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In vitro Models, Endpoints and Assessment Methods for the Measurement of Cytotoxicity

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Introduction

Cells display specific responses upon exposure to diverse chemicals or physical stresses. Currently, the examination of these cellular responses, such as morphological alterations, damage to the membrane, irregularities in biochemical processes, and nuclear condensation, is being performed frequently to assess the potential risks associated with a chemical[1]. It is important to note that the manifestation of these effects is greatly dependent on the length of chemical exposure and mechanism of cytotoxicity. Cytotoxicity is considered primarily as the potential of a chemical to induce alterations in cellular behavior and processes resulting in death[1]. Cytotoxicity test, an alternative method in toxicological sciences, is an *in vitro* study to measure the different parameters involved in the progression of cell death and proliferation. Compared to animal studies, cell-based testing is easier to perform, reproduce and control the experimental conditions, is less ethically ambiguous, and is less expensive. A number of assays based on various cytotoxic endpoints are currently being used to measure the cellular responses to a toxic chemical. Many assays are also being developed to meet the needs of toxicological experimentation. Most of these assays are basically designed to estimate the cellular responses at three different levels: (i) basic cellular responses, such as cell proliferation, viability, and death; (ii) to analyze signal transduction pathways (e.g., ion channels and secondary messengers); (iii) at the transcriptional/translational and DNA level (e.g., proteins, cellular metabolites, and gene)[2].

The aim of the present review is to address the commonly used *in vitro* cell-based models, cytotoxic endpoints, and the assessment or detection methods employed for cytotoxicity analysis. In addition, we will focus on the popular cytotoxicity assays and their comparative study in terms of the sensitivity and endpoints. The major drawbacks of the currently employed cytotoxicity assays and methods will be also briefly discussed. Finally, we will highlight the new approaches and current efforts for cell-based screening to minimize the limitations of the conventional cytotoxicity methods and to advance the techniques of cytotoxicity assays.

In vitro Models for Cytotoxicity Tests

With the increasing demand of cell-based screening in toxicology research, the development of a sensitive *in vitro* model of cell culture system, which can help to generate more comprehensive and reliable data for toxicological risk evaluation, has been encouraged over the recent years. The major aims of *in vitro* systems have been the reproduction of original tissue characteristics and its cell-cell interactions, creation of appropriate microenvironment for the cells, rapid performance of cytotoxicity assays, sensitiveness and accuracy in the identification of endpoints[3]. Consequently, several *in vitro* models, such as 2D cell culture system (monolayer and co-culture model), 3D cell culture system (multilayer model), tissue slice or xenograft model, are specifically developed to perform the cytotoxicity analysis of various chemicals. Furthermore, the recent use of microfabrication technology has fostered the development of a better and more economic *in vitro* cell culture model with potential applications in high-throughput toxicity analysis[4]. The promising advantages of micro-scale cell culture model are rapid cytotoxicity analysis, mimicking the *in vivo* condition more perfectly and high sensitivity. In addition, this model system is useful in controlling cell adhesion, cell shape, intercellular contact, and heterologous cell interactions, providing remarkable benefits over the other conventional *in vitro* model systems. The specific advantages and disadvantages of the common *in vitro* cytotoxicity models are provided in Table 1[4-10].

Table 1. The specific advantages and disadvantages of the commonly used *in vitro* models for cytotoxicity analysis[4-10].

In vitro models	Advantages	Disadvantages
2-D cell culture model (conventional monolayer model)[5,6]	Easy and convenient to maintain, and increased viability	Loss of organ-specific cell-to cell interactions and differentiated functions
2-D co-culture model[7]	Interactions between multiple cell types	Cell-cell competition, complicated and conflicting culture requirements
3-D cell culture model (hydrogels, multicellular spheroids, and multilayer cell culture)[8]	Well-defined geometry, co-cultivation of multiple cell types, mimics the *in vivo* situation very closely, and 3-D scaffold for mechanical stability	Poor viability, diffusion and action of chemicals altered, multicellular resistance, and expensive
Tissue slice/Grafting model[9,10]	3-D representation of cellular environment, preserve cell-to-cell & cell-to-matrix environment, realistic preclinical model, and preserve the functional and morphological heterogeneity	Viability is limited, morphological evaluation alters with the slice, difficult to reproduce, and limited availability for human models
Micro-scale cell culture model (microfluidic cell culture system)[4]	Mimics the *in vivo* situation very closely, controlled microenvironment, allow reproduction of biotransformation *in vitro*, short reaction times, portability, low cost, and small consumption of reagents and cells	Complex fluidic connection and flow control required, expensive detection and analysis system, and formation of air bubble

