

Methods for the measurement of cell and tissue compatibility including tissue regeneration processes

Methoden zur Ermittlung der Zell- und Gewebekompatibilität einschließlich der Geweberegeneration

Abstract

Biocompatibility is one of the main requirements for the safe use of medical devices. Determination of cytotoxicity is part of the initial evaluation stipulated by ISO standards for the biological evaluation of medical devices. The use of cell cultures to test the biocompatibility of drugs, biomaterials or treatment techniques used in various disciplines is gaining in importance. A wide variety of self-initiated and commercially available cell lines has been evaluated and used: cultured fibroblasts from human skin, buccal mucosa, periodontal membrane, embryonic lung, epithelial and HeLa cells; cultures of human keratinocytes and HaCaT cells; different murine cell lines (C3H-L, Balb/c 3T3, L929 and others) as well as murine cells cultured from liver and spleen; T-lymphocytes from lymph nodes and macrophages obtained by lavage. All of the above cells are suitable for use in biocompatibility tests. Nevertheless, the general opinion is that toxicity tests *in vitro* will be more convincing when performed with cells that are homologous with the human tissue concerned. In accordance, appropriate cell lines for use in cytotoxicity and tolerance tests concerning the skin would be human dermal fibroblasts and human epidermal keratinocytes, as they take an active part in the immune response, inflammatory processes, and wound healing.

The evaluation of the *in vitro* cytotoxicity of a biomaterial is often a qualitative analysis based on the morphological examination of cell damage and growth after direct or indirect contact with the material. Different commercial assays based on the determination of nucleic acids, metabolic activity, protein content or membrane integrity are available to measure cell proliferation and cell viability. A small selection – Pico Green® DNA Cell Proliferation Assay, ATPLite™ Luminescence ATP Detection Assay, BC Assay: protein quantitation kit, AlamarBlue™ Proliferation Assay and Live/Dead Staining with SYTO-13 and EthD-2 – are discussed concerning sensitivity, reliability and applicability.

Keywords: biocompatibility, cell proliferation, cytotoxicity, fibroblasts, keratinocytes, skin

Zusammenfassung

Biokompatibilität ist eine der Hauptforderungen für den sicheren Einsatz von Medizinprodukten. Die Bestimmung der Zytotoxizität gehört daher auch zu den Evaluierungsprozessen, die in den ISO Standards für die biologische Beurteilung von medizinischen Produkten festgelegt sind. Die Anwendung von Zellkulturen für die Testung auf Biokompatibilität von Pharmazeutika, Biomaterialien oder Behandlungsmethoden gewinnt dafür zunehmend an Bedeutung. Eine Vielzahl von selbst gewonnenen oder kommerziell erhältlichen Zelllinien kommen dabei zum Einsatz: Fibroblasten isoliert aus humaner Haut, der Mundschleimhaut, der Wurzelhaut oder aus embryonalen Lungengewebe; epitheliale Zellen und HeLA-Zellen; Kulturen von humanen Keratinozyten und HaCaT-

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Zellen; verschiedene murine Zelllinien (C3H-L, Balb/c 3T3, L929 u. a.) sowie Zellen isoliert aus der Milz oder der Leber der Maus und T-Lymphozyten aus Lymphknoten.

Die genannten Zellen sind alle für Biokompatibilitätsuntersuchungen geeignet. Die generelle Meinung ist jedoch, dass *in vitro* durchgeführte Toxizitätstest überzeugender sind, wenn sie mit Zellen durchgeführt werden, die im Zusammenhang mit dem humanen Gewebe stehen an dem die Applikation des Medizinproduktes erfolgt. Dementsprechend eignen sich am besten humane dermale Fibroblasten und humane epidermale Keratinozyten für die Anwendung in Zytotoxizitäts- und Toleranztests die die Haut betreffen, da sie eine wichtige Rolle bei der Immunantwort, inflammatorischen Prozessen und der Wundheilung spielen.

Die Evaluierung der *in vitro*-Zytotoxizität von Biomaterialien ist oft eine qualitative Analyse basierend auf der morphologischen Untersuchung von Zellschäden und Zellwachstum nach direktem oder indirektem Kontakt mit dem Material. Verschiedene kommerzielle Testsysteme sind erhältlich, um die Zellproliferation und Zellviabilität zu messen. Sie beruhen auf der Bestimmung von Nukleinsäure, der metabolischen Aktivität, dem Proteingehalt oder der Membranintegrität. Eine kleine Auswahl, wie Pico Green® DNA Cell Proliferation Assay, ATPLite™ Luminescence ATP Detection Assay, BC Assay: protein quantitation kit, AlamarBlue™ Proliferation Assay und Lebend/Tot-Färbung mit SYTO-13 und EthD-2, soll hier bezüglich ihrer Sensitivität, Verlässlichkeit und Anwendbarkeit vorgestellt werden.

