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Bovine corneal stroma and epithelium reconstructed *in vitro*: characterisation and response to surfactants

PIER PAOLO PARNIGOTTO, VALERIA BASSANI, FRANCESCO MONTESI, MARIA TERESA CONCONI

Abstract

In order to define safety profiles and proper handling procedures for new industrial products, it is essential to determine their potential for ocular irritation. The Draize test is normally employed but it involves using rabbits. There is today a great need for all researchers to limit the use of animals for laboratory experiments and to encourage the development and adoption of alternative *in vitro* methods to evaluate the potential toxicity of new products. This study proposes a three-dimensional model of bovine corneal stroma and epithelium that is not only easy to reproduce but may also be used in the toxicological field as an alternative to animal experimentation. The data presented here show that this model allows the growth of epithelium similar in features to *in vivo* epithelium. Basal cells are cube-shaped, whereas superficial areas are horizontally longer; desmosomes and 64 kDa keratin, as a marker for differentiation of corneal epithelial cells, are both expressed; the basal lamina is synthesised also. The 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay was carried out on the model to evaluate the toxicity of some surfactants: benzalkonium chloride, Triton X-100, sodium dodecylsulphate and Tween 20. Since the *in vitro* data fit very well the results of the Draize test *in vivo* as reported in the literature, the three-dimensional culture may be used to predict the potential cytotoxicity of surfactants.

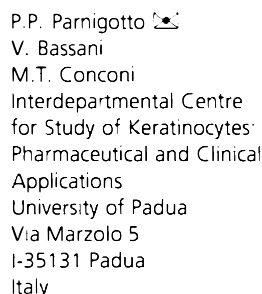
Key words Reconstructed bovine cornea, Corneal epithelium, Keratocytes, Cytotoxicity, Surfactants

In order to define the safety profiles and proper handling procedures of new industrial and pharmaceutical products, it is essential to determine their potential for ocular irritation, at present carried out using rabbits as standard animal models. The whole process is regulated by EEC directive 79/831 of 18 September 1979, which registers not only the methods for determination of physicochemical properties but also the toxicity and ecotoxicity of various substances by *in vivo* tests. The rabbit test is based on parameters established by Draize *et al.*[1] that estimate damage to cornea, conjunctiva and iris after instilling the test substance, in either liquid of powder form, into the lower conjunctival sac of albino rabbit eye.

The European Community has recently approved a directive (93/95/CEE) aimed at limiting the use of animals for laboratory experiments and encouraging the development and adoption of alternative *in vitro* methods for evaluating the potential toxicity of new industrial and pharmaceutical products.

At present, several alternative *in vitro* methods are used, most of them employing cultures of various cell types and, lately, also bioluminescent bacteria. However, these methods have disadvantages – for example, insoluble materials cannot be tested as they do not mix with the culture medium. This obstacle has been overcome by using the hen's egg chorioallantoic membrane test,[2,3] enucleated animal eyes and excised bovine corneas.[4-6] Skin organotypic cultures have recently been further studied in order to test the irritation potential of skin irritants. Various substrates may be used for epithelium growth: nylon nets seeded with fibroblasts,[7,8] de-epidermised dermis[9-11] or collagen matrices containing skin fibroblasts.[11-15] Dermatotoxicity tests performed on these models show fairly good correlation indices with the Draize test.

Osborne *et al.*[16] have recently developed an *in vitro* method for screening ocular irritancy using human skin reconstructed *in vitro* (Skin[2] Model ZK 1200 from Advanced Tissue Sciences). The reconstruction of a three-dimensional model of human cornea is an obvious step in the evolution of *in vitro* test systems as alternatives to the ocular Draize test. Although corneal reconstruction has been proposed by Minami *et al.*[17] for cell biology studies on the proliferation and differentiation



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of the epithelium, and by Ohji *et al.*[18] for clinical applications, it has never been applied to toxicological studies. In the present study, bovine corneal epithelium and keratocytes were cultured both in monolayers and on a three-dimensional matrix, and the irritancy potential of four surfactants of known toxicity tested: benzalkonium chloride, sodium dodecylsulphate, Triton X-100 and Tween 20.

