Comparison of Human Corneal Cell Cultures in Cytotoxicity Testing

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Summary

The cytotoxic pattern of cosmetic or pharmaceutical compounds within different layers of the human cornea is of special interest with respect to ocular safety testing. The aim of this study was to evaluate the ability of a newly developed human corneal keratocyte (HCK) cell line as an in vitro model to predict toxicity towards keratocytes in the corneal stroma. The cytotoxic response of immortalised HCK cultures towards different surfactants was compared to that of primary cultures of human corneal keratocytes. Our studies revealed comparable results for immortalised and primary keratocytes. Furthermore, we quantified surfactant-induced cytotoxic effects on immortalised cultures of corneal epithelium and endothelium. In conclusion, the HCK cell line represents an appropriate model to test keratocyte-specific toxicity and may serve as a useful building block in the construction of three-dimensional human cornea equivalent models.

Zusammenfassung: Vergleichende Zytotoxizitätstests an menschlichen Korneazellkulturen

Für die ophthalmologische Sicherheitsüberprüfung von kosmetischen und pharmazeutischen Produkten ist eine differenzierte Untersuchung der zytotoxischen Wirkung einer Testsubstanz auf die verschiedenen Gewebeschichten der Kornea von großer Bedeutung. Ziel dieser Studie war festzustellen, ob sich die neu entwickelte humane Keratozytenzelllinie HCK als Modellzelllinie zur Vorhersage der Keratozyten-spezifischen Toxizität im kornealen Stroma eignet. Hierzu wurden die zytotoxischen Effekte verschiedener Detergenzien an immortalisierten HCK-Kulturen und an primären Keratozytenkulturen untersucht. Im Vergleich zeigten immortalisierte und primäre Keratozyten eine ähnliche Sensitivität. Ferner wurden die Detergenzien auch an menschlichen Zelllinien des kornealen Epithels und Endothels getestet. Zusammenfassend haben unsere Untersuchungen gezeigt, dass die HCK-Zelllinie ein geeignetes Modell zur Überprüfung der Keratozyten-spezifischen Toxizität darstellt und sich daher als Werkzeug zum Aufbau eines dreidimensionalen humanen Korneaäquivalents eignet.

Keywords: cornea, keratocyte, SV40-immortalisation, WST-1

1 Introduction

The Draize rabbit eye irritation test has been used in regulatory safety testing since 1944 (Draize et al., 1944). During the last 20 years this test has often been criticised for its lack of objectivity and reproducibility, for over-predicting human responses (Sharpe, 1985; Frazier et al., 1987; Wilhelmus, 2001), as well as for reasons of animal welfare. Meanwhile, a variety of refinement and reduction alternatives to animal tests have been developed, but currently none of these alternative methods could be validated to gain regulatory acceptance as a complete replacement for the Draize eye test.

Since human cell lines offer the advantage of permanent access to standardised cell sources, several investigators prospected a notable benefit from the use of human ocular cell lines for the prediction of eye toxicity (Kahn et al., 1993; Araki-Sasaki, 1995). Moreover, artificial human eye tissue, constructed from permanent cell lines, may give useful mechanistic information. Recently, we reported the construction of a three-dimensional corneal model from human cell lines (Zorn-Kruppa et al., 2004; Engelke et al., 2004). These studies are based substantially on the establishment of a new permanent cell line from the human corneal keratocytes (HCK), which were used for the construction of an artificial stromal tissue.

The purpose of the present study was to evaluate the ability of the new human corneal keratocyte cell line to predict eye toxicity. Therefore, the validity of the cytotoxic response towards selected test compounds was compared to that of primary cultures of human corneal keratocytes. Furthermore, the toxicity data were compared to those obtained with immortalised cultures of human corneal epithelium and endothelium in order to determine the keratocyte-specific effects. Both the epithelial and the endothelial cell lines have been described to retain the properties of their respective normal corneal counterparts (Araki-Sasaki et al., 1995; Bednarz et al., 2000) and have already been used in several studies concerning cytotoxicity (Saarinen-Savolainen et al., 1998; Burgalassi et al.,

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2001; Mertens et al., 2002; Huhtala et al., 2003) and apoptosis (Haertel et al., 2003; Thuret et al., 2003).