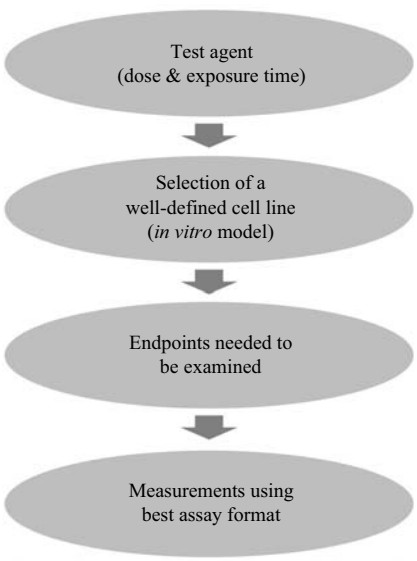

Figure 1. General scheme for the performance of *in vitro* cytotoxicity test.

Common Endpoints for Cytotoxicity Tests

A large number of *in vitro* cytotoxicity assays have been utilized and categorized on the basis of their endpoints. However, choosing a right one from a variety of assays could be a challenging task. Depending upon the experimental need, set-up and the endpoint, various assays are designed and developed to measure the cellular responses that range from cell death to a temporary or often reversible alteration in cellular structure and function. The common endpoints being measured for cytotoxicity analysis are (i) cell number, (ii) cell viability, (iii) membrane permeability, (iv) cellular metabolic function, (v) cellular ATP, (vi) intracellular calcium, (vii) mitochondrial membrane potential, and (viii) nuclear size. The popular assays to measure a particular endpoint are presented in Table 2[11-24].

Cytotoxicity Assays

The selection of a particular assay required for the analysis of cytotoxicity of a chemical depends on several factors, such as (i) *in vitro* cell culture models, (ii) culture platforms, (iii) endpoints, (iv) assessment or detection methods, (v) mechanism of cytotoxicity, (vi) physicochemical properties of a chemical. The basis and methods of common cytotoxicity assays are discussed here in this review.

MTT Assay

The MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay is one of the widely used methods to assess cell viability[13,25]. MTT is reduced by mitochondrial dehydrogenases in living cells to a blue-magenta-colored formazan precipitate. The absorption of dissolved formazan in the visible region correlates with the number of intact alive cells. Cytotoxic compounds are able to damage and destroy cells, and thus decrease the reduction of MTT to formazan.

Table 2. The popular cytotoxicity assays based on various endpoints[11-24].

End points	Cytotoxicity assays
Cell number	methylene blue staining assay[11], resazurin (AB)[12], sulforhodamine B (SRB) assay[13], trypan blue[11], Hoechst[14], ALP assay[15]
Cell viability	LDH assay[13], alamar blue[12], crystal violet[11], calcein-AM[16], fluorescein diacetate[16], redox sensing[17]
Membrane permeability	LDH[13], MTT[13], annexin[16], granzyme-based[18], caspase-based assay[19]
Cellular metabolic function	MTT[13], TMRE[20], WST-1[20]
Cellular ATP	ATP-based luminescent assay[21]
Intracellular calcium	Fluo-3[22], Fluo-4[22]
Total cellular protein	SRB assay[13]
Nuclear size	ethidium homodimer[14], propidium dye[14], BrdU[14], DAPI[14], TUNEL assay[19], Hoechst[14], caspases-based assay[19]
Lysosomal activity	neutral red assay[19], cathepsin D activity assay[23], granzyme-based assay[17]
Glucose	fluorescent glucose analog[24]

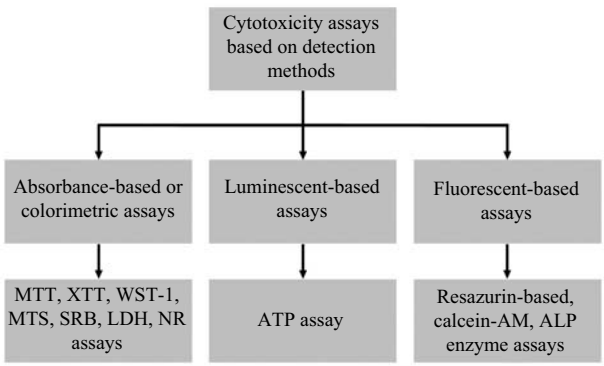

Figure 2. Major types of popular cytotoxicity assays based on detection methods.