Materials and methods

Cell cultures

Eyes from steers (*Bos taurus*, aged 5–6 months) were enucleated and the corneas removed 3 h after slaughter. These were cut into pieces approximately 5 × 5 mm and prepared by removing the Descemet's membrane and endothelial layer with scissors. The pieces were washed in ethyl alcohol 70% (Carlo Erba Reagenti, Italy) and phosphate-buffered saline (PBS; Sigma, St Louis, MO) in sterile conditions. Epithelial cells were obtained by removing the epithelial layer with a thermolysin solution (50 μg/ml in PBS containing 1 mM $CaCl_2$, pH 7.8) (Sigma) for 1 h at 4 °C. Thermolysin is a proteolytic enzyme that selectively digests epithelium-matrix adherens junctions, as described by Germain *et al.*[19] and Walzer *et al.*[20] and yields primary cultures of epithelial cells free of keratocytes. The epithelial sheet was then incubated with 0.05% trypsin (Biochrom, Berlin, Germany) and 0.1% EDTA (Sigma) for 10–15 min at 37 °C to obtain a single-cell suspension. To obtain keratocytes, the epithelium-free corneas were next incubated for 18 h at 4 °C in a 0.25% trypsin solution; stroma fragments (~4 mm^2) were then placed in Petri dishes (Becton Dickinson, UK) and cultured with Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS) and an antibiotic–antimycotic solution containing penicillin G sodium salt (100 IU/ml), streptomycin sulphate (100 μg/ml), gentamicin sulphate (0.5 μg/ml) and amphotericin B (0.25 μg/ml) (Sigma). After 8–10 days, keratocytes migrated from the tissue and colonised the Petri dishes.

Epithelial cells were seeded, according to Rheinwald and Green,[21] on a feeder monolayer of keratocytes previously treated with 40 μg/ml Mitomycin C (Sigma) for 2 h, as described by Rheinwald.[22]

Cultures were incubated at 37 °C in a humidified incubator (Forma Scientific, OH) (90% RH) in an atmosphere containing 5% CO_2 with Supplemental Hormonal Epithelial Medium (SHEM; Sigma) enriched with 10% FCS (Biochrom KG), according to Jumblatt and Neufeld.[23]

Corneal epithelial cells cultured on collagen gel

Collagen was extracted from Wistar rat tails according to Ponec *et al.*[24] Protein concentration was determined by the method of Lowry *et al.*[25] The stock solution was adjusted to 3.5 mg/ml at −20 °C. A suspension of 1.5×10^4 cultured keratocytes, passages 2 to 7, was added to the collagen mixture (1.5 ml), poured into 35 mm culture dishes (Becton Dickinson) and polymerised at 37 °C for 15 min. When the gel had contracted, 0.5×10^6/Petri epithelial cells from the feeder layer of primary cultures were seeded. Then the epithelial surface was exposed to the solution.

Morphological analysis

Histochemistry and immunohistochemistry Corneal epithelial cells cultured on collagen gel with keratocytes were fixed with a 10% formalin–neutral buffer solution (Carlo Erba), routinely processed, and embedded in paraffin (Bio-Optica, Milan, Italy). Deparaffinised sections were either stained with Mayer's hemalum (Merck, Darmstadt, Germany) or immunostained with monoclonal anti-epithelial keratin antibody AE5 (ICN Biomedicals, Costa Mesa, CA) and an anti-mouse IgG (Fc-specific) peroxidase conjugate (Sigma).
Ultrastructure Samples were fixed with 2.5% glutaraldehyde (Merck) in 0.2 M cacodylate buffer (Pro-Labo, Paris, France), pH 7.4, postfixed in 1.0% osmium tetroxide (Bio-Optica), dehydrated and embedded in Epon 812 (Bio-Rad Laboratories, Richmond, CA). Thin sections were stained with uranyl acetate and lead citrate (Bio-Optica) and examined under an H 7000 Hitachi transmission electron microscope.