Schlüsselwörter: Biokompatibilität, Fibroblasten, Haut, Keratinozyten, Zellproliferation, Zytotoxizität

Introduction

Biocompatibility has become the central requirement for the medical application of materials and devices. The determination of cytotoxicity is part of the initial evaluation process stipulated by ISO standards. However, it is not only the absence of a toxic effect that describes the term biocompatibility, but also the presence of a positive influence in terms of biofunctionality (e.g., promotion of wound healing). Cell culture systems may be of value in testing the biocompatibility of drugs, biomaterials or treatment techniques, as they allow the direct measurement of cytotoxicity and effect on cellular growth or the determination of tissue/material interactions. At present, a wide variety of self-initiated and commercially available test methods are used. These include different types of cell lines, such as cultured fibroblasts from human skin, buccal mucosa, periodontal membrane, embryonic lung, epithelial and HeLa cells, cultures of human keratinocytes and HaCaT cells, different murine cell lines (C3H-L, Balb/c 3T3, L929, liver and spleen cell lines, others), T-lymphocytes from lymph nodes and macrophages obtained by lavage [1]. All of these cells are suitable for biocompatibility tests. Nevertheless, the general opinion is that toxicity tests *in vitro* are more convincing when performed with cells that are homologous with the human tissue concerned [1]. In accordance, appropriate cell lines for use in cytotoxicity and tolerance tests concerning the skin would be human dermal fibroblasts and human epidermal keratinocytes.

Fibroblasts are located in the dermis (Figure 1). Their main function is the maintenance of the structural integrity through continuous secretion of macromolecules of the extra-cellular matrix (collagen, glycosamine glycanes and glycoproteins). Tissue damage stimulates fibroblast proliferation and induces the release of cytokines. For experimental purposes, fibroblasts can be isolated from skin biopsies or phimoses through enzymatic digestion of the dermis or by explant method. They are available as the primary cell line NHDF (normal human dermal fibroblasts) from PromoCell (Heidelberg, Germany).

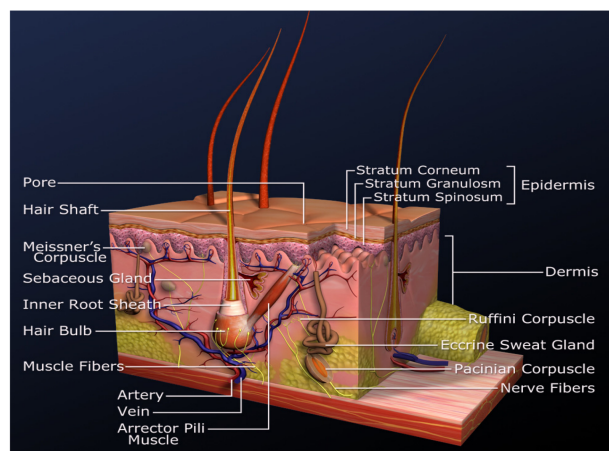


Figure 1: Structure of the skin

Keratinocytes are the most common cell type of the epidermis (Figure 1). They are named after the protein keratin, which they produce. They develop from epidermal stem cells located in the stratum basale (lowest layer of the epidermis) and differentiate into corneocytes during their movement to the stratum corneum (upper layer of the epidermis). There, they form a barrier which protects the body from foreign bodies and germs as well as dehydration. Keratinocytes take an active part in the immune response, inflammatory processes, and wound healing as they are able to synthesize a broad range of cytokines, growth factors, and complement factors. Like fibroblasts, they can be isolated from skin biopsies and phimoses through enzymatic treatment of the epidermis. PromoCell (Heidelberg, Germany) also offers a primary keratinocyte cell line (NHEK, normal human epidermal keratinocytes). Primary cell lines cannot be maintained in culture indefinitely. After a few passages, the cells lose their proliferation ability, probably due to chromosomal alterations. Those developing changes can influence experiment outcomes, which means there is a relatively small time frame for repeating tests. HaCaT cells are spontaneously immortalized keratinocytes. They originate from the isolate of the distal periphery of a melanoma located on the upper half of the back of a 62-year-old male patient and have been described in detail by Boukamp et al. [2]. HaCaT keratinocytes are practically immortal. It is possible to cultivate them for more than 140 passages. While they demonstrate a transformed phenotype *in vitro*, they are not tumorigenic, exhibit a steady expression of specific keratins and other identification markers, and show normal differentiation.