However, there are several inherent disadvantages of this assay, including the safety hazard of personnel exposure to large quantities of dimethyl sulfoxide, the deleterious effects of this solvent on laboratory equipment, and the inefficient metabolism of MTT by some human cell lines. These limitations led to the development of an alternative assay utilizing a different tetrazolium reagent, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-S-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT)[25], which is metabolically reduced in viable cells to a water soluble formazan product. This reagent allows direct absorbance readings, therefore eliminating a solubilization step and shortening the assay procedure. Most human tumor cell lines examined metabolized XTT less efficiently than MTT, however, the addition of phenazine methosulfate (PMS) markedly enhanced cellular reduction of XTT. In the presence of PMS, the XTT reagent yielded usable absorbance values for growth and drug sensitivity evaluations with a variety of cell lines. Similarly, other modified tetrazolium salts like (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) (WST-1)[20,26], (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt) (MTS)[26] are more recent alternative to MTT and have significant advantages over MTT.

Sulforhodamine B Assay

The sulforhodamine B (SRB) protein staining assay was developed for the *in vitro* measurement of cellular protein content of adherent and suspension cultures[13,27]. The dye binds to basic amino acids of cellular proteins and colorimetric evaluation provides an estimate of total protein mass which is related to cell number. For chemosensitivity testing, the SRB assay has many advantages over the MTT assay. The SRB assay shows a wide linear range with cell number, while the MTT test is not linear at high cell densities. In contrast to the MTT assay, SRB staining is not dependent on mitochondrial activity, and this results in a low variation between cell lines and therefore there is less need for cell line-specific optimization. Although both assays are comparable in terms of outcome when used for chemosensitivity testing, the SRB assay is preferred for its lower variation between cell lines, higher sensitivity and larger linear range. Moreover, the SRB assay has some practical advantages over the MTT assay, i.e. the staining is stable for a long period of time and the assay can be interrupted at several steps during the staining protocol.

LDH Assay

The lactate dehydrogenase (LDH) leakage assay is based on the measurement of lactate dehydrogenase activity in the extracellular medium[13,28]. The basic characteristics of this assay are reliability, speed and simple evaluation. The loss of intracellular LDH and its release into the culture medium is an indicator of

irreversible cell death due to cell membrane damage. This assay was originally used to measure neuronal cell death occurring *via* necrosis, but later, it has been shown to accurately measure neuronal apoptosis in cortical cultures.

Neutral Red Assay

The use of neutral red (NR) as a cytotoxic indicator was first described in a viral cytopathogenicity test[19,29]. The NR assay is a reproducible and sensitive assay specifically developed for screening toxic substances. It is based on the *in vitro* incorporation of a supravital dye, neutral red, into lysosomes of living mammalian cells. The NR assay is capable of measuring cell death or injury caused by toxic chemicals. Damage to cell surface or lysosomal membranes by a variety of chemicals results in decreased dye uptake.

ATP Assay

The ATP (adenosine triphosphate) assay is one of the very sensitive and widely used methods to assess cell viability[21,25]. The nucleotide ATP plays a dominant role in energy exchange processes in biological systems. ATP represents the most important chemical energy reservoir in cells and is used for biological synthesis, transport, and movement processes. Cellular ATP is one of the most sensitive end points in measuring cell viability. When lethal cell damage occurs, the ATP level decreases dramatically. Additionally, the ATP availability in the cell determines the type of cell death although there are more intracellular substances controlling the mode of cell death (e.g. superoxide). The ATP assay first developed as a somatic cell viability assay is based on the reaction of luciferin to oxyluciferin catalyzed by the enzyme luciferase in the presence of Mg^{2+} ions and ATP yielding a luminescent signal. A linear relationship exists between the intensity of the luminescent signal and the ATP concentration.

Resazurin-based Assay

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide), a redox-sensitive dye long used for detecting bacteria and yeast, is the primary reporter dye in alamar blue, a proprietary mixture with other compounds (poisoning agents) added to optimize mitochondrial reduction and inhibit non-specific reduction of resazurin[12,30]. Healthy cells reduce resazurin more effectively than do dead or dying cells. Resazurin reduction leads to a loss of oxygen and a gain of hydrogen in the molecule, and its reduced product, resorufin, can be detected both colorimetrically and fluorometrically. Resorufin is a highly fluorescent molecule. Resazurin is a more sensitive reporter than other commonly used mitochondrial reductase dyes such as MTT and XTT. Unlike MTT and XTT, resazurin is non-mutagenic and relatively non-toxic and can be washed free from the cells so that other assays can be performed. The resazurin-based cytotoxicity assay is also relatively insensitive to interference from drugs, serum, and phenol red.