Test substances

Cytotoxicity tests were performed both with conventional submerged cultures and with the three-dimensional corneal model after 24 h of incubation with various concentrations of surfactants: benzalkonium chloride (BAC), sodium dodecylsulphate (SDS), Triton X-100 (TX100) and Tween 20 (Tw20) (Sigma).

MTT assay

Keratocytes (50 000 per well), passages 2 to 7, and epithelial cells (100 000 per well), from the feeder layer of primary cultures, were seeded in 24-well tissue-culture microtitre plates (Becton Dickinson) and treated 48 h after seeding with various surfactant concentrations for 24 h. Three-dimensional corneal models were reconstructed *in vitro* in 35-mm Petri dishes (Becton Dickinson) and, 7 days after seeding, treated with various concentrations of surfactants for 24 h. The medium was then removed and the cultures washed 3 times with PBS (Sigma) to remove the surfactant. The 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay was performed as described by Mosmann.[26] Briefly, the MTT (Sigma) solution (5 mg/ml) was added in a 1:10 ratio to the culture medium and the plates incubated for 4 h; 1 ml of acid propan-2-ol (Sigma) (0.04 M HCL in propan-2-ol) was then added. After a few minutes' shaking, the plates were read on a UV-VIS spectrophotometer (model EL13, Bio-Tek Instruments, Winooski, VT) at a wavelength of 570 nm.

Six concentrations of each surfactant were tested in each experiment. The mean optical density (OD) values were converted into growth percentages; the mean OD values corresponding to the non-treated controls were taken as 100%. The growth percentage for each concentration was plotted against the concentration (μg/ml) of the test substance, and the MTT_{50} value (concentration of test agent reducing OD values by 50%) was determined from the graph. Each concentration was assayed in triplicate.

Results

Cell cultures

Keratocytes Isolated keratocytes were obtained from stromal fragments: after 8–10 days of culture keratocytes migrated from the tissue, adhered to the Petri dishes and began to proliferate. Fig. 1 shows their characteristic shape: cells are lengthened and have many cytoplasmic processes.

Epithelial cells Fig. 2 shows a primary confluent culture of epithelial cells 7 days after seeding on a feeder layer of keratocytes: cells are attached side-to-side, polygonal in shape and homogeneous in size. When the colonies became confluent, keratocytes were replaced by epithelial cell colonies and eliminated by later changes in culture media.

Reconstructed cornea in vitro

Morphological characterisation Fig. 3 shows the stratification of corneal epithelium (E) induced by the three-dimensional model after 7 days of culture. The epithelial basal layer is in close contact with the substrate and is made up of cuboidal cells, while the cells of the superficial layer are flattened and lengthened. Seven days after seeding the whole surface of the gel is epithelialised, although the number of cell layers varies slightly; the typical number ranges from 2 to 7. Structural integrity is maintained for at least 2 weeks, after which the layers begin to decay and the epithelium is no longer vital (data not shown).

Reconstructed epithelial cells adhere to a basal lamina (Fig. 4) like those of *in vivo* cornea; the lamina lucida (LL) and lamina densa (LD) are visible in the figure. Superficial layers of cells of reconstructed epithelium (Fig. 5) are also very similar to those *in vivo*, being attached not side-to-side but connected by a few desmosomes (D).

Immunohistochemical analysis Immunohistochemical analysis was performed on sections of *in vitro* reconstructed cornea. Monoclonal antibody AE5, which specifically reacts with 64 kDa keratin, a differentiation marker of corneal epithelium, was used as primary antibody. Fig. 6 shows that, after 7 days of culture, *in vitro* reconstructed cornea expresses this protein, revealing an advanced stage of epithelial differentiation. As a negative control, a three-dimensional cornea was used 3 days after seeding. The model had only slightly layered, actively proliferating epithelial cells (two or three layers), which were not yet expressing 64 kDa keratin.

Toxicological tests in vitro

Fig. 7 shows dose–response curves in keratocytes, epithelial cells and reconstructed cornea, after 24 h of incubation with surfactants. The sensitivity of the reconstructed cornea model to surfactants is higher than that of keratocyte cultures but similar to that of epithelial cell cultures.

