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Cell-based Cytotoxicity Methods
Hücre Bazlı Sitotoksisite Yöntemleri

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Abstract

Cell-based cytotoxicity methods are an essential part of cancer research and drug discovery. Cancer is characterized by increase in the cells proliferation and decrease in cell death. Likewise, drugs can interfere with many cellular functions that involve different cell death pathways. These changes in the physiology of the cells can be qualified with several available methods. Many assays rely on numerous cell functions such as metabolic activity and cellular Adenosine 5′-triphosphate (ATP), membrane integrity, DNA cleavage, caspase activity, protein content and mitochondrial changes. Some assays are considered suitable for high throughput screening due to the rapid evaluation of many samples in a cost-effective way, in addition to low sample and reagent consumption. Selecting the right methods is critical to obtain reliable results. There are several unique molecular signaling pathways involved in cell death and every assay gives specific insights into the processes of apoptosis in the cells. This enforces and facilitates the data interpretation for a better understanding of specific cell death mechanisms for in vitro analysis. This review gives an overview of the most commonly used methods to assess cell death.

Keywords: Cytotoxic assays, In vitro, Apoptosis

Özet

Hücre temelli sitotoksisite yöntemleri kanser araştırma ve ilaç keşfinin önemli bir parçasıdır. Kanser hücre proliferasyonunda artış ve hücre ölümünde azalma ile karakterizedir. Ayrıca, ilaçlar farklı hücre ölüm yolaklarını da içeren birçok hücresel fonksiyonla etkileşime girebilir. Hücre fizyolojisindeki bu değişimler mevcut birkaç yöntemle nitelendirilebilir. Birçok test metabolik aktivite ve hücresel adenozin 5'-trifosfat (ATP), membran bütünlüğü, DNA ayrılması, kaspaz aktivitesi, protein içeriği ve mitokondriyal değişimler gibi çok sayıda hücre fonksiyonuna dayanmaktadır. Bazı testlerin çok sayıda örneğin uygun maliyetle hızlı değerlendirilmesinin yanısıra az miktarda örnek ve reaktif kullanımı nedeniyle yüksek verimli tarama için uygun olduğu düşünülmektedir. Doğru metot seçimi güvenilir sonuçlar elde etmek için çok önemlidir. Hücre ölümü ile ilgili birkaç kendine özgü moleküler sinyal yolu mevcuttur ve her test hücrelerdeki apoptozis süreçleri hakkında spesifik bilgiler verir. Bu durum in vitro analizlerde spesifik hücre ölüm mekanizmalarının daha iyi anlaşılması için verilerin yorumlanmasını güçlendirir ve kolaylaştırır. Bu derleme hücre ölümünün değerlendirilmesinde yaygın olarak kullanılan metotlara genel bir bakış sunmaktadır.

Anahtar kelimeler: Sitotoksik testler, İn vitro, Apoptozis

1. INTRODUCTION

Unlike normal cells, cancer cells are characterized by unlimited proliferation and resistance to death. Proliferation and cell death can be easily measured using cell-based assays to evaluate the physiological differences between the normal and cancer cells especially in any response to cytotoxic compounds (Kepp, Galluzzi, Lipinski, Yuan, J., & Kroemer, 2011). In basic science and drug development laboratories, it is essential to know at the end of the study how many viable cells are remaining to determine if the drugs have effects on cell proliferation or display any direct cytotoxic effects on the cells for a scientific analysis. Many assays are specific to healthy living cells to produce a proportional number of viable cells, based on biochemical events such as metabolic activity and cellular ATP, membrane integrity, DNA damage, caspase activity, protein content in living cells and colony formation (Mery et al., 2017). The screen can cover the basal function of the cell or the special function in the cell (Elmore, (2007) (Figure 1). This review offers an overview of the most commonly used cell-based cytotoxicity assays in vitro.

1.1. ATP Measurement Assay

The active cells that proliferate have high metabolic activities, but the cells that treated with drugs or under certain abnormal conditions will have low metabolic activities. There are many assays for measuring levels of important metabolic proteins such as ATP or use the reduction of either tetrazolium salts or resazurin dyes (Berridge, Herst & Tan, 2005). ATP is generated by living cells, however during death process, the ability of cells to synthesize ATP decreases (Vives- Bauza, Yang, & Manfredi, 2007). Thus, measuring the ATP amount in tissue culture cells is used to quantify cell viability. Sensitive measurement of intracellular ATP is attained by a luciferase-based assay that is based on the ability of firefly luciferase to generate a luminescent signal. The luminescent ATP detection delivers a robust data and it is easy to perform. For these reasons, it has become the technique of choice for quantifying cell viability (Ulukaya, Ozdikicioglu, Oral, & Demirci, 2008). In addition, the ATP assay is a sensitive microplate assay for identifying viable cells in culture as less than 10 cells per well to be labeled. The ATP reagent must be mixed properly to lyse the cells in 96 well plates to produce accurate ATP values.

