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(54) Title: CORNEAL ORGAN CULTURE SYSTEM FOR CHEMICAL TOXICITY TESTS OF CONSUMER PRODUCTS

(57) Abstract

Mammalian eyes, corneas, or cornea-containing portions thereof are cultured under defined conditions as an organ culture system. The system of the invention, compared to cell culture, far more closely resembles an *in vivo* testing system in terms of maintaining the corneal architecture and natural interaction between epithelial cells and other cells residing in the ocular surface. The organ culture system of the invention is sensitive, efficient, economical and reliable for evaluating the potential of a chemical or formulation to cause eye irritation or injury and can be used in the safety tests of consumer products.

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TITLE OF THE INVENTION

5 CORNEAL ORGAN CULTURE SYSTEM FOR CHEMICAL TOXICITY TESTS OF CONSUMER PRODUCTS

CROSS REFERENCE TO RELATED APPLICATIONS

10 This application claims priority under 35 U.S.C. §119(e) of provisional patent application 60/080,492, filed April 2, 1998, the disclosure of which is hereby incorporated by reference herein.

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STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

--None--

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BACKGROUND OF THE INVENTION

There is a great demand for minimizing the use of animals in chemical toxicity tests and for developing replacements for such standards as the Draize eye test, a 25 standard procedure using live rabbits for identifying potential human eye irritants. To date, much of the effort to satisfy this demand has revolved around the development of cell culture systems. However, data obtained from testing substances on cultured cells are often not relevant to their 30 actual effects in humans, since cells in intact tissue may respond differently from those in culture. Furthermore, there are no commonly accepted parameters or end-points for

determining the effects of a specific irritant in existing cell culture systems.

SUMMARY OF THE INVENTION

In the system of the invention described herein, a mammalian whole eye or a cornea-containing portion thereof, or, preferably, a cornea or portion thereof, is cultured under defined conditions as an organ culture system for chemical toxicity tests, e.g., of consumer products. Corneas or corneal tissue may be advantageously cultured together with a limbal-conjunctival ring. Most preferably, bovine or porcine corneas are used (or corneas from any other mammal wherein sufficient test material is available). When a compound to be tested is applied onto the surface of the cultured mammalian eye, cornea, or portion thereof, the system of the invention enables the toxic effects of the compound to be evaluated. The *ex vivo* organ culture system of the invention, compared to cell culture, far more closely resembles an *in vivo* testing system in terms of maintaining the corneal architecture and natural interaction between the epithelial cells and other cells residing in the ocular surface, including keratocytes in the stroma and limbal epithelial cells. Another key advantage of this invention is that end-points or reference standards of specific tests can be precisely characterized. Examples include such tests as determining the activation of well-known, stress-responsive transcription factors in response to chemical stimuli, or assessing corneal structural and functional changes caused by chemical exposure. Other parameters that can be measured include changes in, e.g., corneal transparency or opacity, transepithelial permeability and

electrical resistance, leakage of cellular enzymes into culture media, and epithelial wound-healing. The system of the invention is sensitive, efficient, economical and reliable for evaluating the potential of a substance, e.g., a chemical or a formulation, to cause eye irritation or injury, and can be used in the safety tests of consumer products. Kits for using the system are also provided. Such kits include, e.g., quantitation controls such as bovine or porcine eyes in culture and ready for testing.

BRIEF DESCRIPTION OF THE DRAWINGS

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims, taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a diagram of the corneal organ culture model system for chemical toxicity testing according to the method of the invention;

Fig. 2 depicts the corneal organ culture system at different stages of the method (2A-2E);

Fig. 3 depicts an electrophoresis mobility shift assay (EMSA) for NF-κB activation in organ-cultured, corneal epithelial cells exposed for 1 hour to different chemical compounds; and

Fig. 4 depicts an EMSA for AP-1 activation in organ-cultured, corneal epithelial cells exposed for 1 hour to different chemical compounds.

DETAILED DESCRIPTION OF THE INVENTION

The *ex vivo* organ culture system of the invention, for the sensitive and specific assay of chemically induced activation of stress-responsive transcription factors or other changes in a mammalian eye or cornea, may be used to replace the Draize eye test, a standard procedure using live rabbits for identifying potential human eye irritants. Specific details of the *ex vivo* system are illustrated in Figs. 1 and 2. This new system is based on organ culture of, e.g., bovine or porcine corneas, whole eyes, or a corneal tissue-containing portion thereof, and allows the effects of tested substances to be evaluated. Exemplary substances include chemical compounds or mixtures of compounds (hereinafter commonly referred to as "chemicals"). They may be naturally derived, extracted, or synthesized. Typical evaluation methods include detecting changes in the electrophoresis mobility shift assay determination of NF-κB or AP-1 activation. Changes in other parameters can be measured as well, e.g., at the ultrastructural and physiological level. The system of the invention serves as an effective tool for the prediction of ocular irritations caused by diverse classes of chemicals.