The WST-1 was used for cytotoxicity testing. This is a colorimetric assay for the non-radioactive quantification of cell proliferation and viability, based on the cleavage of the tetrazolium salt (4(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulphonate) to formazan, which only occurs in living cells. Three surfactants were used as test compounds: benzalkonium chloride (BAC), sodium dodecyl sulphate (SDS) and Triton-X-100. The test compounds were selected according to the guidelines for oculotoxic safety testing of the European Center for Ecotoxicology and Toxicological Chemicals (ECETOC Reference Chemicals Data Bank, June 1998).

2 Materials and methods

2.1 Human corneal keratocyte (HCK) cultures

2.1.1 Primary culture of human corneal keratocytes

The primary human corneal keratocytes were isolated from eye bank donor material (University Eye Hospital, D-Hamburg). A human donor cornea that was unsuitable for transplantation due to low endothelial cell density was first organ-cultured before the initiation of the keratocyte culture, which was carried out according to Dropkova et al. (1999) with slight modifications. Descement membrane and endothelium were stripped off and the epithelial part was dissected using a hockey-knife. The remaining stroma was cut into tissue pieces of approximately 1 mm^3. These fragments were placed in 6-well cluster plates containing 0.5 ml basal medium F99, a 1:1 mixture of Ham's F12 and medium 199 (M199, Life Technologies, Karlsruhe, Germany) supplemented with 5% fetal calf serum (FCS) (Gibco BRL, Paisley, Scotland) and 1% (v/v) antibiotic/antimycotic solution containing penicillin 10,000 U/ml, streptomycin 10,000 mg/ml and amphotericin B 25 µg/ml (Gibco BRL, Paisley, Scotland). After attachment of the tissue pieces, 2 ml fresh medium were added. As soon as the pieces had produced a "halo" of outgrowing keratocytes, the culture was passaged by standard trypsin dispersion and resuspended in F99 culture medium. Primary cultures of human keratocytes (HCKp) survived 4 months and were subcultured once a week for 5 passages.

2.1.2 Immortalised human corneal keratocytes

To obtain a human corneal keratocyte strain with an extended life span, primary keratocytes were transfected with a construct containing the SV40 early region as reported previously (Zorn-Kruppa et al., 2004). Cultures of transformed cells (HCKt) were subcultured twice a week for 23-29 passages until entering crisis. During the next two months 6 of these cultures died, whereas colonies of immortalised HCK (HCKi) appeared in one culture. The post-crisis cells could be subcultivated in regular intervals of 3 days and have now undergone 73 passages.

2.2 Immortalised human corneal epithelial cell line (HCE)

The immortalised human corneal epithelial cell line used in this study was established by Araki-Sasaki et al. (1995) via infection of primary cells with a recombinant SV40-adenovirus vector followed by cloning three times to obtain a continuously growing cell line. This cell line, originally designated HCE, was a generous gift from the Araki-Sasaki research group. HCE culture growth medium consists of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Paisley, Scotland) and Ham's nutrient mixture F-12 (Ham's F-12, Life Technologies, Karlsruhe, Germany) supplemented with 15% (v/v) FCS (Gibco BRL, Paisley, Scotland), 1% (v/v) antibiotic / antimycotic solution containing penicillin 10,000 U/ml, streptomycin 10,000 mg/ml and amphotericin B 25 µg/ml (Gibco BRL, Paisley, Scotland), 2 mM L-glutamine (Gibco BRL, Paisley, Scotland), 5 µg/ml insulin (Sigma-Aldrich, Steinheim, Germany), and 10 ng/ml human epithelial growth factor (Sigma-Aldrich, Steinheim, Germany). The cells were grown in a 5% CO_2 atmosphere at 37°C and serially passaged at a split ratio of 1:4 three times a week.