Measurement of cell and tissue compatibility

Human HaCaT keratinocytes (kindly provided by Prof. N.E. Fusenig, DKFZ, Heidelberg, Germany) are cultured in Dulbecco's modified Eagle's medium (DMEM, PromoCell, Heidelberg, Germany), supplemented with 10% fetal calf serum (FCS, PromoCell, Heidelberg, Germany) and 1% antibiotic-antimycotic mix (PSF, PromoCell, Heidelberg, Germany) at 37 °C in a humidified atmosphere containing 5% CO₂. Primary fibroblasts (NHDF, PromoCell, Heidelberg, Germany) and keratinocytes (NHEK, PromoCell, Heidelberg, Germany) can be maintained in appropriate basal media supplemented with growth factors but without antibiotics under the same conditions. For experiments, the cells are seeded at the required density into 96-well plates, 12-well plates, object slides etc. After allowing the cells to attach to their new surface, they are incubated with the test substances. Therefore, the cell culture medium is replaced by either fresh culture medium (negative control/medium control) or the test substance dissolved in culture medium or the material extract prepared according to the DIN standard EN ISO 10993-12.

The subsequent assessment of the *in vitro* cytotoxicity of the substance is often a qualitative analysis based on

the morphological examination of cell damage and growth after direct or indirect contact with the material. In general, methods involving the measurement of cellular growth (cell counting or confluency evaluation) are more sensitive and discriminating than those in which the test materials are placed directly in the cell cultures to determine the zone of growth inhibition [3].

Cell counting with a haemocytometer is the most direct way of quantifying cell numbers. It is often done in conjunction with trypan blue exclusion staining. Trypan blue is not able to penetrate viable cells because of the membrane potential. However, dead cells lose their membrane integrity and are stained blue. This method is inexpensive and easy to perform, but unsuitable for high-throughput screening; it is only recommendable for suspension cells, as adherent cells need to be trypsinized prior to counting.

Determination of cell proliferation and cell viability has become the key technology to assess the growth behavior in a cell culture. A wide variety of commercial assays are available to this end. They are based on the determination of nucleic acids, metabolic activity, and protein content, or membrane integrity. A small selection (Table 1), such as Pico Green[®] DNA Cell Proliferation Assay, ATPLite[™] Luminescence ATP Detection Assay, BC Assay: protein quantitation kit, AlamarBlue[™] Proliferation Assay and Live/Dead Staining with SYTO-13 and EthD-2, is discussed here.

PicoGreen (Molecular Probes Inc, OR, USA) is a reagent for quantifying dsDNA, which provides a stable measurement parameter. The measurement is based on fluorescence enhancement of the dye upon binding to dsDNA. The relative fluorescence units measured correlate with the number of cells present (Figure 2). Cell number and PicoGreen fluorescence exhibit a linear relationship for low and medium cell densities, but which is lost for high cell densities. On the other hand, the assay shows a high sensitivity for low cell numbers, as it is able to detect dsDNA concentrations as low as 25 pg/ml.

Equally sensitive is ATPLite (Perkin Elmer Life Sciences, MA, USA), an ATP monitoring system based on the detection of light generation by firefly luciferase. In an ATP-dependent reaction, the enzyme converts D-luciferin to oxyluciferin. The amount of emitted light is directly proportional to the ATP concentration (Figure 2) and can be measured with a luminometer. ATP is present in all metabolically active cells and declines rapidly when cells undergo necrosis and apoptosis. Therefore, it constitutes an equally stable parameter as dsDNA. However, it allows only the measurement of viable cells and not the quantification of the total cell number.