Table 1 shows MTT_{50} values, defined as the concentration of test agent reducing the OD value by 50%, in comparison with control cultures. Every experiment revealed the same cytotoxicity rank order: benzalkonium cholride > Triton X-100 > sodium dodecylsulphate > Tween 20.

Discussion

In this preliminary study, we carried out *in vitro* reconstruction of epithelial and stromal layers of bovine cornea, revealing the presence of the basal lamina with its ultrastructural components: lamina lucida and lamina densa. Epithelial cells were seeded in a gel of collagen fibres and keratocytes.[17,18] The importance of both collagen and keratocytes in the differentiation of epithelial cells, and in the synthesis of the basement membrane, led us to choose these two components to obtain a three-dimensional *in vitro* cornea model. Kawamura and Ichihara[27] demonstrated that an extracellular matrix, especially of collagen, induces proliferation of thyroid follicle cells; Toda and Sugihara[28] used a three-dimensional collagen gel culture to obtain differentiated prostate epithelial cells. As regards keratocytes, Chan and Haschke[29] showed that the presence of keratocytes is essential: in co-cultures, this type of cell produces growth factors and stimulates epithelial cell differentiation. In a previous work Parnigotto *et al.*[30] demonstrated that a keratocyte feeder layer is more effective than other types of feeder layers or conditioning media in stimulating the growth and differentiation of bovine corneal epithelial cells.

Keratocytes also express a keratinocyte growth factor that acts specifically on epithelial cells as a paracrine mediator.[31] This is similar to what happens in epidermal cells, in which an interaction between epithelial cells and stromal fibroblasts take place, as some authors have already shown on human[32] and murine keratinocytes.[33,34] Moreover, according to Goldyne,[35] interactions between keratinocytes and fibroblasts occur by means of prostaglandin E_2 (PGE_2) and interleukin-1 reciprocal exchanges. Conconi *et al.*[36] showed that the autacoid seems to play an important role in the growth of rat keratinocytes, although its effect is probably affected by fibroblasts and their products. Bonhert *et al.*[37] had already stressed the importance of fibroblasts, which

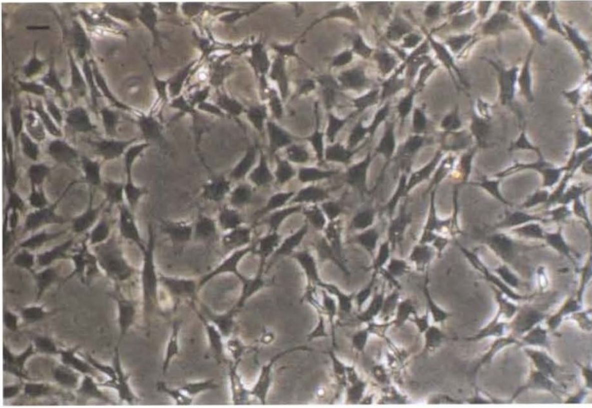

Fig. 1. *Phase-contrast micrograph of corneal bovine keratocytes at third passage. Scale bar represents 20 μm.*

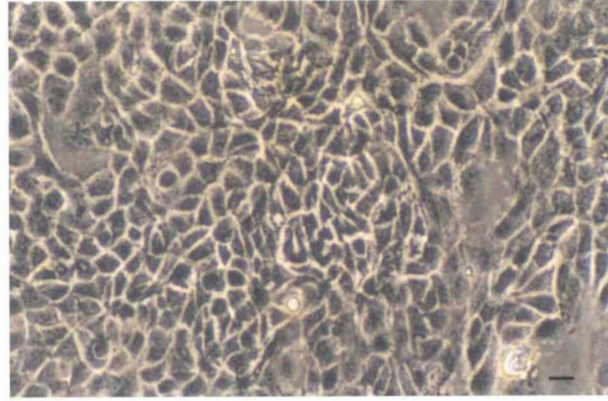

Fig. 2. *Phase-contrast micrograph of corneal bovine epithelium grown for 7 days with a keratocyte feeder layer. Scale bar represents 10 μm.*