Eye irritation that results from chemical exposure affects the anterior ocular surface, primarily the cornea. Approximately 75% of the historically relevant, Draize rabbit eye irritation test scores were derived directly from corneal effects. Thus, a logical component to the *ex vivo* assessment of chemically induced damage to the ocular surface is the evaluation of corneal effects. There are three cell types in the cornea: the stratified epithelium covering the anterior surface, the keratocytes interspersed in the stroma, and the single cell layer of the endothelium.

The first line of cellular defense of the cornea against eye irritants is the impermeable barrier of the epithelium, which is vulnerable to chemical insults. Thus, measurement of the cellular response of the epithelium to irritants provides a reliable parameter for testing chemical toxicity. Severe irritation causing complete epithelial cell loss will result in anterior stromal damage, including increased thickening of the stroma and apoptosis of keratocytes (Jester, Petroll et al., 1998; Jester, Li et al., 1998).

Organ culture serves as an appropriate model for chemical safety tests, the corneal architecture being well maintained via the use of air interface organ culture techniques and the development of, e.g., agar, agarose, agar-collagen or agarose-collagen gel for filling the endothelial corneal concavity. Several parameters have been determined as indicating corneal integrity. These parameters can be separately or simultaneously measured in the method of the invention to determine the stress-related responses of the *ex vivo* corneal model system to diverse classes of chemicals.

Cellular responses to stress and toxicants are usually the result of transcriptional activation, leading to alterations in gene expression. The protooncogens c-*fos* and *c-jun* are members of the immediate early response class, whose expression is rapidly and transiently induced following a wide variety of stimuli. The transcription factor AP-1 (activation protein-1) is formed by either *jun-jun* homodimers or *jun-fos* heterodimers. Increase of AP-1 activity, or expression of c-*fos* or c-*jun* genes, in response to chemical exposure in a variety of cells has been reported. The transcription factor NF-κB (nuclear factor-κB) acts as a central, coordinating regulator. Cells

treated with a variety of chemical agents elicit NF-κB binding activity within minutes. The activation of these two transcription factors in the corneal organ culture system of the invention, in response to toxicants can be measured with a standard electrophoresis mobility shift assay (EMSA). A dosage response curve for each chemical tested can be obtained from the measurement. Complementary assays determining NF-κB activation (by nuclear staining of activated forms) and AP-1 expression (by PCR determination of c-jun and c-fos mRNA levels) can also be assessed.

In addition to determining the appearance of transcription factors (e.g., NF-κB, AP-1, or p53), the activation of other kinds of genes may also be the subject of chemical safety testing: heat shock proteins such as Heat shock protein 70, cytokines such as interleukin 1 - alpha and interleukin 8, and enzymes such as cyclooxygenase 2, a key enzyme with prostaglandin synthase activity. The activation of these genes can be determined by measuring their promoter activity by a reporter gene,(e.g. luciferase, β-galactosidase, green fluorescence protein), or by measuring their enzymatic activity. Since each distinct group of genes may be involved in a unique response process, the measurement of gene expression and/or activation from different groups should be considered as multiple, but intrinsically related, end-points.

Organ culture also permits assessing other parameters, e.g., the effects of substances such as chemicals on the ocular surface. Some parameters may include changes in corneal morphology, transepithelial permeability and transepithelial electrical resistance, and corneal transparency, as well as epithelial wound healing and leakage of cellular enzymes (e.g., transketolase) into the

culture medium. These parameters comprise mechanistically relevant parameters for the evaluation of toxicity of consumer products to the ocular surface. For example, anatomic abnormality can be induced by chemicals into the corneal culture system of the invention and detected by histological examination of cryostat section of the sampled cornea. In another example, chemicals can cause programmed cell death (apoptosis) of keratocytes in organ cultures. Individual apoptotic cells in corneal cryostat sections can be detected by the TUNEL assay (Moore et al., 1998). The percentage of apoptotic keratocytes would directly correlate to the toxicity of a chemical. In addition, toxic chemicals may cause cloudiness or opacity in the cornea, which is normally transparent. (See Fig. 2E, a color photo showing a normal, white cornea and an opaque, cream-colored cornea.) The transparency of the test corneas in organ culture can be measured and directly related to the toxicity of a chemical. This direct testing of corneal opacity is especially facilitated by the use of a phenol red-free medium and/or pure agarose instead of agar in the invention, which allows better detection of even faint color changes in the cornea that reflect increased opacity (e.g., from white to cream or a yellow hue).