Table 1: Cell quantification and cytotoxicity assays

Assay	Advantages	Disadvantages
Cell proliferation tests		
<i>PicoGreen DNA Cell Proliferation Assay</i> (Molecular Probes Inc., OR, USA)	<ul style="list-style-type: none"> dsDNA is a stable measurement parameter very sensitive (detection limit of 25 pg/ml dsDNA according to the provider) 	<ul style="list-style-type: none"> dye toxicity – special disposal regulations non-linear relationship of cell number and fluorescence for high cell densities interference with other fluorescent substances
<i>ATPLite Luminescence ATP Detection Assay</i> (Perkin Elmer Life Sciences, MA, USA)	<ul style="list-style-type: none"> measurement of viable cells high sensitivity (detection limit of 5 CHO cells in 100 µl medium according to the provider) long-lived luminescent signal seldom interference with other substances no special disposal regulations to follow 	<ul style="list-style-type: none"> as universal energy carrier ATP is everywhere, contamination of samples and reagents must be avoided no determination of the total cell number, only of living cells
<i>BC Assay protein quantitation kit</i> (Uptima Interchim, France)	<ul style="list-style-type: none"> very sensitive stable reagents compatible with many substances 	<ul style="list-style-type: none"> reaction needs to be read at a precisely defined time and temperature conditions, as the colored complex will continually develop (no true end-point-method) low cell numbers do not provide the necessary protein concentration
<i>AlamarBlue Proliferation Assay</i> (Biosource, CA, USA)	<ul style="list-style-type: none"> non-destructive method non-toxic high sensitivity data can be recorded using fluorescence or absorbance 	<ul style="list-style-type: none"> reaction needs to be read at a precisely defined time and temperature conditions, as the resorufin will continually develop linear range and lower limit of detection are dependent on the cell type and the ability to reduce the redox indicator
Cytotoxicity Assays		
<i>Live/Dead Staining with SYTO-13 and EthD-2</i> (Molecular Probes Inc., OR, USA)	<ul style="list-style-type: none"> direct count of viable and dead cells quantification via microscopic evaluation or plate fluorometer 	<ul style="list-style-type: none"> dye toxicity – special disposal regulations
<i>Cytotoxicity Detection Kit</i> (Roche, IN, USA)	<ul style="list-style-type: none"> quick and easily adaptable 	<ul style="list-style-type: none"> large interference and background from compounds with inherent LDH activity (serum supplemented media) indirect measurement of cell death
<i>Caspase-Glo® Assays</i> (Promega GmbH, Mannheim, Germany)	<ul style="list-style-type: none"> high sensitivity high signal stability high specificity (verifiable with different specific caspase inhibitors) 	<ul style="list-style-type: none"> assay performance is temperature-dependent – use of constant test conditions necessary chemical environment (different culture media) affects the enzymatic activity and luminescence intensity

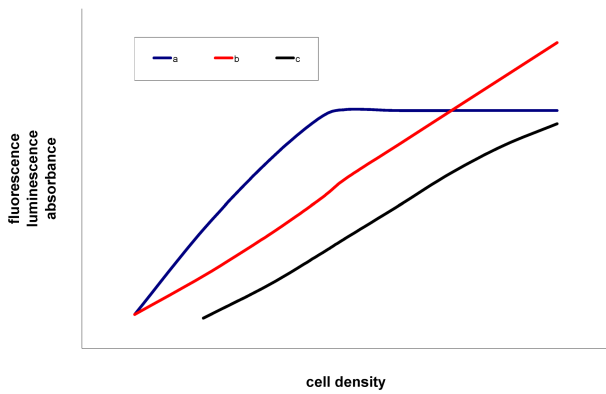


Figure 2: Correlation of cell number and fluorescence intensity (blue), luminescence (red) and absorbance (black)

Another possibility is the determination of the protein content, which naturally corresponds directly to the cell number (Figure 2). The BC Assay (Uptima Interchim, France) is derived from the Biuret reaction ($\text{Cu}^{2+} + e^- \rightarrow \text{Cu}^+$) using bicinchoninic acid. It is based on the measurement of the formation of a water soluble, purple-colored complex of bicinchoninic acid and Cu^+ . The test is sensitive, but not as sensitive as the PicoGreen or ATP assay, and low cell numbers do not provide the necessary protein concentration.

In contrast to the first three methods, the AlamarBlue Proliferation Assay (Biosource, CA, USA) is a non-destructive method. The test uses the indicator dye resazurin, which is dark blue in color and possesses little intrinsic fluorescence until it is reduced by the metabolic activity of viable cells to resorufin, which is pink and highly fluorescent. Non-viable cells rapidly lose their metabolic capacity, do not reduce the dye, and thus do not generate a fluorescent signal. The assay allows simultaneous incubation and measurement but requires precisely defined time and temperature conditions, as the resorufin will continually develop. This presents a serious problem for assay handling. Figure 3 shows a cell standard curve incubated with AlamarBlue measured after 3 h (Figure 3a) and 5 h (Figure 3b).

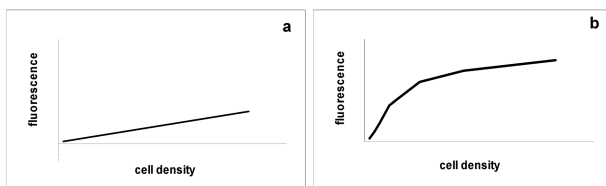


Figure 3: Measurement of the same cell standard curve after 3h (a) and 5h (b) incubation with AlamarBlue

In conclusion, all of the cell quantification methods discussed have advantages and disadvantages (summarized in Table 1), and the most suitable test method for a given study must be chosen. Besides, it is often necessary and always recommendable to verify the test results with at least two different assays. In that way, it is possible to exclude outside influences or false interactions of the components.

The assays can be used to determine the cell proliferation over a particular time period (Figure 4). So far, we have been able to demonstrate proliferative as well as cytotoxic effects of various test substances on keratinocytes and fibroblasts. Figure 5 and Figure 6 present examples of our results.