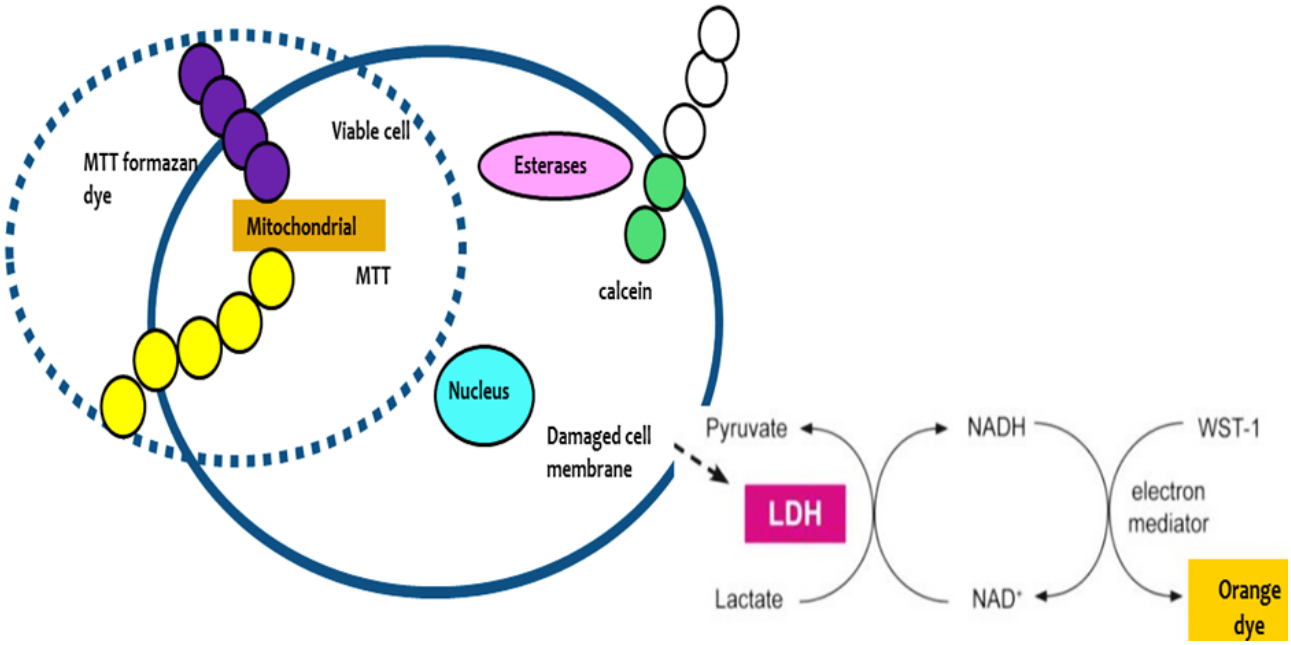

Figure1. Different mechanisms can be used for measuring the cell viability in vitro. MTT, conversion of MTT to a water-insoluble colored formazan which is then solubilized in stop solution and give an estimation of the number of healthy cells. In damaged cell membrane, LDH is released and reduced NAD+ to NADH, and then converted a tetrazolium dye to a soluble range-colored formazan. Calcein AM, a non-fluorescent, cell permeable compound that easily invades live cells and gives green fluorescence which counted by flow cytometry.

ATP quantification is not always linked directly with cell viability as the decrease in intracellular ATP concentrations may happen from nonlethal perturbations, such as termination of proliferation like senescence and inhibited mitochondrial respiration (Ziegler, Wiley, & Velarde, 2015).