Transepithelial permeability and transepithelial electrical resistance are two physiological relevant parameters for evaluating the barrier function of the corneal epithelium, i.e., corneal health. These properties are likely to be affected by damage caused by chemicals added to the organ culture system during testing. Transepithelial permeability can be assessed by measuring the retention of fluorescein (which usually is not permeable through corneal epithelium) (Tchao 1988), and

transepithelial electrical resistance can be measured using an Epithelial Voltohmmeter (Kruszewski et al., 1997; Ward et al., 1997; Gautheron et al., 1992).

Chemical safety tests require that the relevant products or their ingredients be evaluated over a large range of concentrations. The use of bovine or porcine eyes or cornea in an organ culture test system offers several advantages for *ex vivo* chemical tests. For example, bovine and porcine eyes are the by-products of the meat industry; no live animal would be sacrificed for the tests. These eyes are readily available resources worldwide; therefore, large-scale tests are possible. In addition, both bovine and porcine eyes are very similar to those of humans, anatomically and biochemically. Thus, the data obtained by using these eyes are relevant to the human response to chemicals. Furthermore, human eyes from eye banks might be used in a similar fashion for some key tests. Finally, bovine and porcine eyes both provide enough corneal epithelial cells for easy use in the required tests outlined in this application.

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These examples are not intended in any way otherwise to limit the scope of the disclosure.

EXAMPLE I

Preparation of mammalian corneas or whole eyes for organ culture and exposure to test agents

Bovine or porcine eyes are obtained from the abattoir within several hours (e.g. five) of slaughter. To prevent damage to the corneal epithelium, the eyes are placed individually onto a container similar to egg cartons with 5 the epithelial-side up, and they are transported to the laboratory on ice in a moisture chamber (or cooler). For long-term culture, (i.e. up to 3 weeks) the ocular surface should be disinfected using 0.1% povidine-iodine solution. It is optional when the eye or cornea is tested shortly 10 after preparation.

A. Culture of corneas

Corneal-scleral rims, with approximately 5 mm of the limbal conjunctiva present, are excised and rinsed in sterilized phosphate buffered saline as described in Foreman 15 et al., 1996. (See Fig. 2A.)

The excised corneas are placed epithelial-side down into a sterile "cup" containing minimal essential medium (MEM) to prevent drying of the epithelium. The cups, made with silicone rubber, are designed to allow the scleral rims 20 to rest on the cup edges without damage to the corneal epithelium. (See Figs. 2B and 2C.) The endothelial corneal concavity is then filled with MEM containing 0.5-1% agar or, preferably, agarose, with or without 1 mg/ml of rat tail tendon collagen maintained at 37 °C. This mixture is 25 allowed to gel. Corneas are then inverted and then transferred to a 60 mm dish, as illustrated in Fig. 1 and Fig. 2D. Medium, with or without test substance(s), is added dropwise to the surface of the corneal epithelium until the limbal conjunctiva are covered. The dish then is 30 placed in a humidified 5% CO_2 incubator. To moisten the epithelium, 100 µl of the same medium is added dropwise to

the surface of the corneal epithelium every 12 hr during culture and every 30 minutes during chemical testings. Corneas are cultured in the test medium for typically 0.5-6 hours, preferably about 1 hour.

5 Alternatively, a portion of the cornea immersed in a medium containing a test substance, e.g., a chemical, for approximately 0.5-6 hours, preferably about 1 hour, is another way the corneal culture system of the invention may be used to test toxicity of a substance.

10

B. Culture of whole eyes

The whole eye may also be used for corneal organ culture. In this case, the posterior part of the eye is immersed in a culture medium that covers the eye up to the
15 limbal conjunctiva region, leaving the corneal epithelium exposed to the air. The test chemicals are added to the medium and applied to the surface of the epithelium and incubated for a time within 0.5-6 hours, pref. about 1 hour.

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EXAMPLE II

Preparation of corneal epithelial extract for electrophoresis mobility shift assays

25 At the end of the desired incubation time, preferably 1 hour, the corneal surface is washed with PBS, and the epithelial cells are removed from the corneas and transferred to a 1.5-ml tube using a scalpel blade. Cells are resuspended in a buffer containing 20 mM Hepes-KOH (pH
30 7.0), 1 mM DTT, 1 mM EDTA, 200 mM KCl, 20% glycerol, 0.1 mM phenylmethyl-sulfonyl fluoride (PMSF) and 0.1% NP40 by pipetting the cells up and down. The cell suspensions are

left on ice for about one hour, with occasional mixing, and then centrifuged for 10 min at 4 °C. The supernatants are transferred to new tubes and can be used, e.g., for protein determination and for the electrophoresis mobility shift assay. They can also be stored in liquid N_2 for future use.

