBlue. Subsequently, absorption of the oxidised form of the dye was assessed using an ELISA-Reader.

Results: Cell best survival rates in series 1 after 15 min were found for Lacrimal® (0.89), Wet Comod® (0.84) compared to PBS (0.66) and in series 2 for Vidisept® EDO (0.57) and Lacrimal® (0.56) compared to PBS (0.01). After 45 min highest survival was seen in series 1 for Lacrimal® (0.46) and Lacophthal® sine (0.36) compared to PBS (0.33) and in series 2 for Lacrimal® (–0.06) and Arufil (–0.16) compared to PBS (–0.23).

Conclusions: Both cell lines tested showed different susceptibility towards desiccation and the artificial tears showed differences in preventing cells from desiccation.

key words: **cell protection • epithelium • cornea • conjunctiva • dry eye • cell culture • ocular surface**

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Background

Dry eye syndrome is a disorder of the ocular surface that is characterized by loss of natural function of the corneal and conjunctival epithelial barrier. Moreover, cytomorphological studies have revealed microchanges in the epithelium comprising changes in size of surface cells and number of goblet cells as well as occurrence of snake-like chromatin [1–3]. The resulting deficit in wetting is compensated by use of tear substitutes that also should protect the mucosa against desiccation [1,4–7]. There are three categories of artificial tears according to the product description table: artificial tears with preservative, artificial tears with disappearing preservative and artificial tears without preservative. Despite the fact that various products improve the situation of dry eye-patients, examinations demonstrating objectively the protective effects are rare [8–11]. Recently we have tested *in vitro* the effectiveness of different agents to prevent desiccation (eg. Artelac® EDO, Vidisic® EDO, Vidisic Fluid® EDO, Acuolens®, Viscofresh® and Hyal Drops® SDU). For this purpose we used human epithelial cells in a standardised fashion [12]. In the present study we studied the protective effect of another set of lubricating eye drops (e.g. Arufil® uno, Arufil®, Lacrimal® O.K., Lacophtal® sine, Siccaprotect®, TEARS AGAIN®, Vidisept® EDO, Vistil™, WET-COMOD®) that were also widely applied in drye eye disease in order to compare the results with established data previously.

Material and Methods

Different pharmacological substances were tested for their capability to prevent desiccation of the conjunctival epithelial cell line Chang 1-5c-4 (CCL-20.2 American Type Culture Collection®; series 1) and corneal cell culture line 2.040 pRSV-T (2.040pRSV-T American Type Culture Collection®; series 2) as described previously [12]. On confluent cell growth, cells were incubated (20 min) with the artificial tears (see below). Incubation with PBS (100 µl) served as a negative control and positive control was incubation with unsupplemented medium (100 µl). After incubation artificial eye drops were discarded and the cell cultures were exposed to a constant air flow for 0, 15, 30 and 45 minutes. To assess the amount of vital cells, cultures were incubated with the vital dye alamarBlue (Biosource, Camarillo, USA). Absorption of the oxidised form of the dye was measured using an ELISA-Reader, in order to detect the amount of live epithelial cells still present [13].

Cell culture

Cell culture was done as described previously [12].

Medicines evaluated in this test

The following medicines were used: Arufil® uno povidone 20 mg, Arufil® povidone 20 mg, Lacrimal® O.K. polyvinyl alcohol 14 mg, povidone 6 mg, Lacophtal® sine povidone 20 mg, Siccaprotect® dexpanthenol 30 mg, polyvinyl alcohol 14 mg, TEARS AGAIN® soy lecithine 10 mg, Vidisept® EDO povidone 20 mg, Vistil™ polyvinyl alcohol 14 mg, WET-COMOD® povidone 20 mg); PBS as negative control (100 µl).

Viability testing

Cells (1.5×10^5 cells/100 µl culture medium/well) were cultured in 96-well plates (Nunc, Wiesbaden) overnight (37°C, respective culture conditions). For experiments involving the 2.040.pRSV-T cells the 96-well plates were coated 2 hours before use with 1 ml solution [0.01 mg/ml fibronectin (SIGMA, St. Louis, USA) and 0.03 mg/ml vitrogen 100 (Invitrogen, Karlsruhe)].

When cells were confluent the medium was removed and 1 to 2 drops test solution were added to the cells, followed by incubation at 37°C for 20 min. Per test solution 4 wells were used. After removal of respective agents, cells were dried (with continuous air flow) for 0 min, 15 min, 30 min and 45 min, respectively. After washing [3 times with 100 µl PBS (1×PBS)] the cells were incubated for 4 hours at 37°C with respective medium and 10% Alamar Blue (Biosource). The absorption of the oxidised dye was measured using an ELISA-Reader (Anthos, Eugendorf, Austria) at 570 nm and 630 nm.