2.3 Immortalised human corneal endothelial cells (HCEC)

The human corneal endothelial cell line was established by Bednarz et al. (2000). Immortalisation was achieved by transfection with the pRNS plasmid containing an early region of SV40. HCEC cells were cultivated in basal medium F99, supplemented with 5% (v/v) FCS and 1% (v/v) antibiotic / antimycotic solution. The cells were serially passaged at a split ratio of 1:4 twice a month to preserve monolayer formation by keeping the cells in a contact-inhibited environment.

2.4 Treatment of cell cultures for cytotoxicity testing

The cells were seeded in 96-well microtiter plates (Corning GmbH, Wiesbaden, Germany) at a density of 1.5×10^4 cells per well. The medium was discarded after 24 h and the cells were incubated for 60 min in the absence or presence of increasing concentrations of the following test substances diluted in culture medium: sodium dodecyl sulphate (SDS; Sigma, Taufkirchen, Germany), Triton X-100 (Serva, Heidelberg, Germany) and benzalkonium chloride (BAC; FeF Chemicals A/S, Koege, Denmark). The medium was removed after treatment and the cells were rinsed once with basal F99. Cells were incubated with 100 µl fresh growth medium for a recovery period of 24 hours, after which the WST-1 test was initiated.

2.5 WST-1 test

Mitochondrial activity was determined using the WST-1 reduction assay for fast, spectrophotometric quantification of cell growth and viability. The WST-1 reagent (Roche Diagnostics, Mannheim, Germany) was added in a volume of 1:10 and incubated for at least 60 min in a humidified 5% CO_2 atmosphere at 37°C. The microtiter plates were shaken thoroughly for 1 min prior to the absorption measurement (450 nm) against a background control with an automated MRX microtiter plate reader (Dynatech Medical Products Ltd, West Sussex, England).

2.6 Statistics

Cytotoxicity data were obtained from two independent measurements with cells from different passages. It was en-

sured that the effect of passage number (inter-experimental variation) was not significant. At least 8 wells were tested for each concentration. The mean of the optical density values gained for the non-treated controls was taken as 100%. The results of treated cultures were expressed as percentages of non-treated control cultures. The EC_{50} value (%) was determined as the concentration of the test agent that decreased the WST-1 reduction values to 50% of the control value. EC_{50} values were calculated from fitted dose response curves using sigmoidal curve analysis (Origin® 7SRI, OriginLab Cooperation, Northhampton, USA). The statistical significance of the differences in EC_{50} values was determined by one-way ANOVA with Bonferroni's post test (GraphPad Prism, GraphPadTM, San Diego, USA). Differences were considered significant for $p<0.05$.

3 Results

Figures 1 and 2 illustrate typical dose response curves obtained with the WST-1 cytotoxicity data of the tested compounds. Tables 1 and 2 summarise the half-maximal effective concentrations (EC_{50}), calculated from the dose response curves.

Figure 1 illustrates the concentration-dependent effects of BAC, SDS and Triton X-100 on primary (HCKp), transfected (HCKt) and immortalised (HCKi) keratocyte cultures. All compounds caused a concentration-dependent decrease in WST-1 formazan formation subsequent to an exposure time of one hour. The statistical analysis of the corresponding EC_{50} values listed in Table 1 reveals no significant differences for BAC and Triton X-100 between the three different keratocyte cultures. In contrast, SDS was less toxic to HCKp cells ($EC_{50} \cong 0.0135$) compared to HCKt ($EC_{50} \cong 0.009$) and HCKi cells ($EC_{50} \cong 0.0053$).

The comparison of the cytotoxic response of keratocytes (HCK), epithelial (HCE) and endothelial (HCEC) cell lines is illustrated in Figure 2. Typical dose response curves with relatively small ranges from minimum effects of 0.001% (w/v) to maximum effects of 0.02% (w/v) were observed for all tested compounds. As expected, the endothelial cell line responded more sensitively towards Triton than the other cell lines (Tab. 2). One hour exposure to 0.003% (w/v) Triton caused a 50% decrease in HCEC-cell viability, which differs significantly from the EC_{50} values obtained with the epithelial and keratocyte cell cultures ($p<0.0001$).