EXAMPLE III

Electrophoresis mobility shift assay

For detection of NF-κB and AP-1 DNA binding activities, a 20 μl volume containing 3-4 μg of epithelial extract is mixed with 4 μl of 5 x binding buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 1 mM DDT, 2.5 mM $MgCl_2$, 20% Ficoll), 2 μg of poly(dI-dC) as nonspecific competitor DNA, 2 μg of bovine serum albumin, and 10,000-15,000 cpm Cerenkov radiation of the labeled oligonucleotide. After a 30-min binding reaction at room temperature, samples are loaded on a 4% non-denaturing polyacrylamide gel and run in 0.5 x TBE buffer (89 mM Tris, 89 mM boric acid, 1 mM EDTA), pH 8.3.

The sequences of the oligonucleotides used to detect the DNA binding activity are:

for NF-κB: 5'-AGCTTCAGAGGGGATTTCCGAGAGG-3'; and

for AP-1: 5'-CGCTTGATGAGTGAGCCGGAA-3'.

The oligonucleotides are annealed with their complementary strands and labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP. The labeled probes are separated from free nucleotides by ethanol precipitation.

This is a standard technique used in research and industrial laboratories investigating DNA-binding activity.

Since the labeled oligonucleotides are in excess, the amount of oligonucleotides with shifted mobility (shifted to the top part of a gel) is dependent on the concentration of activated transcription factors. The activation of NF-κB or AP-1 in each test sample can be quantitated by PhosphoImager analysis and presented as activity relative to the control, untreated corneal epithelium.

The data obtained by the assays described above should quantitatively reflect the changes in the corneas caused the chemical exposure. Although a detected alteration may not be considered as a direct cause(s) of "pain" or "irritation," any identified quantitative correlation could be used as an *ex vivo* biochemical endpoint/marker for predicting ocular irritation.

A. EMSA for NF-κB activation

Fig. 3 shows an EMSA for NF-κB activation in organ-cultured epithelial cells in response to various chemicals, in accordance with the invention. The excised corneas in organ culture were treated with benzalkonium chloride (BAK), isopropanol (ISO), or H_2O_2, or left untreated (in medium as control C) and incubated for about 1 hour at 37°C and 5% CO_2. The corneal epithelial cells were then collectd by scraping. NF-κB activation was determined using radiolabeled oligonucleotides encompassing the consensus motifs of NF-κB. The gel binds in Fig. 3 indicate NF-κB binding. The relative intensity of each band, reported as a numeric value at the bottom of Fig. 3, was measured with a PhosphoImager. NFκB DNA-binding activity was significantly increased in response to H_2O_2 or decreased in response to BAK or isopropanol, and the observed changes were concentration-dependent.

B. EMSA for AP-1 Activation

Fig. 4 depicts an EMSA for AP-1 activation in organ-cultured corneal epithelial cells in response to various chemicals, in accordance with the invention. The excised corneas in organ culture were treated with benzalkonium chloride (BAK), isopropanol (ISP), or H_2O_2, or left untreated (in medium as control C), and incubated for approximately 1 hour at 37°C and 5% CO_2. The epithelial cells were collected by scraping. AP-1 activation was determined by electrophoresis mobility shift assay using radiolabeled oligonucleotides encompassing the consensus motifs of AP-1. The gel bands in Fig. 4 indicate AP-1 binding. The relative intensity of each AP-1 band reported as a numeric value at the bottom of Fig. 4, was measured with a PhosphoImager. AP-1 DNA-binding activity was significantly increased in response to H_2O_2 or decreased in response to BAK or isopropanol, and the observed changes were concentration-dependent.

EXAMPLE IV

Leakage of cellular enzymes

Extracellular leakage of metabolic enzymes into culture medium has been used as an indicator of cytotoxicity (Grant et al, 1992; Grant et al, 1996). We have reported that mammalian corneal epithelium contains a high concentration of transketolase ("TKT"), approximately 10% of total cellular proteins (Sax et al, 1996; Guo et al, 1997). To evaluate cell membrane and cell junctions integrity, a toxicity test based on TKT leakage measurements may be used. Since TKT is located in the wing and basal layers but not the apical layer (Guo et al, 1997), extracellular presence

of this enzyme in the culture medium indicates probable damage to the plasma membrane, cell junctional complexes and intercellular adhesions of the epithelial cells. TKT leakage is measured as follows. Cultured bovine corneas are exposed to test substances for 1 hr and the culture media collected. TKT levels in the collected media are assayed by colorimetric determination of 7-sedoheptulose phosphate production (Sax et al, 1997; Takevchi, et al, 1984). A dose- dependent curve of TKT leakage for each test chemical can be derived, allowing the evaluation of both eye irritation and recovery of the epithelium.