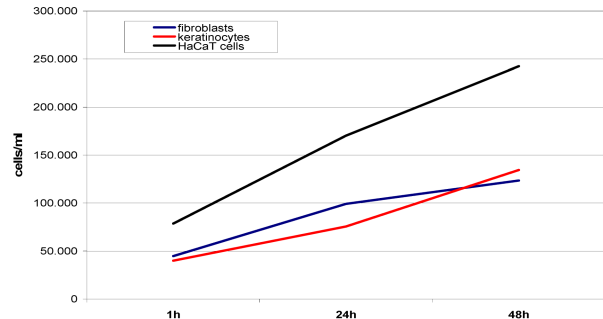


Figure 4: Proliferation of HaCaT cells (black), primary keratinocytes (red) and fibroblasts (blue) over a cultivation period of 48 h determined by ATP quantification with the ATPLite Luminescence ATP Detection Assay

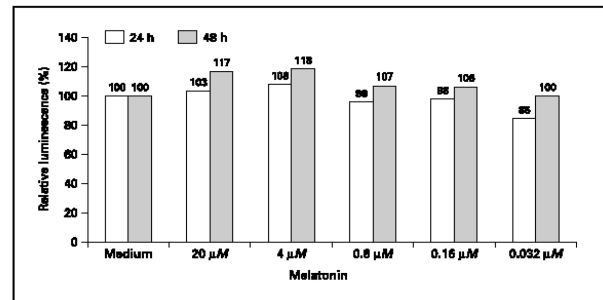


Figure 5: Proliferation of HaCaT keratinocytes depending on the melatonin concentrations after 24 and 48 h incubation. The ATP bioluminescence assay was performed using melatonin in different concentrations [9].

Cell proliferation tests are able to express toxic effects via loss of proliferation ability or reduction of living cells. These negative effects can be quantified by methods that determine cytotoxicity through the measurement of necrosis or apoptosis (Table 1).

Staining with SYTO-13 and Ethidiumhomodimer-2 enables the direct count of viable and dead cells. SYTO-13 and Ethidiumhomodimer-2 (Molecular Probes Inc, OR, USA) are green and red fluorescent dyes that bind to nucleic acids. While SYTO-13 can permeate membranes and is therefore able to stain living cells, Ethidiumhomodimer-2 cannot, and can only enter dead cells, but possesses a higher binding affinity to DNA than SYTO-13. Treatment of the cells with a toxic substance leads to a reduction in the ratio of green to red cells (Figure 7).

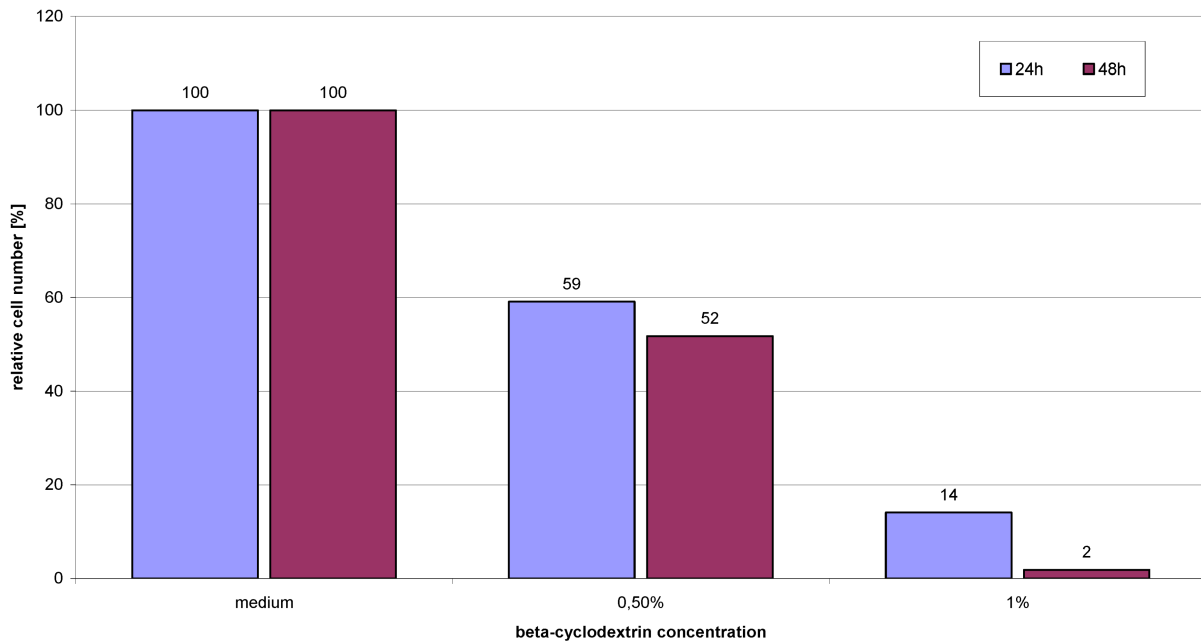


Figure 6: β -cyclodextrin in concentrations of 0.5 and 1% (w/v), respectively, exert a significant negative effect on HaCaT keratinocyte proliferation [10].