Among the three tested compounds, BAC had the highest cytotoxic potential, while SDS and Triton were less toxic and yielded similar results.

4 Discussion

Several studies have shown that corneal tissue equivalent models are useful for the evaluation of ocular irritation and may support the reduction or the replacement of traditional animal-based safety testing (Minami et al., 1993; Zieske et al., 1994; Schneider et al., 1997; Stern et al., 1998; Griffith et al., 1999; Germain et al., 2000; Tegtmeyer et al., 2001). In our previous report, we presented a human corneal equivalent model constructed from cell lines of the three main corneal cell types (Zorn-Kruppa et al., 2004). Beside the construction of the epithelial and the endothelial layers, the main effort of our previous study was the development of a new immortalised keratocyte strain (HCK) to complete an artificial stroma.

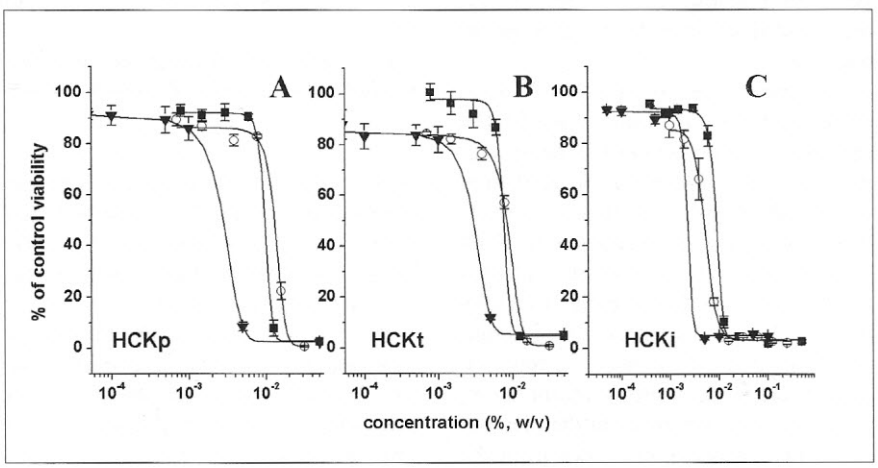

Fig. 1: Viability of primary (HCKp, A), transformed (HCKt, B), and immortalised (HCKi, C) human corneal keratocytes after exposure to SDS (○), BAC (▼), and Triton (■).

Tab. 1: Toxicity of BAC, SDS, and Triton-X-100 on primary (HCKi), transformed (HCKt) and immortalised (HCKi) human corneal keratocytes

Testsubstance	WST-1 EC_{50} values % (w/v) (MEAN±SEM)			One-way analysis of variance
	HCKp	HCKt	HCKi	*P*-value (summery)
SDS	0.01348 ± 0.00081	0.00899 ± 0.00031	0.00527 ± 0.00012	<0.0001 (***)[1]
BAC	0.00287 ± 0.00002	0.00312 ± 0.00036	0.00228 ± 0.0257	0.9996
Triton X-100	0.00983 ± 0.00031	0.00755 ± 0.00086	0.00839 ± 0.00071	0.1998

[1] P values of Bonferroni's Multiple Comparison Test: HCKp versus HCKt $P < 0.05$; HCKp versus HCKi $P < 0.001$; HCKt versus HCKi $P < 0.05$.