EXAMPLE V

Effects of irritants on epithelial wound healing

Normally, corneal epithelium heals a wound rapidly. Damage caused by irritants could inhibit or even prevent wound closure. In cultured epithelial cells, exposure to test chemicals prolonged healing times of a scraping wound (Jung et al, 1993). The cytotoxicity of test chemicals is evaluated by assessing their effects on the healing of an epithelial debridement wound. The epithelial debridement wound can be made with a 5-mm trephine as described in Guo et al, 1998 or Yu et al, 1998. In normal bovine organ culture, this wound is re-covered by epithelium within 36 hours (Yu et al, unpublished results). The effects of test chemicals may be assessed under two different sets of conditions: (i) corneas in culture are pretreated with a substance of chemical in MEM, followed by epithelial wounding and wound healing in the absence of the test chemical; or (ii) a wound of normal epithelium is allowed to heal in the presence of the test chemical. The first system

may be used to indetify acute toxicity caused by high-irritancy chemicals or by high concentrations. The second system would be more useful for identifying prolonged effects of low-dose or mildly irritating chemicals. The wounded corneas are allowed to heal in culture with no chemical treatment (37°C in a humidified 5% CO_2 incubator), as the control. Any remaining corneal defect after a suitable incubation time (e.g. 32 hr) are stained with Richardson's stain and the corneas photographed. The diameter of the remaining defect can be measured, and the size of the uncovered area can then be calculated, yielding the rate of wound healing. From this, one can determine the concentration of test substance that permit wound closure (permissive, or practically non-irritating chemical), inhibit wound closure (inhibitory, or minimal or mild irritant), or prevent wound closure (cytotoxic, or severe to extreme irritant). This approach allows chemicals and concentrations to be ranked according to their toxicity, and these rankings can be compared to those published in the literature for *both in vitro* and *in vivo* tests, and statistical correlations can be established. This sensitive, functional test might become an important endpoint for *our ex vivo* system in the evaluation of the irritancy potential of consumer products.

USE

The corneal organ culture system of the invention can permit the reduction or elimination of the use of animals for safety tests and allow for easy manipulation of test parameters under well-defined conditions, in a similar manner to cell culture. The *ex vivo* organ culture and testing system of the invention, however, is much more physiologically relevant to the human condition than conventional cell cultures, and the data obtained are much more reliable in terms of predictability for human *in vivo* response. The end-points permitted by *ex vivo* tests, as described here, range from cellular response to ultrastructure alternations. Combination of these parameters should give realistic assessments of the toxicity of a chemical to the eye (eye irritation).

The assays described are commonly used and easily standardized. A set of internal controls can be established, allowing quantitation and elimination of differences from different experiments. Many different products, such as consumer products, chemicals, formulations, solvents, powder and dusts, can be tested for eye irritation with the *ex vivo* system of the invention. Therefore, this system has applications in cosmetic, pharmaceutical, chemical, biotechnology, food and environmental industries, as well as other industries.

While the present invention has been described in conjunction with a preferred embodiment, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and other alterations to the compositions and methods set forth herein. It is therefore intended that the

protection granted by Letters Patent hereon be limited only by the definitions contained in the appended claims and equivalents thereof.

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REFERENCES

Foreman, D.M., S. Pancholi, J. Jarvis-Evans, D. McLeod, and
M.E. Boulton. 1996. A simple organ culture model for
10 assessing the effects of growth factors on corneal re-epithelialization. Exp Eye Res. 62:555-564.

Fried, M.G. 1989. Measurement of protein-DNA interaction
parameters by electrophoresis mobility shift assay.
15 Electrophoresis 10:366-376.

Gautheron, P., M. Dukic, D. Alix, and J.F. Sina. 1992. Bovine corneal opacity and permeability test: an in vitro assay of ocular irritancy. Fundam Appl Toxicol. 18:442-449.

Guo, J., C.M. Sax, J. Piatigorsky, and F.X. Xu. 1997.
20 Heterogeneous expression of transketolase in ocular tissues. Curr. Eye Res. 16:467-474.

Guo, J., G. Thinakaran, Y. Guo, S.S. Sisodia, and F.X. Yu.
1998. A role for amyloid precursor-like protein 2 in corneal
25 epithelial wound healing. Invest Ophthalmol Vis Sci. 39:292-300.