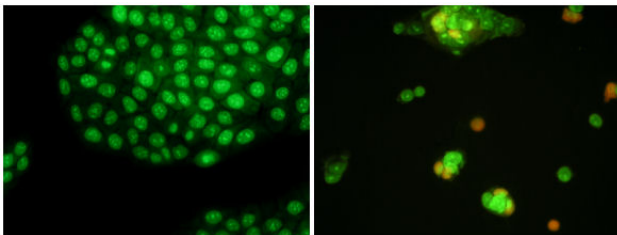


Figure 7: HaCaT keratinocytes not treated (left) and treated (right) with an antiseptic solution (Lavasept® Fresenius, Bad Homburg, Germany)

Lactate dehydrogenase (LDH) in the supernatant is a sign for necrotic degradation of the cells depending on the release of LDH from the cytosol after cell membrane damage. The Cytotoxicity Detection Kit (Roche, IN, USA) is a colorimetric assay which measures the activity of released LDH. Cytotoxic substances which affect membrane integrity cause a rapid release of LDH compared to the medium control (Figure 8). This process is accompanied by a rapid decline of cell viability (Figure 8). This method is quick and easily adaptable for different types of cells and test conditions, but the assay is sensitive to interference from compounds with inherent LDH activity, such as serum-supplemented culture media.

Frequently, the treatment with a substance does not kill the cells directly, but induces cellular damage that subsequently leads to pathological events. Apoptosis is the term for the induction of the programmed cell death. The members of the caspase family play a key effector role in mammalian cell apoptosis. The Caspase-Glo 3/7, 8 and 9 Assays (Promega GmbH, Mannheim, Germany) provide specific luminogenic substrates that form a luminescence signal proportional to the amount of caspase activity present (Figure 9).

In most cases, the release of inflammatory cytokines accompanies the reaction of the cells to the test substance (Figure 10). Cells do not function independently from each other, they communicate through the secretion of interleukins such as IL-6 and IL-8. Interleukin 6 is a cytokine that provokes a broad range of cellular and physiological responses, e.g. inflammation, haematopoiesis and neuronal differentiation. Interleukin 8 is a member of the superfamily of chemokines and acts as a chemo-attractant for neutrophils; it plays a crucial role in many inflammatory events. The cytokine concentration in the supernatant can be easily measured by ELISAs specific for IL-6 and IL-8 (Milenia biotec, Bad Nauheim, Germany).

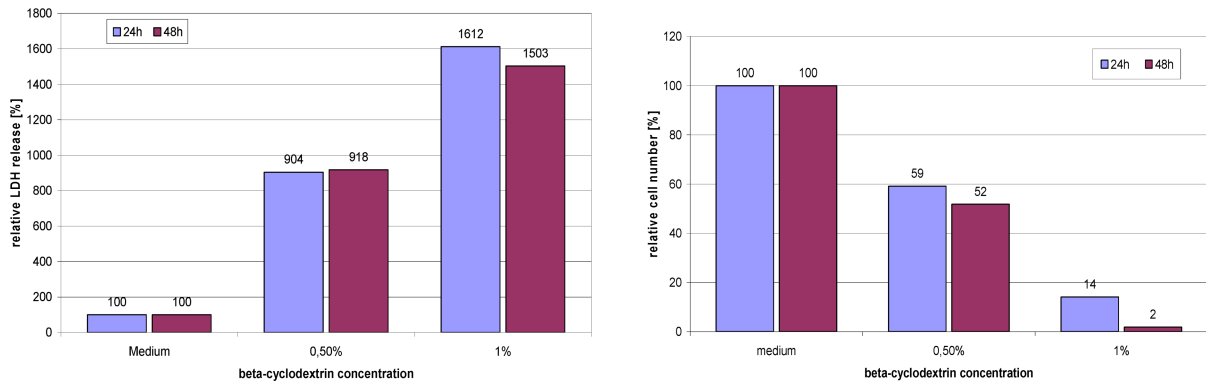


Figure 8: The release of LDH under the influence of toxic β -cyclodextrin concentrations correlates with the reduction of cell viability measured by ATP content [10].