Tab. 2: Toxicity of BAC, SDS, and Triton-X-100 on immortalised cell lines of human corneal keratocytes (HCK), human corneal epithelium (HCE) and human corneal endothelium (HCEC)

Testsubstance	WST-1 EC_{50} values % (w/v) (MEAN±SEM)			One-way analysis of variance
	HCK	HCE	HCEC	*P*-value (summery)
SDS	0.00527 ± 0.00012	0.00594 ± 0.00023	0.00427 ± 0.00256	0.7296
BAC	0.00228 ± 0.0257	0.00203 ± 0.00168	0.00171 ± 0.00133	0.9996
Triton X-100	0.00839 ± 0.00071	0.009 ± 0.0007	0.00319 ± 0.0001	<0.0001 (***)[1]

[1] P values of Bonferroni's Multiple Comparison Test: HCK versus HCE P > 0.05; HCK versus HCEC P < 0.001; HCE versus HCECP < 0.001.

Corneal keratocytes are known to be essential to obtain a differentiated prostate epithelium, due to production of specific growth factors (Chan and Haschke, 1983). In addition, Parnigotto et al. (1996) stressed the importance of a stromal keratocyte layer within a cornea equivalent model for growth stimulation and differentiation of the epithelial cells (Parnigotto et al., 1996; Parnigotto et al., 1998). Considering the role of corneal keratocytes in the process of wound healing (Wilson et al., 2003), the HCK cell line may also facilitate studies on the cell biology of corneal injury response.

The aim of the present study was to demonstrate, that the immortalised HCK cell line developed in our laboratory represents a valid model for the prediction of keratocyte-specific toxicity. Therefore, the cytotoxic response of HCK monolayer cultures towards selected compounds with known corneal toxicity (BAC, SDS and Triton X-100) was compared to that of primary cultures of human corneal keratocytes. Furthermore, we studied the difference in the cytotoxic response between cell lines from the endothelial, epithelial and stromal part of the cornea.

We used the WST-1 assay to measure cell viability as the cytotoxic endpoint. Unlike the MTT test, which is widely used to measure cell viability and proliferation, the WST-1 test yields water-soluble cleavage products and does not require any additional solubilisation steps before photometric measurements. Compared with MTT it turned out that WST-1 is even more stable, more sensitive, and more exact in cell cultures and fresh allografts (Alotto et al., 2003). In recent studies, the WST-1 test served as a cytotoxicity assay for certain ophthalmic adjuvants in ocular cultures. (Daniels and Khaw, 2000; Burgalassi et al., 2001; Jiang et al., 2002; Mannerström et al., 2002; Huhtala et al., 2003).

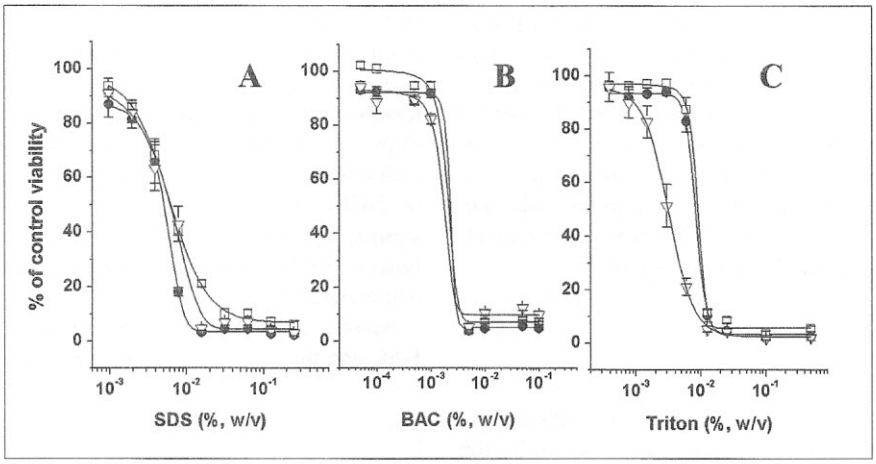

Fig. 2: Viability of immortalised epithelial cells, HCE (□), endothelial cells, HCEC (▽) and keratocytes, HCK (●) after exposure to SDS (A), BAC (B), and Triton (C).

We used EC_{50} values calculated from dose response curves to demonstrate the differences in the sensitivity of the cell lines and the cytotoxic potential of the investigated compounds.