1.2. MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl-tetrazolium bromide) Assay

MTT has been commonly accepted and still popular in cancer research as proven by countless of publications. Tetrazolium reduction assay is suitable to measure the activity of cell metabolism where NAD (P) H-dependent cellular oxidoreductases possibly reflect the number of viable cells existing in the end of the experiment. Mitochondrial dehydrogenases in the cytochrome b and c sites of living cells cleave the tetrazole ring, and the water-soluble MTT (yellow color) is reduced to produce a purple crystalline formazan in the cytoplasm, and to a lesser extent in the mitochondria and cell membrane (Berridge et. al., 2005). The amount of emerged crystals represents a positive correlation to the metabolic activity and number of cells. Inactive cells cannot convert MTT into formazan. Therefore, color formation serves as a marker of active and healthy cells. The MTT reagent is soluble in dimethyl sulfoxide and routinely used at a final concentration of 0.2 - 0.5mg/mL. This is followed by adding the stop solution (20% SDS and 37%HCL in H_2O).

The assay can be performed in a 96-well format and the cells are incubated with MTT substance for 1 to 4 hours and the absorbance is identified at 570 nm by plate reading spectrophotometer. It is very robust assay and can be used for most cell lines (van Meerloo, Kaspers, & Cloos, 2011). The assay has the ability to detect accurately from 200 to 100,000 cells per well. Although, MTT assay is considered as the standard method for cells viability analysis, the drawback is that the conversion to formazan crystals relies on the metabolic degree and the number of mitochondria because some viable cells might have a low metabolic activity which may interfere with the results (Twentyman, & Luscombe, 1987). Another important issue in MTT is the length of incubation time as it depends on the cell type and the compounds used in the application. Short incubation sometimes produces too little formazan and this gives incorrect outcomes leading to inaccurate results.

1.3. MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) Assay

MTS is a colorimetric assay for quantification of viable cells that are based on formazan which is similar to MTT, but in the presence of phenazine methosulfate (PMS) or phenazine ethyl sulfate (PES), MTS is able to form a colored formazan product that is soluble in cell culture media (Goodwin, Holt, Downes, & Marshall, 1995). PMS and PES are intermediate electron acceptor reagent that can penetrate live cells to change the tetrazolium to soluble formazan. MTS powder is dissolved in DPBS to 2 mg/ml to produce a clear golden-yellow solution, then PES powder is dissolved in MTS solution pH6.0 and it is kept in dark at 4 °C. The cells can be seeded in 96-well plates containing a final volume of 100 µl per well. MTS then can be used at final concentration of 0.33 mg/ml per well and incubated at 37 °C for 1-4 hours. The absorbance of the produced formazan dye may be measured at 490 nm. It is considered as a rapid cytotoxicity assay because the reagent can be directly added into the media of tissue culture cells without the requisite for washing or solubilization, which are essential in the MTT assay. This assay is very sensitive for measuring the cell viability, but it requires the optimization for each cell type (12).

1.4. XTT (2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) Assay

XTT is a tetrazolium derivative that is similar to MTT and MTS. The quantification of cell viability is based on the activity of mitochondrial enzymes in living cells that reduce XTT. Unlike the insoluble formazan in MTT, XTT is reduced to a highly water-soluble orange-colored product. Therefore, there is no solubilization step needed, but 96-well microtiter plates can be quantified at 475 nm after a 2 – 5 hours required incubation period at 37 °C. Similar to MTS, XTT is also reduced appropriately by the living cells in the presence of phenazine methosulfate. XTT is a sensitive viability assay and less cytotoxic than MTT (Jost, Kirkwood, & Whiteside, 1992).

1.5. Water Soluble Tetrazolium Salts (WSTs) Assay

This assay is also based on mitochondrial metabolic activity but tetrazolium dyes are reduced outside cells in the presence of phenazine methosulfate. Water soluble Tetrazolium Salt (WST) is another descriptive assay based on mitochondrial metabolic activity, but tetrazolium dyes are reduced through a chemical reaction relying on the presence of phenazine methosulfate outside of the cells and produce a water-soluble formazan dyes. Incubation time range is between 1 to 4 hours at 37 °C (Peskin, & Winterbourn, 2000). The absorbance read is obtained at 490 nm. There are many commercial kits containing the solutions of MTS, XTT and WST with the intermediate electron acceptor reagents. However, in general, this class of tetrazolium reagents is prepared at 1-2mg/ml concentration for better solubility, but the concentration of the intermediate electron acceptor depends on the kits provider (Bommer, & Ward, 2016).