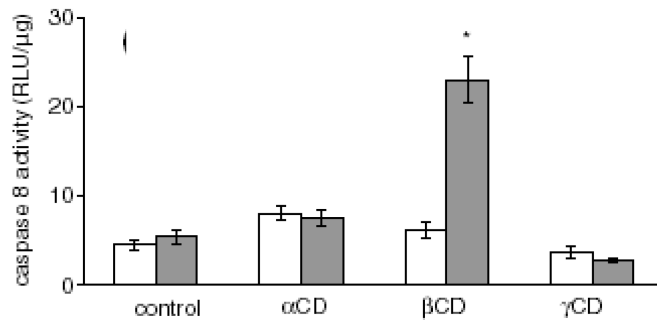


Figure 9: β -cyclodextrin induces apoptosis in human keratinocytes via caspase-8 activation. The caspase-8 activity was determined using the bioluminescent Caspase-Glo™ 8 Assay kit (Promega GmbH, Mannheim, Germany) [11].

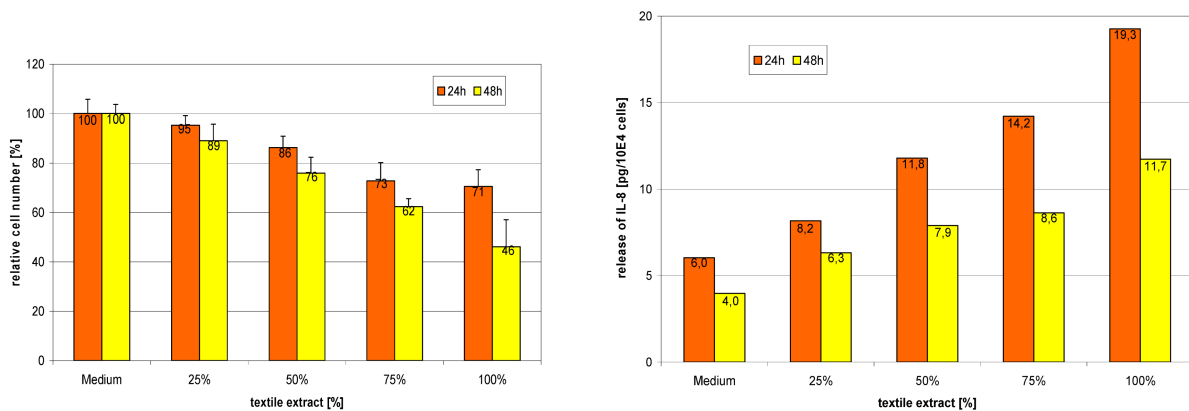


Figure 10: Cultivation of HaCaT keratinocytes with textile extracts (cotton) has a negative effect on cell proliferation determined by dsDNA content and triggers the release of the cytokine IL-8.

Determination of regeneration processes

Materials and substances used, for example, in the treatment of wounds do not only influence cell proliferation and viability, but also affect their migration capacity. Fibroblasts and keratinocytes enable the maintenance of the barrier function of normal skin. Upon wounding, this shield is disrupted and fibroblasts and keratinocytes migrate into the wound area to regenerate the skin. Therefore, the cells must dissociate from one another and break the cell-matrix contacts to detach from the basal lamina. Migration into the wounded area entails remodelling of the extra-cellular matrix by its proteolytic

degradation as well as by *de novo* synthesis and deposition of newly formed matrix components. Both degradation and reformation of the matrix are functions performed by dermal fibroblasts and epidermal keratinocytes. The mechanical scratch wounding of confluent monolayers (Figure 11) serves as a model to study cell migration at the wound margins [4]. The method allows the direct measurement of cell migration and layer regeneration. Using the scratch apparatus described by Büth et al. [4], the scratches can be inflicted with high precision and in reproducible fashion.

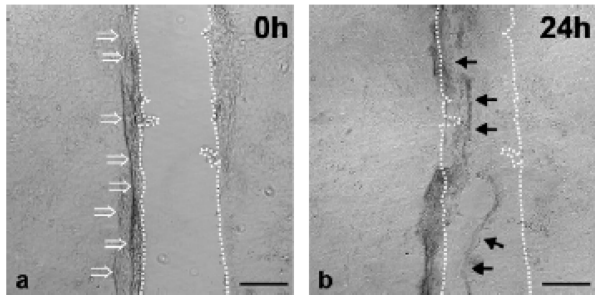


Figure 11: Conventional scratch wounding. Phase-contrast micrographs of HaCaT keratinocytes were taken directly (a) and 1 day (b) after scratch wounding. Keratinocytes were grown on non-coated cover glasses. Scratch rims are denoted by dotted lines. Bars: 100 μm [4]

So far, all featured test methods utilize cell monolayers which are an exclusively *in vitro* condition. Evidence suggests that cells behave differently within these two dimensions than in their familiar 3-dimensional *in vivo* environment. For instance, fibroblasts reach confluency in monolayer cultures with a high proliferation rate and display protein biosynthesis levels that do not resemble the *in vivo* situation. On the other hand, fibroblasts embedded in 3-D collagen gels exhibit lower biosynthetic activity, regulated growth, and stellate shape; they also have protruding dendritic extensions and a state of differentiation close to the *in vivo* situation.