Primary cell cultures have been thought to be better candidates for toxicity testing than cell lines, because native cells are thought to be more sensitive than respective cell lines (Borenfreund and Borrero, 1984). Currently, there are no studies comparing the cytotoxicity results of primary human keratocytes to those of an immortalised keratocyte cell line under similar test conditions. In the present study, the transfected and the immortalised keratocyte cell line as well as the primary keratocyte culture were sensitive to the investigated compounds in a comparable concentration range. Statistically significant differences between the immortalised and the native culture were observed in case of SDS, whereas BAC and Triton X-100 treatment resulted in comparable EC_{50} values in all three cell cultures. Our results suggest that the newly developed HCK cell line is a reliable model of keratocyte toxicity, since both primary and immortalised cell lines were in most cases equally sensitive to the tested substances.

HCK, HCE, and HCEC cell lines derived from the three corneal tissues differ in morphology and function. In the human cornea, the stromal tissue is located between the outermost stratified epithelium and the innermost monolayer of specialised endothelial cells. In *in vivo* experiments with rabbit corneas, Jester et al. (1998) identified quantifiable differences in the distinct corneal layers for surfactants producing different degrees of ocular irritation. It was questionable whether these differences are trans-

ferable to *in vitro* experiments with monolayer cultures of the three cell lines. However, our results revealed no differences between the sensitivity of epithelial, endothelial and stromal cell lines for BAC and SDS, while endothelial cells proved to be more sensitive in case of Triton X-100.

The surfactants used in this study represent different types according to their chemical structures: SDS is the most widely used of the anionic surfactants, which are generally used as the prime surfactants in foaming toiletries. Non-ionic surfactants, like Triton-X-100, are mostly used as emulsifiers and solubilisers in cosmetic applications. The cationic surfactant BAC is a preservative and ingredient of topical ophthalmic solutions, such as those used for glaucoma treatment.

Among the tested compounds, BAC was found to be the most toxic, which is in accordance with other results (Parnigotto et al.,1998). It is well known that cationic surfactants are the most irritating to the rabbit eye, followed by anionic and non-ionic detergents (Draize and Kelly, 1952). Corresponding to our results, *in vitro* studies using primary cultures of rabbit corneal epithelial cells revealed a comparable ranking from highly toxic to less toxic, that is cationic > anionic > non-ionic surfactants. However, the cytotoxic potential of Triton was comparable to that of anionic surfactants. Moreover, the measured *in vitro* rankings corresponded well to the reported *in vivo* Draize rabbit eye test data (Grant et al., 1992).

The mechanism of surfactant-induced cytotoxicity has been evaluated in various cell lines (Kruszewski et al., 1997; Jester et al., 1998; Jelinek and Klocking, 1998; Xu et al., 2000). There seemed to be no uniform pathway and no correlation with the surface-active properties of the detergents. Most of the surfactants caused non-enzymatic membrane damage, which would lead to apoptosis at low concentrations or necrotic cell death at high doses (Jelinek, 2001).

Although the presented cytotoxicity studies must be considered preliminary, we believe that three-dimensional corneal models consisting of the tested cell lines will result in more differentiated toxicity responses towards distinct test substances.

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References

Alotto, D., Ariotti, S., Graziano, S. et al. (2003). The role of quality control in a skin bank. Tissue viability determination. *Cell Tissue Bank. 3*, 3-10.

Araki-Sasaki, K., Ohashi, Y., Sasabe, T. et al. (1995). An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest. Ophthalmol. Vis. Sci. 36*, 614-621.

Bednarz, J., Teifel, M., Friedl, P. and Engelmann, K. (2000). Immortalization of human corneal endothelial cells using electroporation protocol optimized for human corneal endothelial and human retinal pigment epithelial cells. *Acta Ophthalmol. Scand. 78*, 130-136.

Borenfreund, E. and Borrero, O. (1984). In vitro cytotoxicity assays. Potential alternatives to the Draize ocular allergy test. *Cell Biol. Toxicol. 1*, 55-65.