1.6. Alamar Blue Assay:

Alamar Blue is a cell-permeable reagent with blue color. Once it enters the cells; resazurin is reduced to resorufin where the color changes to red. Alamar Blue is non-toxic reagent and stable in tissue culture media. The advantage of this assay is that it can be measured by both fluorimetric and colorimetric plate readers (Back, Khan, Gan, Rosenberg, & Volpe, 1999). It also has very high sensitivity where it can measure 50 cells with very a reproducible signal. The linear reading is ranging from 50–50,000 cells. It is considered being a robust, simple, and more sensitive than tetrazolium-based methods (Obrien, Wilson, Orton, & Pognan, 2000). When 1/10 volume of alamarBlue reagent added into each well in 96-well plates, an immediate color change is observed. Incubation time is around 1-4 hours at 37 °C with the protection from direct light. The reduction for alamarBlue measured at 570-600 nm (Patel, Zaveri, Zaveri, Shah, & Solanki, 2013).

1.7. Lactate Dehydrogenase (LDH) Assay

One of the most reliable methods for studying cell death is Lactate Dehydrogenase (LDH) assay. During apoptosis or necrosis, the membrane integrity is altered and LDH enzyme is released. LDH is a ubiquitous soluble enzyme that is present in the cytoplasm. The enzymatic activity of LDH is easily detected in cell culture media. It is a fairly stable enzyme that is able to convert lactate to pyruvate. During this conversion, LDH reduces NAD to NADH and this result in reducing the tetrazolium salt to a red color formazan. This formazan can be measured at 490nm (Decker, & Lohmann-Matthes, 1988). Typically, the LDH quantification is a positive marker to identify the presence of cytotoxicity (Howell, McCune, & Schaffer, 1979). Kits for the fluorometric or colorimetric detection of LDH are commercially available. However, a major inconvenience of these techniques is that physicochemical factors such as pH variations in the culture medium may interfere with the activity of these enzymes (Chan, Moriwaki, & De Rosa, 2013). Subsequently, the activity of LDH may decrease over time at outside environments, which might mask the detection of the type of cell death (Neri, Mariani, Meneghetti, Cattini, & Facchini, 2001).

1.8. Calcein-acetoxymethylester (Calcein-AM) Assay

Calcein-AM is a commonly used membrane-permeable dye to detect cell viability in eukaryotic cells; its acetoxymethyl ester is hydrolyzed through intracellular esterases which then converted to a green-fluorescent calcein. Dead cells with damaged cell membranes do not maintain the dye. The fluorescent dye can be detected by using flow cytometric analysis with excitation and emission wavelengths of 495/515 nm as well as using fluorescent microscopy and the right filter (Neri et al., 2001).

1.9. Propidium Iodide (PI) Assay

PI is a membrane impermeant dye and thus cannot enter live cells having intact membrane, but only the dead cells. Once, PI gains access and intercalates to nucleic acids, its fluorescence escalates and this serves to detect dead cells (Riccardi, & Nicoletti, 2006). The fluorescence molecules intercalating with nucleic acidsin dead cells with compromised membrane can be measured by flow cytometry and fluorescence microscopy. However, to differentiate apoptotic cells from nonapoptotic cells, PI is combined with Annexin-V where apoptotic cells are Annexin-V positive but with intact plasma membranes (Rieger,Nelson, Konowalchuk, & Barreda,z2011). For the reason that the cells in death process lose

their ability to keep phosphatidylserine enzyme active, therefore, this enzyme remain on the outside and is detected by Annexin-V at an early apoptotic stage. This allows the distinction between healthy (Annexin V and PI negative), early apoptotic (Annexin V positive and PI negative), necrotic cells (Annexin V and PI positive). The reagents are applied to live cells for 15-30 min followed by washing and fixation, after that the results are quantified by flow cytometry (Sawai, & Domae, 2011).

1.10. DNA cleavage

The programmed cell death, apoptosis has many changes in the nuclear morphology, particularly chromatin fragmentation. The breakdown of cellular chromosomes is an important phase in apoptosis, and simple gel electrophoresis can assess the DNA fragmentation as apoptotic cells display a characteristic DNA ladder, whereas necrotic cells show a smear of randomly degraded DNA. However, this method is not qualitative, but it only shows the DNA breakdown (Lee, Costumbrado, Hsu, & Kim, 2012). Enzyme-linked Immunosorbent Assays (ELISAs) have been utilized to quantify BrdU (bromolated deoxyuridine)-labeled fragments of DNA in the supernatants of cell culture. BrdU is a synthetic nucleoside that has the ability to incorporate into DNA fragments that are released in the cytoplasm of apoptotic cells. BrdU-labeled DNA fragments can be detected by using antibody against BrdU and provides an estimate of apoptotic cells (Darzynkiewicz, Galkowski, & Zhao, 2008). There are many ELISA kits available where you can easily use to measure BrdU-incorporated in the cytoplasm of damaged cells after the exposures to cytotoxic compounds. These kits were designed to be fast, simple and stable in quantifying the amount of BrdU-labeled DNA in cell culture using microplate and measuring with a colorimetrical plate reader.