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

Grant, R.L., and D. Acosta. 1996. Prolonged adverse effects
of benzalkonium chloride and sodium dodecyl sulfate in a primary culture system of rabbit corneal epithelial cells. Fundam Appl Toxicol. 33:71-82.

Jester, J.V., W.M. Petroll, J. Bean, R.D. Parker, G.J. Carr, H.D. Cavanagh, and J.K. Maurer. 1998. Area and depth of
surfactant-induced corneal injury predicts extent of subsequent ocular responses. Invest Ophthalmol Vis Sci. 39:2610-2625.

Jester, J.V., H.F. Li, W.M. Petroll, R.D. Parker, H.D. Cavanagh, G.J. Carr, B. Smith, and J.K. Maurer. 1998. Area and depth of surfactant-induced corneal injury correlates with cell death. Invest. Ophthalmol Vis Sci. 39:922-936.

Jung, E.H., K.F. Sheu, and J.P. Blass. 1993. An enzymatic
and immunochemical analysis of transketolase in fibroblasts from
Wernicke-Korsakoff syndrome. Journal of the Neurological Sciences.
114:123-127.

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

Moore, A., C. Donahue, K. Bauer, and J. Mather. 1998. Simultaneous measurement of cell cycle and apoptotic cell death. Methods in Cell Biology. 57:265-278.

Sax, C.M., C. Salamon, W.T. Kays, J. Guo, F.X. Yu, R.A. Cuthbertson, and J. Piatigorsky. 1996. Transketolase is a major protein in the mouse cornea. J Biol Chem. 271:33568-33574.

5 Takeuchi, T., K. Nishino, and Y. Itokawa. 1984. Improved determination of transketolase activity in erythrocytes. Clin Chem. 30:658-661.

10 Tchao, R. 1988. Transepithelial permeability of fluorescein in vitro as an assay to determine eye irritants. In Progress, in In Vitro Toxicology, Alternative Methods in Toxicology. Vol. 6. A.M. Goldberg, editor. Mary Ann Liebert, New York. 271-283.

15 Ward, S.L., T.L. Walker, and S.D. Dimitrijevich. 1997. Evaluation of chemically induced toxicity using in vitro model of human corneal epithelium. Toxicol. In Vitro. 11:121-139.

Yu, F.X., J. Guo, and Q. Zhang. 1998. Expression and 20 distribution of adhesion molecule CD44 in healing corneal epithelia. Invest. Ophthalmol Vis Sci. 39:710-717.

CLAIMS

What is claimed is:

1. A method for testing the toxicity of a chemical, said method comprising the steps of:

providing a member from the group consisting of a whole mammalian eye, a cornea-containing portion of a mammalian eye, a mammalian cornea, and a portion of a mammalian cornea, in an *ex vivo* organ culture system;

culturing said member under defined conditions;

applying a composition comprising a compound to be tested to a surface of said cultured member;

incubating said cultured member in the presence of said compound to be tested for a defined period of time;

determining the experimental value of a test parameter in said incubated cultured member; and

comparing said determined experimental value for said compound to be tested to a standard value for said test parameter in the absence of said compound to be tested in said organ culture system.

2. The method of claim 1, wherein said member in said organ culture system is of bovine or porcine origin.

3. The method of claim 1, wherein, in said culturing step, said defined conditions comprise culturing said mammalian cornea along with a limbal-conjunctival ring attached to said mammalian cornea.

4. The method of claim 1, wherein said determining step further comprises, following said incubation period, removing a sample of cells from said incubated cultured

member; and determining the experimental value of said test parameter using said sample of cells.

5. The method of claim 4, wherein said test parameter is the extent of activation of a stress-responsive transcription factor in said sample of cells from said incubated cultured member.

6. The method of claim 5, wherein said stress-responsive transcription factor is NF-κB, AP-1, or p53.

7. The method of claim 5, wherein the extent of activation of said stress-responsive transcription factor is determined in an electrophoresis mobility shift assay.

8. The method of claim 4, wherein said test parameter is the extent of activation of an enzyme, a heat shock protein or a cytokine.

9. The method of claim 1, wherein said test parameter in said determining step is the extent of a structural or a functional change in corneal morphology in said incubated cultured member.

10. The method of claim 9, wherein said change in corneal morphology is selected from the group consisting of: an increase in corneal opacity; a change in transepithelial permeability; a change in transepithelial electrical resistance; and a change in epithelial wound healing rate.

11. The method of claim 4, wherein said test parameter is the extent of extracellular leakage of a cellular enzyme from said incubated cultured member.