Analysis

The survival rate was assessed with the following formula (alamarBlue™ Assay Booklet by Biosource page 17):

$$\text{Survival rate} = \frac{(\varepsilon_{ox})\lambda_2 A\lambda_1 - (\varepsilon_{ox})\lambda_1 A\lambda_2 \text{ test solution} \times 100}{(\varepsilon_{ox})\lambda_2 A°\lambda_1 - (\varepsilon_{ox})\lambda_1 A°\lambda_2 \text{ untreated positive control}}$$

λ_1 =570 nm
λ_2 =630 nm
$(\varepsilon_{ox})_1$ =80,586 (molar extinction coefficient of Alamar Blue (oxidised) at wave length 570 nm)
$(\varepsilon_{ox})_2$ =34,798 (molar extinction coefficient of Alamar Blue (oxidised) at wave length 630 nm)
$A\lambda_1$ = absorption of the sample at 570 nm
$A\lambda_2$ = absorption of the sample at 630 nm
$A°\lambda_1$ = absorption of the positive control at 570 nm
$A°\lambda_2$ = absorption of the positive control at 630 nm

Data analysis

The medians for all substances tested were depicted in the graph in dependence of exposure times for the respective cell line (overall means) allowing a rapid comparison of the different cell survival rates and the protective effect of the substances tested. 1.0 is the value given to the positive control (culture medium).

Results

All artificial tears reduced the quantity of vital cells in the examined cell cultures with increased drying time periods. The baseline values (after 0 min) for live pRSV-T cells were better compared to the Chang cells. In addition, protection against desiccation was more effective in the pRSV-T cells than in the conjunctival Chang cells that is in accordance to previous studies [12]. After 30 or 45 min of desiccation significant protective effects were seen only for 2 substances, namely Lacrimal® and Lacophthal® sine. After the maximum exposure time of 45 min, the overall means from the 3 sets of experiments were very close, with a very high proportion of living cells compared to the other test substances.

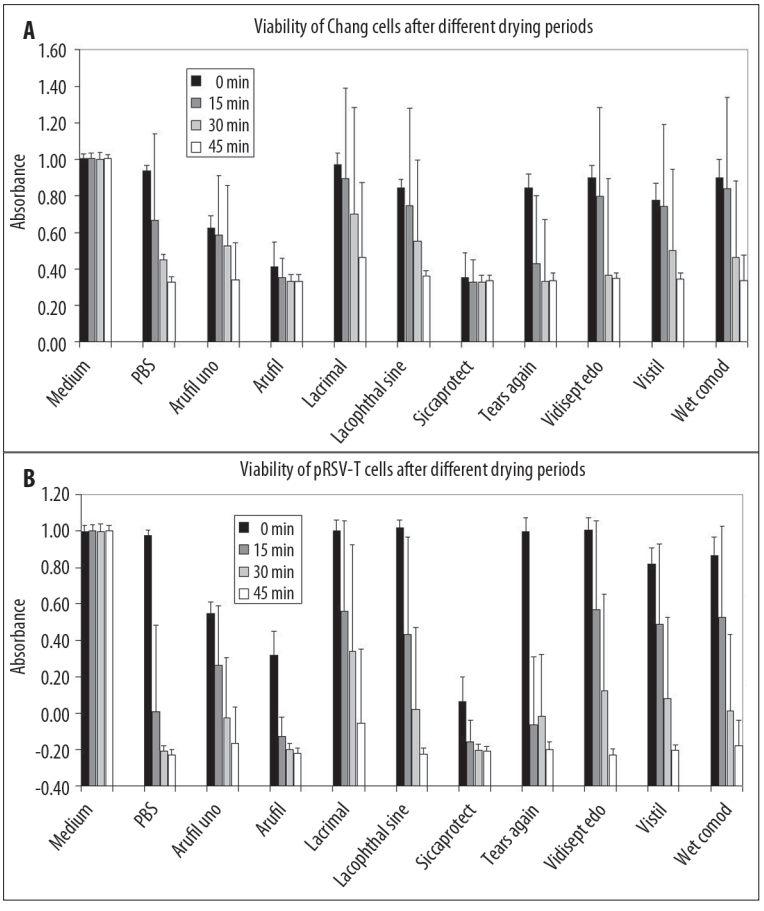

Figure 1A,B. The viability values of conjunctival cells protected with various lubricating tears and exposured to constant air flow, as determined by Alamar Blue assay. The values in Lacrimal® OK are significantly different from each other. Cell viability at different time points after exposition with air flow. 45 minutes after air exposition (desiccation time) the viabilities values of cells protected with Lacrimal® OK increased significantly with control.