Burgalassi, S., Chetoni, P. and Saettone, M. F. (2001). Cytotoxicity of potential ocular permeation enhancers evaluated on rabbit and human corneal epithelial cell lines. *Toxicol. Lett. 122*, 1-8.

Chan, K. Y. and Haschke, R. H. (1983). Epithelial-stromal interaction: specific stimulation of corneal epithelial cell growth in vitro by factor(s) from cultured stromal fibroblasts. *Exp. Eye Res. 59*, 385-392.

Daniels, J. T. and Khaw, P. T. (2000). Temporal stimulation of corneal fibroblast wound healing activity by differentating epithelium in vitro. *Invest. Ophthalmol. Vis. Sci. 41*, 3754-3762.

Draize, J. H., Woodard, G. and Calvery, H. O. (1944). Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J. Pharmacol. Exp. Ther. 82*, 377-390.

Draize, J. H. and Kelly, E. A. (1952). Toxicity to eye mucosa of certain cosmetic preparations containing surface-active-agents. *Proc. Sci. Sec. Toilet. Goods Assoc. 17, I*, 1-4.

Dropcova, S., Denyer, S. P., Lloyd, A. W. et al. (1999). A standard strain of human ocular keratocytes. *Ophthalmic Res. 31*, 33-41.

Engelke, M., Patzke, J., Tykhonova, S. and Zorn-Kruppa, M. (2004). Assessment of ocular irritation by image processed quantification of cell injury in human corneal cell cultures and in corneal constructs. *ATLA*, in press.

Frazier, J. M., Gad, S. C., Goldberg, A. M. and McCulley, J. P. (1987). A critical evaluation of alternatives to acute ocular irritation testing. In A. M. Goldberg (ed.), *Alternative Methods in Toxicology*, Vol. 4 (pp. 136). New York: Mary Ann Liebert.

Germain, L., Carrier, P., Auger, F. A. et al. (2000). Can we produce a human corneal equivalent by tissue engineering? *Prog. Ret. Eye Res. 19*, 497-527.

Grant, R. L., Yao, C., Gabaldon, D. and Acosta, D. (1992). Evaluation of surfactant cytotoxicity potential by primary cultures of ocular tissues: I. Characterization of rabbit corneal epithelial cells and initial injury and delayed toxicity studies. *Toxicology 76*, 153-176.

Griffith, M., Osborne, R., Munger, R. et al. (1999). Functional human corneal equivalents constructed from cell lines. *Sience 286*, 2169-2172.

Haertel, S., Zorn-Kruppa, M., Tikhonova, S. et al. (2003). Staurosporin-induced apoptosis in human corneal epithelial cells in vitro. *Cytometry 55*, 15-23.

Huhtala, A., Alajuuma, P., Burgalassi, S. et al. (2003). A collaborative evaluation of the cytotoxicity of two surfactants by using the human corneal epithelial cell line and the WST-1 test. *J. Ocul. Pharmacol. Ther. 19*, 11-21.

Jelinek, A. and Klocking, H. P. (1998). In vitro toxicity of surfactants in U937 cells: cell membrane integrity and mitochondrial function. *Exp. Toxicol. Pathol. 5*, 472-476.

Jelinek, A. (2001). In-vitro-Toxizität grenzflächenaktiver Substanzen: Wirkung auf Zellmembran, mitochondriale Funktion und Apoptose, Martin-Luther-Universität Halle-Wittenberg: Rer. nat. Dissertation.

Jester, J. V., Li, H. F., Petroll, W. M. et al. (1998). Area and depth of surfactant-induced corneal injury correlates with cell death. *Invest. Ophthalmol. Vis. Sci. 39*, 922-936.

Jiang, S., Moriarty, S. E., Grossniklaus, H. et al. (2002). Increased oxidant-induced apoptosis in cultured nondividing human retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci. 43*, 2546-2553.