Calcein-AM Cytotoxicity Assay

Acetoxymethyl diacetylester of calcein (calcein-AM), a derivative of fluorescein, is an indicator of cell viability and cellular cytotoxicity due to its superior cell retention and relative insensitivity of its fluorescence to pH in the physiologic range[16,25]. Calcein-AM is a highly lipophilic vital dye that rapidly enters viable cells, is converted by intracellular esterases to calcein that produces an intense green (530 nm) signal, and is retained by cells with intact plasma membrane. The unhydrolyzed substrates and their fluorescent products are rapidly extruded from dying or damaged cells with compromised membrane integrity. In recent times, the calcein-AM assay has replaced the conventional ^{51}Cr-release method for assessment of cell viability and cytotoxicity, and has been applied to quantitate apoptosis.

Alkaline Phosphatase Enzyme Assay

The alkaline phosphates (ALP) enzyme, a mostly membrane bound enzyme found in nearly all human cell types, assay is a rapid and accurate method to assess cell growth based on a sensitive fluorogenic enzyme assay[15]. The non-fluorogenic substrate, 5-methylumbelliferylphosphate, reacts with all forms of the enzyme to produce a free phosphate and 5-methylumbelliferylerone which is a highly fluorogenic compound. Cell number can be rapidly and efficiently estimated '*in situ*' by measuring the activity of alkaline phosphatase using a fluorogenic enzyme assay. A limitation of the ALP assay is that it cannot be utilized to count cells when chemical agents have been used that may modulate the expression of alkaline phosphatase activity. One of the main advantages of the ALP assay is the speed with which cell growth from a large number of cultures can be estimated, thus alleviating the tedium of counting cells using the standard methods, such as haemocytometer, Coulter counter or thymidine labeling. Another advantage of the ALP assay is its sensitivity since as few as 10^4 cells can be accurately estimated. This assay appears to be more rapid, simpler to perform and more accurate than the other commonly used assays (MTT, XTT and NR assays).

Assessment or Detection Methods

The treatment of cells with a chemical results in a variety of cell fates. The fate of a cell following treat-

ment with a chemical is mostly dependent on the dose and exposure time. Cells may undergo necrosis or may activate internal programmed pathway (apoptosis) to induce death. It is suggested that cells following a chemical insult proceed through a chain of biochemical responses which may be initially shared between apoptosis and necrosis, but the final outcome could be a result of progressive exclusive choices. Therefore, the identification of endpoints is very crucial to examine the fate of a cell. However, the transient nature of endpoints and kinetic differences associated with cell death progression render the analysis more difficult and may cause to inaccuracy in the outcome. Therefore, it is very necessary to assess the suitability of a particular assessment or detection method for the identification of certain endpoints before employing any type of cytotoxicity assay. Although the assays have been specifically designed for the analysis of certain endpoints, their sensitivity and accuracy much vary depending upon the detection method. Broadly, cytotoxicity assays can be divided into three major categories based on detection methods; (i) absorbance-based or colorimetric assays, (ii) luminescent-based assays, (iii) fluorescent-based assays. Absorbance-based or colorimetric assays, such as MTT, XTT, WST-1, MTS, SRB, LDH, NR, basically rely on the absorbance of the dyes which is measured by a spectrophotometer. However, in recent years, the development of *in vitro* cytotoxicity assays has been driven by the need of simple, convenient, rapid, inexpensive, automated, homogeneous and highly sensitive assays. Absorbance-based assays lack the consideration of certain demands of modern *in vitro* cytotoxicity assays. To meet such demands, luminescent- or fluorescent-based assays have been prolifically developed over recent years. Bioluminescent detection of ATP using the firefly luciferase enzyme provides a very simple, highly reproducible and extremely sensitive assay. Similarly, a fluorescent dye, resazurin, stands well to the demands of modern cytotoxicty screening. The test is very simple to perform, requiring the addition of only one reagent to the cell culture supernatant (therefore providing a homogeneous assay). Resazurin is non-mutagenic and relatively non-toxic and can be washed free from the cells so that other assays can be performed. The resazurin-based cytotoxicity assay is also relatively insensitive to interference from drugs, serum, and phenol red. Furthermore, luminescent- and fluorescent-based cytotoxicity assays are assessed to be very much suitable for high-throughput screening (HTS) and high-content screening (HCS)[31]. HTS system offers ultra- high-speed measurements (using luminescence- and fluorescence-based assay), excellent sensitivity, and rapid screening of a chemical due to assay miniaturization, parallel processing, and advanced assay technologies. The promising HTS systems are 96-, 384-, 1536-well plate formats in combination with laboratory robots, and microfluidic devices (biochip technology). In addition, the development of fluorescence microscopy integration with digital and charge-coupled device (CCD) has led to a revolution in basic and cytotoxicity research, as they created the opportunity to measure subcellular structures and genetic alterations providing even spatial and quantitative data. Consequently, the development of an automated fluorescent imaging system in combination with the high cost of HTS systems yielded a new multiplexed screening technique, high content screening. HCS is defined as multiplexed functional screening based on imaging multiple targets in the physiologic context of intact cells by extraction of multicolor fluorescence information. HCS is applied to identify multiple endpoints on cell samples, exploring the mechanism of cytotoxicity and cellular processes very extensively.