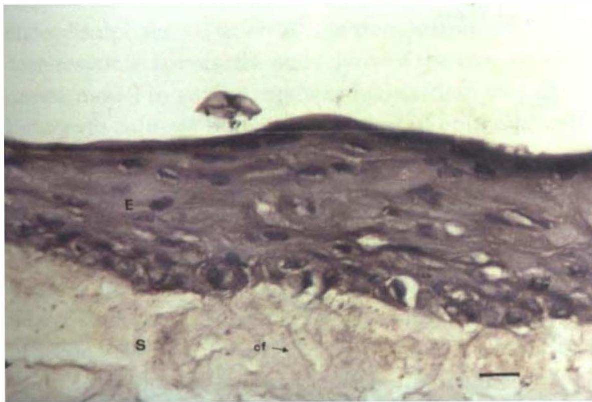

Fig. 3. *Vertical paraffin section (5 μm) of cornea reconstructed* in vitro *grown for 7 days (×1000). Mayer's hemalum solution stain. S, substratum; cf, collagen fibres; E, multilayered epithelium. Scale bar represents 10 μm.*

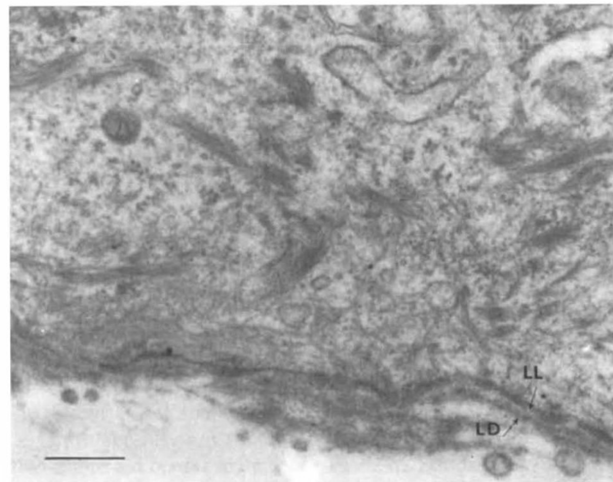

Fig. 4. *Transmission electron micrograph of cornea reconstructed* in vitro. *Cells synthesise lamina lucida (LL) and lamina densa (LD) on collagen gel. Scale bar represents 1 μm.*

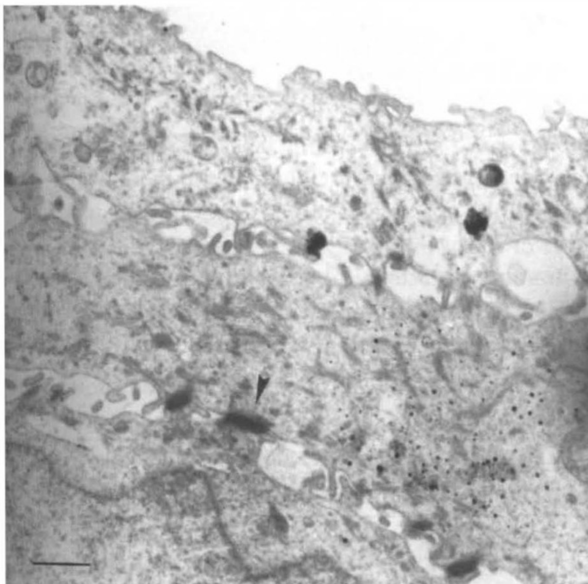

Fig. 5. *Transmission electron micrograph of a thin section of cornea reconstructed* in vitro *after 7 days of culture. Superficial layers are less compact and contain a few desmosomes (arrowhead). Scale bar represents 1 μm.*