5 12. The method of claim 11, wherein said cellular enzyme is transketolase.

13. The method of claim 1, wherein, in said determining step, the experimental value of each of a plurality of test 10 parameters is determined and, wherein further, in said comparing step, for each of said plurality of test parameters, said determined experimental value for said compound to be tested is compared to a standard value for said test parameter in the absence of said compound to be 15 tested.

14. The method of claim 1, wherein said method is used to test the toxicity of a consumer product, a chemical, a formulation, a solvent, a powder, or a dust.

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15. A kit comprising components for carrying out a method for testing the toxicity of a chemical, said method comprising the steps of providing a member from the group consisting of a whole mammalian eye, a cornea-containing 25 portion of a mammalian eye, a mammalian cornea, and a portion of a mammalian cornea, in an *ex vivo* organ culture system; culturing said member under defined conditions; applying a composition comprising a compound to be tested to a surface of said cultured member; incubating said cultured 30 member in the presence of said compound to be tested for a defined period of time; determining the experimental value of a test parameter in said incubated cultured member; and

comparing said determined experimental value for said compound to be tested to a standard value for said test parameter in the absence of said compound to be tested in said organ culture system,

5 said kit components comprising said member in an organ culture system.

16. The kit of claim 15, wherein said kit further comprises instructions for carrying out said method.

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17. The kit of claim 16, wherein said instructions comprise instructions for determining the experimental value of a test parameter.

15 18. The kit of claim 17, wherein said instructions further comprise information giving a standard value for said test parameter in the absence of said compound to be tested.

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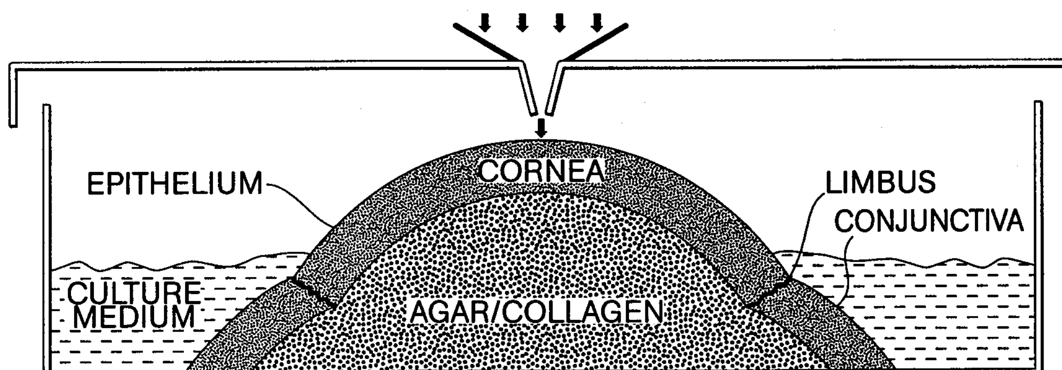