To mimic the conditions which cells experience *in vivo*, the use of 3-D cell cultures is becoming increasingly popular. There are two types of matrices used for cell culture. "Ordered collagen matrices" (ORC) use the long range assembly properties of type I collagen monomers, and the cells are seeded afterwards into the collagen gel formed [5]. The "dermal equivalent" (Figure 12) is formed as a tissue-like structure from a collagen gel contracted by fibroblasts [6]. With 3-D cultures it is possible to study collagen degradation as well as polymerization and deposition of newly synthesized collagen. They allow the study of tissue remodelling *in vitro* and provide an assay system for wound contraction. It is even possible to prepare skin-equivalent tissue by co-culturing with keratinocytes. Of course, these are time-consuming and elaborate preparations that require operator experience and the appropriate evaluation methods.



Figure 12: A contracted dermal equivalent formed from a gel containing 7.2×10^4 fibroblasts cast in square mold after 5 days. Collagen concentration was 1.8 mg/ml [6].

Assessment of the potential of testing physical wound treatment techniques

It has been reported that the use of physical wound treatment techniques, such as wIRA (water filtered infrared A) and HVPC (high-voltage pulsed current), have a positive effect in terms of accelerated wound healing [7] or additional antibacterial activity [8]. For the measurement of the effect of these physical methods *in vitro* or for the application of a new plasma device on cells, several considerations must be taken into account to design an appropriate study. For one, it is necessary to scale the *in vivo* application parameters to the *in vitro* use on cell monolayers (Figure 13).

For assessment of the bactericidal activity of HVPC, cotton pieces were soaked with bacterial suspension, placed on a steel plate, and covered with the electrode (42 mA, 128 Hz, 30 min) [8]. Cells need stable culture conditions that guarantee the establishment of an appropriate cell environment during the experiment. Therefore, *in vitro* studies that have been performed to assess the bactericidal activity of HVPC cannot be applied one-to-one on human cell cultures.

In conclusion, for testing physical treatment methods, the choice will probably tend to the use of a 3-D culture system which more closely resembles the *in vivo* situation, although cellular effects are far easier to detect and quantify in cell monolayers than in 3-D cultures. Hopefully, this summary of methods for measuring cell and tissue compatibility will provide ideas for the design of conclusive *in vitro* studies.

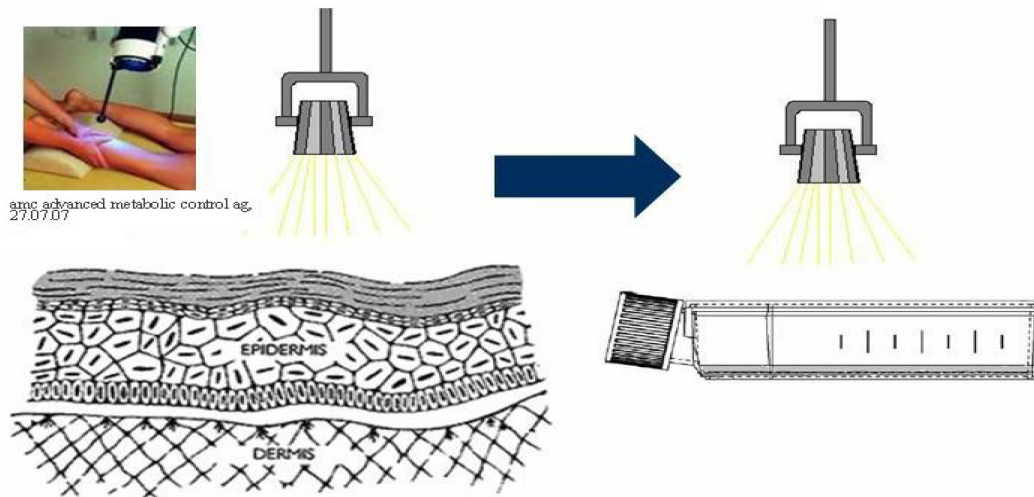


Figure 13: For the investigation of the effects of WIRA, the scaling of the light intensity and application distance from the clinical use to the in vitro system is necessary.

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Please cite as

Wiegand C, Hipler UC. *Methods for the measurement of cell and tissue compatibility including tissue regeneration processes.* *GMS Krankenhaushyg Interdiszip.* 2008;3(1):Doc12.

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