Kahn, C. R., Young, E., Lee, I. H. and Rim, J. S. (1993). Human corneal epithelial primary cultures and cell lines with extended life span: in vitro model for ocular studies. *Invest. Ophthalmol. Vis. Sci. 36*, 3429-3441.

Kruszewski, F. H., Walker, T. L. and DiPasquale, L. C. (1997). Evaluation of a human corneal epithelial cell line as an in vitro model for assessing ocular irritation. *Fundam. Appl. Toxicol. 36*, 130-140.

Mannerstrom, M., Zorn-Kruppa, M., Diehl, H. et al. (2002). Evaluation of the cytotoxicity of selected systemic and intravitreally dosed drugs in the cultures of human retinal pigment epithelial cell line and of pig primary retinal pigment epithelial cells. *Toxicol. In Vitro 16*,193-200.

Mertens, S., Bednarz, J. and Engelmann, L. (2002). Evidence of toxic side effects of perfluorohexyloctane after vitreoretinal surgery as well as in previously established in vitro models with ocular cell types. *Graefes Arch. Clin. Exp. Ophthalmol. 240*, 989-995.

Minami, Y., Sugihara, H. and Oono, S. (1993). Reconstruction of cornea in three-dimensional collagen gel matrix culture. *Invest. Ophthalmol. Vis. Sci. 34*, 2316-2324.

Parnigotto, P. P., Bassani, V., Gottardo, A. et al. (1996). Growth, morphology, morphometry and keratins pattern of corneal epithelial bovine cells cultured in vitro. *Ann. Anat. 178*, 545-551.

Parnigotto, P. P., Bassani, V., , Montesi, F. and Conconi, M. T. (1998). Bovine corneal stroma and epithelium reconstructed in vitro: characterisation and response to surfactants. *Eye 12*, 304-310.

Saarinen-Savolainen, P., Järvinen, T., Araki-Sasaki, K. et al. (1998). Evaluation of various ophthalmic drugs, eye drop excipients and cyclodextrins in an immortalized human corneal epithelial cell line. *Pharmacol. Res. 15*, 1275-1280.

Schneider, A. I., Maier-Reif, K. and Graeve, T. (1997). The use of an in vitro cornea for predicting ocular toxicity. *In Vitro Toxicol. 10*, 309-318.

Sharpe, A. R. (1985). The draize test – motivation for change. *Food Chem. Toxicol. 23*, 139-143.

Stern, M., Klausner, M., Alvarado, R. et al. (1998). Evaluation of the EpiOcular tissue model as an alternative to the Draize eye irritation test. *Toxicol. In Vitro 12*, 455-461.

Tegtmeyer, S., Papantoniou, I. and Müller-Goymann, C. C. (2001). Reconstruction of an in vitro cornea and its use for drug permeation studies from different formulations containing pilocarpine hydrochloride. *Eur. J. Pharm. Biopharm. 51*, 119-125.

Thuret, G., Chiquet, C., Herrag, S. et al. (2003). Mechanisms of staurosporine induced apoptosis in a human corneal cell line. *Br. J. Opthalmol. 87*, 346-352.

Wilhelmus, K. R. (2001). The Draize Eye Test. *Surv. Ophthalmol. 45*, 493-515.

Wilson, S. E., Netto, M. and Ambrosio, R. Jr. (2003). Corneal cells: chatty in development, homeostasis, wound healing, and disease. *Am. J. Ophthalmol. 36*, 530-536.

Xu, K. P., Li, X. F. and Yu, F. S. (2000). Corneal organ culture model for assessing epithelial responses to surfactants. *Toxicol. Sci. 58*, 306-314.

Zieske, J. D., Mason, V., Wasson, M. E. et al. (1994). Basement membrane assembly and differentiation of cultured corneal cells: importance of culture environment and endothelial cell interaction. *Exp. Cell Res. 214*, 621-633.

Zorn-Kruppa, M., Tykhonova, S., Belge, G. et al. (2004). A human cornea equivalent constructed from SV40-immortalized corneal cell lines. *ATLA*, in press.

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