In detail, cell survival rates in series 1 after 0, 15 30, 45 min were (0.62;0.58;0.52;0.34) for Arufil® Uno, (0.41;0.35;0.33;0.33) for Arufil®, (0.97;0.89;0.70;0.46) for Lacrimal®, (0.84;0.75;0.55;0.36) for Lacophthal® sine, (0.35;0.33;0.32;0.34) for Siccaprotect®, (0.84;0.43;0.33;0.34) for Tears Again®, (0.90;0.80;0.37;0.35) for Vidisept® EDO, (0.78;0.74;0.50;0.34) for Vistil®, (0.90;0.84;0.46;0.34) for Wet Comod®, (0.94;0.66;0.45;0.33) for PBS and in series 2 (0.55;0.26;–0.03;–0.16) for Arufil Uno®, (0.32;–0.13;–0.20;–0.22) for Arufil®, (1.0;0.56;0.34;–0.06) for Lacrimal®, (1.02;0.43;0.02;–0.22) for Lacophthal sine®, (0.06;–0.16;–0.20;–0.21) for Siccaprotect®, (1.0;–0.06;–0.02;–0.20) for Tears Again®, (1.0;0.57;0.12;–0.23) for Vidisept® EDO, (0.82;0.49;0.08;–0.21) for Vistil®, (0.86;0.53;0.01;–0.18) for Wet Comod® and (0.98;0.01;–0.21;–0.23) for PBS.

A similar decrease of cell viability in both cell lines was seen after treatment with PBS, Arufil® uno, Arufil®, Lacrimal® O.K., Lacophtal® sine, Siccaprotect®, Tears Again®, Vidisept® EDO, Vistil™, and Wet-comod®. Moreover, in comparison to other test substances and the negative controls the overall means for Lacrimal® were not above average after exposure to air. A decrease of survival rate was assessed during increasing drying time for the individual substances tested in both cell cultures. Except Lacrimal® O.K. both cell cultures showed an initially good tolerability of the test substances (Figure 1A, B).

The graph demonstrates the substantial protective effect of Lacrimal® O.K. on Chang cells exposed to the air before drying. After the maximum exposure time of 45 min, the overall means from the 3 sets of experiments were very close, with a very high proportion of living cells compared to the other test substances. In the Chang cell culture the baseline value for Lacophtal® sine was somewhat lower compared to Lacrimal® O.K. When the proportion of living cells after a drying time of 0 to 45 minutes is taken into account, Tears Again® in the Chang cell culture tends to show a lower protective effect on the cell culture to compared PBS. Lacrimal® O.K. and Lacophtal® sine have a significantly better protective effect compared to the other preparations before the drying of both cell lines tested. Both artificial tears with benzalconiumchloride (BAC) were associated with significantly less cell survival. Vistil™ with Oxyd™ as a biodegradable preservative system showed statistical significant difference, whereas Arufil® and Siccaprotect® with BAC caused higher rate of apoptocic cells (after desiccation time 15 min and 30 min). Combined artificial tears from polyvinyl alcohol and povidone without preservative system had the highest rates of cell viability after 45 min desiccation time.

Discussion

We have tested the efficacy of another series of pharmacological substances to prevent desiccation of cultured human conjunctival and corneal cell lines. Lacrimal® and Wet Comod® were most effective on Chang 1-5c-4 cells (series 1) while Vidisept® EDO (0.57) and Lacrimal® showed the best protective effect on 2.040 pRSV-T corneal cell line after 1–15 min of desiccation. After 45 min highest survival was

seen in series 1 for Lacophthal® sine (0.36) and Lacrimal® (0.46) and in series 2 for Lacrimal® (–0.06) and Arufil (–0.16) compared to PBS (–0.23).

The cell culture system was previously described and shown to be suitable to assess the protective effect of artificial tears *in vitro* [12]. The former study investigated Artelac® EDO, Vidisic® EDO, Vidisic Fluid® EDO, Hyal Drops® SDU, Artelac® 10 ml with preservative, Systane® 10 ml, Aculens® EDO, Viscofresh® EDO) and it turned out that Vidisic Fluid® EDO and Vidisic® EDO showed significantly higher survival rates or markedly lower cell loss on epithelial cells [12]. The present observations are in accordance to our previous study [12]. For instance cell viability decreased progressively after constant air flow exposure during few minutes. When drying time increased (more than 15 min) no or only slight increase in cell loss was seen. Although it is to assume that cells die by necrosis the underlying mechanism of cell death especially after 1 or 15 min of drying remains to be explored.