1.11. Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling (TUNEL) Assay

It is a method to assess DNA fragmentation that generates a multitude of DNA double-strand breaks (DSBs) with accessible 3'-hydroxyl (3'-OH) groups. Labeled nucleotides are added to the end of DNA fragments enzymatically, where TdT is used to add fluorescent or colorimetric labels to the blunt ends of DNA. This assay is sensitive and there are many commercial kits available that can be used to detect apoptotic cells, however, the drawbacks of this assay is that it needs the cells to be fixed overnight and require several washing steps (Darzynkiewicz et al., 2008).

1.12. Caspase Activity Assays

Caspases are family of cytosteine proteases that are divided into initiator caspases and effector caspases. These two groups of caspases have different structure and functions. Initiator caspases are activated by dimerization, able to send signals to the proteolytic activity which then activate effector caspases in cells undergoing apoptosis. This generates events eventually contributing to the breakdown of the cell which can be easily detected as a marker of caspase activation. Either cleavage or activation of caspases can be quantified to obtain the estimation of apoptosis in the cells (Taylor, Cullen, & Martin, 2008). There are many caspase assays where the detection of apoptosis can be achieved by adding caspase (luminescent, fluorescent, colorimetric) substrates into cells cultured in a microplate format.

Luminescent caspase substrates are based on a bioluminescence compound such as firefly luciferase prosubstrate containing the DEVD tetrapeptide sequence recognized by caspase-3 and caspase-7. The cells need to be seed in white-walled plates to perfume this assay. Upon activation of caspase-3 or -7, the DEVD peptide is cleaved, and the liberated aminoluciferin reacts with luciferase to generate measurable light that is proportional to the caspase activity in the cells. Even though caspase 8 and caspase 3 were reported to be also involved in other nonlethal functions by Yi & Yuan (2009), the detection of apoptosis using caspase enzymes remains reliable and widely used. It is very useful assay for quantification of apoptotic cells and many commercial kits are available. Caspase activity can also be detected using fluorescent caspase substrate where the active caspase is target for example caspase-3, which is considered as the key proenzyme that initiates apoptosis in cell by cleaving the substrate and this generates fluorescent signals from apoptotic cells. This can be measured using a fluorescence reader. Black microplate 96 wells are generally used for this assay to avoid the high fluorescence background (Crawford & Wells, 2011; Gurtu, Kain, Zhang,

1997; Liu et al., 1999). Following the same principle, the colorimetric caspase substrates form a colored product after the cleavage by caspase that absorbs light in the visible range and quantified by a spectrophotometer. This method needs to be performed in a clear plate (Poreba, Strozyk, Salvesen, & Drąg, 2013). The detection can also be done using western blots (de Moraes, Carvalho, Maia, & Sternberg, (2011) and ELISAs for cleaved caspases which normally performed on cell extracts or tissues by immunohistochemistry (Saunders et al., 2000). Several high-quality antibodies for the major caspases such as caspase-3 caspase-7, caspase-8, and caspase-9 are available to assess active and inactive caspases based on the fragments size (Sabine, Faratian, Kirkegaard-Clausen, & Bartlett, 2012). The Bcl-2 family comprises both pro- and anti-apoptotic proteins that regulate the mitochondria-mediated apoptosis. Quantifying the total levels of Bcl-2-family proteins is not a sign of apoptosis, nevertheless their phosphorylation state or conformational alterations are essential indicator for apoptosis. ELISA and western blot can thus be utilized to assess the alterations (Gross, McDonnell, & Korsmeyer, 1999; Hardwick, Chen, & Jonas, 2012; Ola, Nawaz, &Ahsan, 2011).

1.13. Sulforhodamine B (SRB) Assay

Sulphorhodamine B is a water-soluble dye that has ability to bind cellular proteins of vital cells. Cells can be seeded in 96 wells plate, allowed to adhere 24 hours, and then cells fixed with a final concentration of 10 % trichloroacetic acid for one hour at 4 °C after which plates need to be washed with tap water 5 times, air-dried and stained for 30 minutes with 0.4% SRB dissolved in 1% acetic acid. Unbound dye is detached by 1% acetic acid and air dried, but the bound protein solubilized with 10 mM buffered Tris base [tris (hydroxymethyl) aminomethane] for viable cells which can be quantified at 492nm. This measurement provides an accurate estimation of the cell viability. SRB is a colorimetric assay that is considered being a sensitive and inexpensive method for measuring the total cellular protein content of adherent but it has low sensitivity in suspension cells (Keepers et al., 1991; Skehan et al., 1999).