Fig. 1

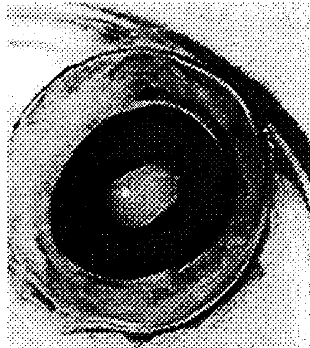

Fig. 2A

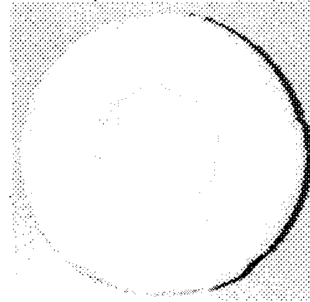

Fig. 2B

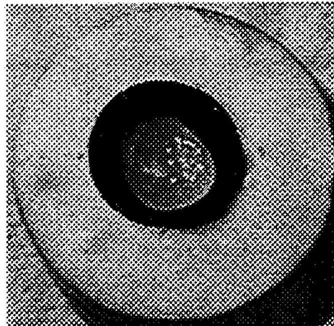

Fig. 2C

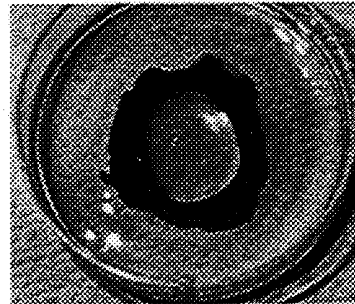

Fig. 2D

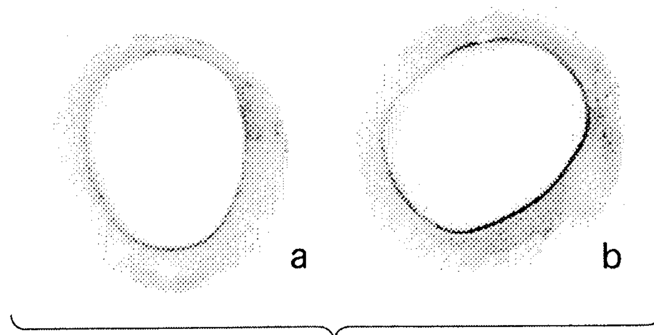

Fig. 2E

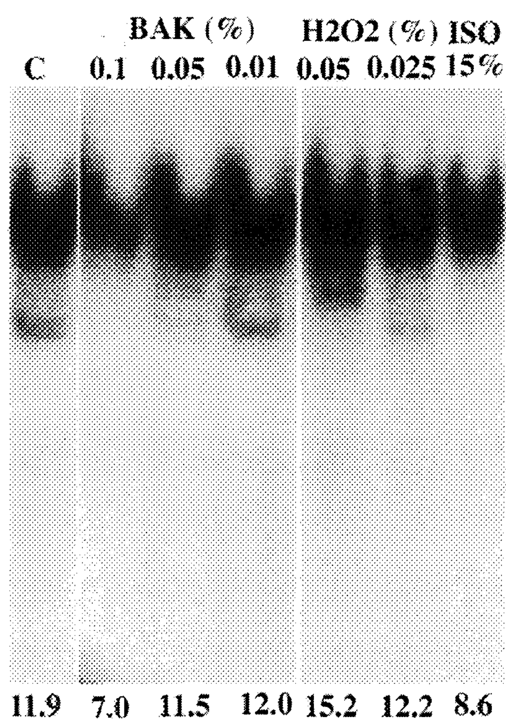

Fig. 3

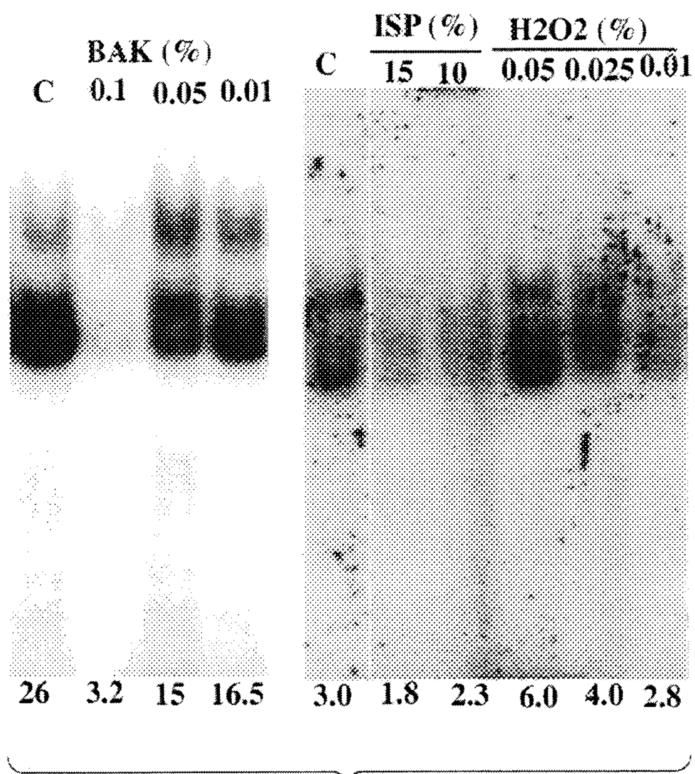

Fig. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07277

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/00; C12N 5/00, 5/02
US CL :435/4, 325
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DOUGHTY, M.J. Evaluation of short-term bovine eye storage protocol for the enucleated eye toxicity test. Toxicology in Vitro. 1997, Vol. 11, pages 229-240, see entire document.	1-18
Y	US 4,959,319 A (SKELNIK et al.) 25 September 1990, see entire document.	1-18
Y	FOREMAN et al. A simple organ culture model for assessing the effects of growth factors on corneal re-epithelialization. Experimental Eye Research. 1996, Vol. 62, pages 555-563, see entire document.	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 JUNE 1999	16 JUL 1999

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	IREM YUCEL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN (CAPLUS), DIALOG (MEDLINE, BIOSIS, SCISEARCH, PASCAL)

Terms: inventor name, eye?, ocul?, cornea? tissue, organ, cultur?, screen?, test?, assay?, effect? toxic?, compound?, chemical?, mammal?, bovine?, procine? limbal conjunctival ring, transcript? factor?