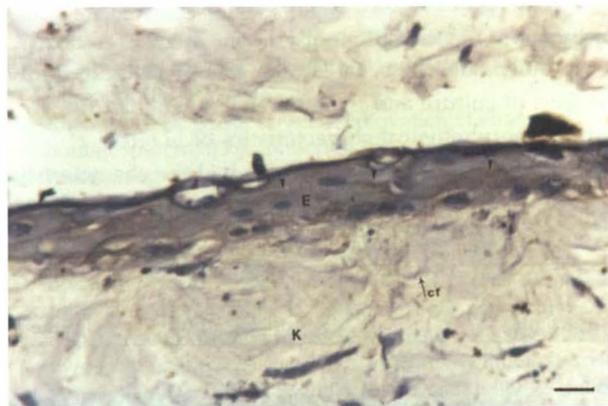

Fig. 6. *Immunohistochemical staining of cornea reconstructed* in vitro *after 7 days of culture. Arrowheads indicate zones positive to AE5 monoclonal antibody. E, epithelium; K, keratocytes; cf, collagen fibres. Scale bar represents 10 μm.*

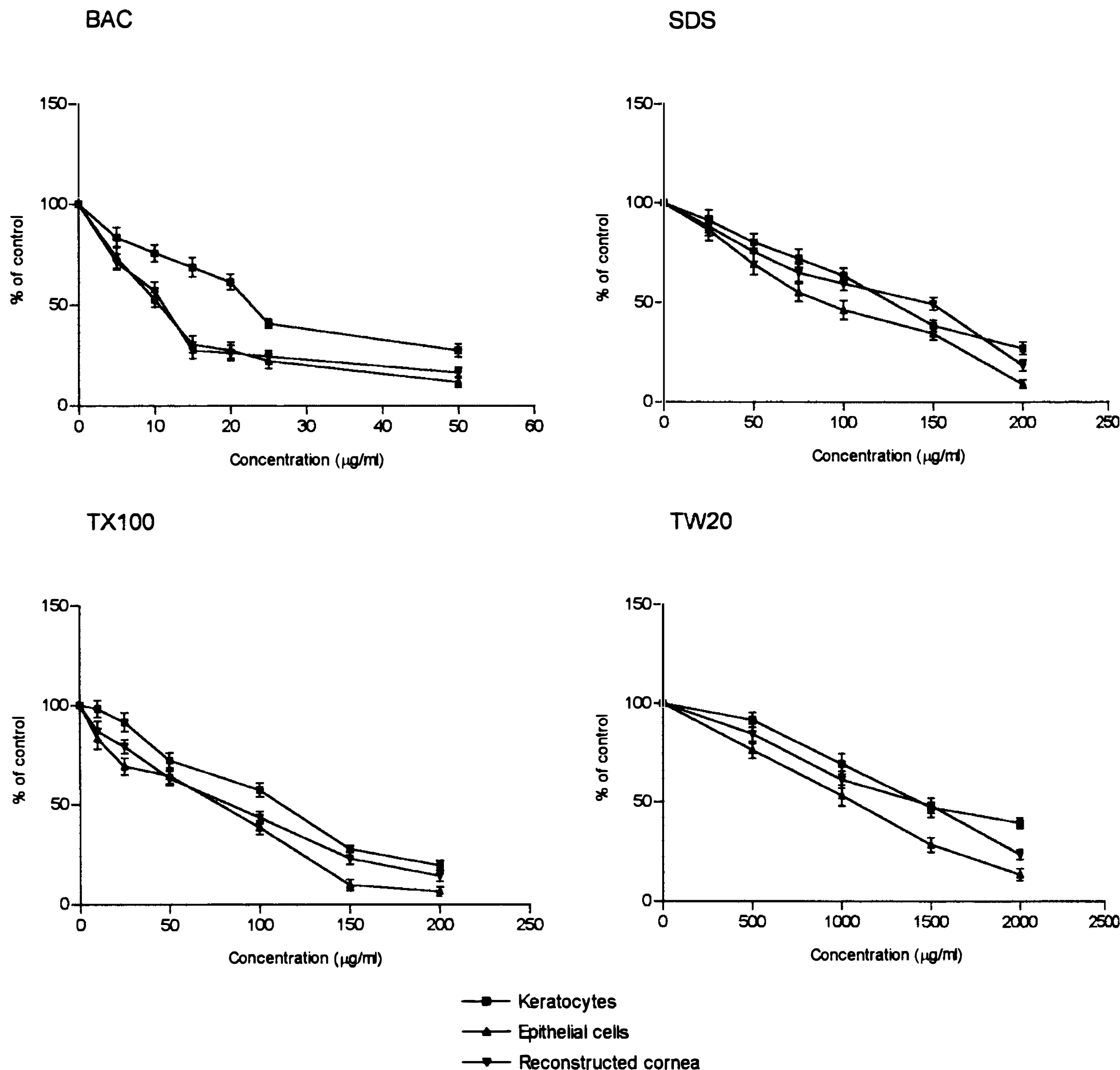

Fig. 7. *Dose–response curves for 24 h exposures of keratocytes, epithelial cells and reconstructed cornea to benzalkonium chloride (BAC), Triton X-100 (TX100), sodium dodecylsulphate (SDS) and Tween 20 (TW20). Vertical bars represent the SD.*

synthesise some components of the basal membrane, stimulate epithelial cells to grow, and synthesise elements of the membrane itself.

In our model the basal lamina was present after only 10 days of culture and the epithelial layers had the typical morphological characteristics of *in vivo* epithelium: (1) the basal layer cells had the characteristic cuboid shape of proliferating cells; (2) suprabasal layer cells were lengthened horizontally; (3) there were intercellular spaces and desmosomes in the superficial layer of cells.

Immunohistochemical analysis with AE5 also demonstrated an advanced stage of epithelial differentiation. According to Schermer *et al.*,[38] AE5 – a

Table 1. *Comparative toxicity of surfactants with cultures of keratocytes, epithelial cells and* in vitro *reconstructed cornea*

	MTT_{50}[a]			
Surfactants	Keratocyte cultures	Epithelial cell cultures	Reconstructed cornea	Ocular irritancy[b]
Benzalkonium chloride	22.89	11.00	12.37	Extreme
Triton X-100	110.60	76.33	82.67	Severe
Sodium dodecylsulphate	126.00	88.75	145.00	Severe–moderate
Tween 20	1425.00	1052.33	1425.00	Mild

Results are expressed as mean values of single MTT assays.
[a]MTT_{50} concentration of surfactant (μg/ml) inhibiting 50% of control MTT conversion.
[b]Kennah *et al.*[41] was used as the source for *in vivo* data.