In the present study Lacrimal O.K. was defined in both cell culture lines by the highest survival rates of epithelial cells. When drying time increased up to 45 minutes, no or only slight increase in cell loss could be observed. Substantial cytotoxic effects on the cultured cells were observed when preparations with established preservatives were used. Therefore, it is to suppose that cytotoxicity is caused by preservatives. This adverse effect possibly could be alleviated with biodegradable preservative systems. To compare with BAC preservative substances, artificial tears with OXYD™ led to maintain better cell viability and barrier function of human conjunctival and corneal epithelial cells. Though, these decomposing preservatives also prevent the potential protective effect against drying, the effect demonstrated by preservative-free substance such as Lacrimal O.K. Accordingly preservative-free wetting agents offer, as expected, the best possible preconditions for an effective protection against drying in the highly differentiated epithelium at the ocular surface, whether normal or damaged.

Previously, cell viability in an immortalized corneal epithelial cell line (T-HEC) showed a 4% to 11% increase in apoptotic cells after treatment with 3 different contact lens multipurpose solutions. Moreover the same solutions led to disturbed expression of tight junction proteins ZO-1 and occludin. Otherwise another multipurpose solution did not affect cell viability or expression of tight junction proteins [14].

Although the *in vitro* test systems yield interesting results on cytotoxicity of different medicines, the results have to be interpreted carefully since cultured cells are quite different from the normal ocular surface epithelium. For example, the cell culture model does not consider the stratified character of the conjunctival barrier, drug diffusion, conjunctival blood supply, mucin production and composition and tear fluid. Therefore, *in vitro* studies cannot exactly predict the properties of pharmaceuticals during *in vivo* use [15–16,22]. However the present study is in line to previous studies employing cell culture models for *in vitro* ocular toxicological studies in order to understand mechanisms of some external eye diseases [6,14–16]

As a major disadvantage artificial tears often contain potentially toxic preservatives, stabilizers, and other additives that can cause further problems to the compromised cornea in the dry eye condition [17,28,30]. Although the concentration of preservatives is usually low, high frequency of use may result in a cumulative effect and damage of the ocular surface. This problem can be prevented by using preservative-free unit-dose artificial tears [30,24,31–33]. Therefore, it is useful to objectively assess corneal-protective effects of artificial tears and to compare the effects of products that contain different components.

Conclusions

In conclusion, the results of the present study suggest that the *in vitro* fluorometric system comprising resazurin (Alamar Blue) microplate assay with human corneal and conjunctival cell culture would be a valuable potential *in vitro* screening approach in the product development of artificial eye drops.

Substances evaluated in this test

Arufil® uno: povidone 20 mg, *2H_2O disodium edetate, disodium phosphate 2H_2 O, sodium 2H_2O, sodium chloride, water f. Inj-purposes;*
Arufil®: povidone 20 mg, *2H_2O disodium edetate, disodium phosphate 12H_2 O, sodium 2H_2O, sodium chloride, water f. Inj-purposes, benzalkonium chloride 0.03 mg;*
Lacrimal® O.K.: polyvinyl alcohol 14 mg, povidone 6 mg, *natriumchloride;*
Lacophtal® sine: povidone 20 mg, *natriumchloride, natriumhydroxide, boric acid, water f. Inj-purposes;*
Siccaprotect®: dexpanthenol 30 mg, polyvinyl alcohol 14 mg, *benzalkoniumchloride, kaliumdihydrogenphosphate, kaliummonohydrogenphosphate;*
Tears Again®: soy lecithine 10 mg, *natriumchloride 8 mg, ethanol 8 mg, phenoxyethanol 5 mg, retinyl palmitate 0.25 mg, α-Tocopherol 0.02 mg;*
Vidisept® EDO: povidone 20 mg, *boric acid, natriumchloride, natriumhydroxide;*
Vistil™: polyvinyl alcohol 14 mg, *OXYD™, edetinacid, dinatriumsaline;*
Wet-Comod®: povidone 20 mg, *natriumhydroxide, citrate buffer, sorbitol;*
PBS as negative control (100 µl);
Unsupplemented medium as a positive control (100 µl).

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