New Approaches

In recent years, cytotoxicity assays have been extended to a very advance level in terms of efficiency, integration with real-time monitoring system, multiplexing and sensitivity. However, most of the traditional assays are based on single endpoints which only detect very specific cellular changes and do not give information on kinetic progression of cell death. Moreover, these assays entirely rely on labeling dyes to measure certain endpoints which are transient in nature. Therefore,

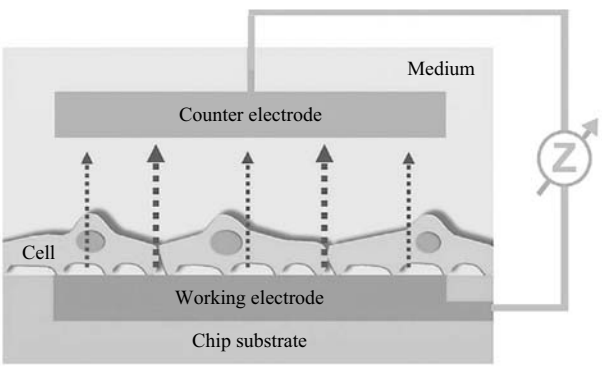

Figure 3. Representative micrographs showing the measuring principles of microelectrode-based impedance spectroscopy. The frequency-dependent alternating current produced by a small alternating voltage flows from a working electrode through or between the cells to an opposite counter electrode. The resulting current between both electrodes is analyzed by the impedance instrument and the impedance magnitude is calculated. Adapted from the reference 33.

there is an urgent need of label-free detection methods for *in vitro* cytotoxicity assays that must allow for online monitoring of living cell behavior over a long period of time providing information about cell status before and after exposure as well as kinetic data of the cell death progression. In consequence, electronic-based approach has been emerged as one of the most interesting label-free technologies which facilitates real-time monitoring and kinetic analysis of cell behavior. Electrochemical impedance spectroscopy (EIS) plays a major role in this new approach of analytic systems[32,33]. The electric cell-substrate impedance sensing (ECIS) technique was first utilized in the development of a biosensor for cell morphology monitoring[32]. However, this system is now reported to be useful in monitoring cell response to physiological and electrical stimulations as well as in studying the cytotoxicity effects on V79 cells after exposure to mercury chloride and trinitrobenzene. Furthermore, the modification of this system is also developed to improve the efficacy and sensitivity of the analysis: Interdigitated Electrode Structures (IDES) for monitoring cell adhesion, and "real-time cell electronic sensing" (RT-CES) for the dynamic measurement of cell responses to cytotoxicants[32]. Electronic-based system offers real-time, non-invasive and automatic impedance measurements with very high reproducibility, sensitivity and cell number capability. In addition, their results (RT-CES system) are found to be well correlated with the standard luminescence-based methods and NR assay results.

Summary

Cells respond rapidly to toxic substances by altering the morphology, growth, behavior, and biochemical processes controlling basic cell functions. *In vitro* cytotoxicity analysis, an alternative to animal-based testing, is an important tool to specifically screen the chemicals and form a comprehensive and reliable toxicological data useful for predicting the toxic effects on humans. Consequently, the increasing demand of cell-based screening system led to the development of various innovative *in vitro* model systems, novel endpoints, variety of cytotoxicity assays, and advanced assessment or detection methods. Although the cell-based screening has come to a very advanced level capable of providing adequate information on kinetic progression of cell death and dynamic interaction between cells and toxicants, the certain limitations of the existing model systems and methods render the results with high variability and questionable relevance. Therefore, the multiplexed assay approach based on multiple endpoints should be preferred for the evaluation of toxicity of a chemical and to estimate the actual cause and mechanism of cell death. In addition, the existing *in vitro* cytotoxicity studies provide the collection of toxicological data useful for the determination of acute effects of a substance. These data don't well suit to analyze the chronic effects of a chemical. Therefore, there is a strong need to improve the existing model systems and methods which could be applicable for the measurement of the time-course of molecular changes and complex sequences of biochemical events involved in chronic toxicity *in vivo*.

Acknowledgements

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