monoclonal antibody specific for 64 kDa corneal keratin – is a differentiation marker of the cornea, since cultured basal cells express the 50/58 kDa keratin pair, exponentially growing cells also synthesise the 48/56 kDa keratin pair, and only post-confluent, stratified cells express the 55/64 kDa keratin pair. Morphological integrity of the three-dimensional model was maintained for about 2 weeks, after which gradual decay of reconstructed tissue was observed (data not shown).

Lastly, preliminary cytotoxicity studies were carried out on a small number of surfactants, representative of the various classes of such substances and of the various degrees of irritation described in the Draize eye test, whose mechanism of toxicity is well known.[39,40] The MTT mitochondrial reduction cytotoxicity test highlighted the different cytotoxicity potentials of the four surfactants tested, following the rank order: benzalkonium chloride > Triton X-100 > sodium dodecylsulphate > Tween 20. As demonstrated by the dose-response curves, the sensitivity of the reconstructed cornea model to surfactants was higher than that of keratocyte cultures but similar to that of epithelial cell cultures. These *in vitro* data correlate with the Draize test results reported by Kennah *et al.*[41] and suggest that submerged cultures of both epithelial and corneal keratocytes and three-dimensional cultures may be used to assess the cytotoxicity potential of chemicals, although a large number of substances with various mechanisms of action will have to be tested to evaluate the validity of this model. Moreover, since corneal epithelial and keratocyte cell cultures are cheaper, quicker and have a shorter life-span than the three-dimensional model, we believe that they are more suitable for preliminary short-term toxicity screening. The latter model, being more complex and preserving its structural integrity for a long period, may be used to study the long-term effects of exposure to toxic substances.

We believe that the three-dimensional cornea model may be developed further, by means of exposure to the air-fluid interface, so that the cytotoxicity of insoluble substances, the application of which is difficult in *in vitro* experimentation, may be properly assessed.

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