1.14. Mitochondrial Membrane Potential Assay

Changes in the mitochondrial membrane potential (MMP) is one of the indicators of the cell death that can be assessed using several cell membrane permeable fluorescent dyes; 3, 3′-dihexyloxacarbocyanine iodide [DiOC6 (3)], rhodamine-123 (Rh123), tetramethylrhodamine methyl and ethyl esters (TMRM and TMRE), and JC-1, are currently available to measure changes in MMP (Brand, & Nicholls, 2011). These dyes are typically lipophilic and cationic where they can accumulate in active and healthy mitochondria owing to their relative negative charge. Nonetheless, the dyes decrease or fail to accumulate on inactive and unhealthy mitochondria. There are many commercial kits available where the assay can be performed in 96-well, 384-well, or 1536-well plate formats to test the mitochondrial toxicity in cells, which can be assessed by fluorescence microscopy or FACS (Derick et al., 2017).

1.15. Cytochrome C Release Detection

During apoptosis, cytochrome C is translocated from mitochondria to the cytosol. It is a crucial part of the electron transport chain. This protein is located in the space between the inner and outer mitochondrial membrane of live cells. Apoptosis triggers the cytochrome c release into the cytoplasm, where it binds to Apaf-1 (apoptotic protease activating factor-1) which then cleaves and activates caspase-9 and another downstream caspase (Ott, Robertson, Gogvadze, Zhivotovsky, & Orrenius, 2002). Hence, assessment of cytochrome C release is a beneficial tool for determining an early event in apoptosis. There are many different detection techniques, and they vary in their sensitivity, accuracy, and the duration of the assay. Western blotting of cellular fractions is a useful method to detect changes in cytochrome C in the cells. In addition, immunocytochemistry is also used with the cytochrome C antibody. However, these techniques are labor intensive, and appropriate controls are needed during cell fractionation process to avoid contamination of cellular compartments. There are many available commercial kits where the cells are combined with selective permeabilization of the plasma membrane by digitonin and assessed by Flow cytometry. This provides a rapid detection of cytochrome C that has been translocated to the cytoplasm (Campos et al., 2006).

1.16. Poly ADP Ribose Polymerase (PARP) Cleavage assay

PARP is a nuclear enzyme that is involved in DNA repair and makes poly ADP ribose from NAD+ in response to DNA damage. Cellular apoptosis is stimulated via an intracellular signaling cascade when the cells are exposed to damaging agents. During this process, activated caspase 3 cleaves PARP and generates 24 kDa N-terminal DNA binding domain, and 89 kDa C-terminal catalytic domain fragments. These cleaved fragments of PARP can be easily detected by western blotting as indication of apoptosis in the cells (Boulares et al., 1999).

1.17. Changes in cell morphology

Light microscopy or transmission electron microscopy can be used to quantify the morphological changes in cells such as cell size reduction, nuclear condensation and fragmentation, the formation of large clear vacuoles and membrane leaking. Such alterations in cells reveal the basic toxicity and gives valuable information about the pathological stages occurring in response to exposures to toxic compounds. Live cell time-lapse imaging allows real-time, automated monitoring of cell proliferation and morphology by high-definition phase-contrast images after DNA staining (Hollville & Martin, 2016).

1.18. Trypan Blue

This is one of oldest and widely used method in routine tissue culture laboratories and a very simple way to check the cell viability. The healthy and viable cells have intact membranes that prevent trypan blue from entering cells. However, trypan blue can easily diffuse into the dead cells which are eventually recognized as blue-stained dead cells. Cells are placed in hemocytometers and counted manually under light microscopes based on the colors. At the present time, there are numerous recently developed and diversely automated cell counters that reduced the consumed time for counting (Strober, 2001).

1.19. Neutral red assay

Neutral red assay is a quantitative assay that provides a precise estimation of the active and healthy cell number against drug toxicity. The assay is performed based on the binding ability of viable cells to water-soluble, weak cationic and supravital dye in the lysosomes (Repetto, Del Peso, & Zurita, 2008). The related dye penetrates cell membranes by nonionic diffusion; accumulates intracellularly in the lysosomes and incorporates with the anionic site in the matrix of the lysosomes. The amount of dye release reflects the number of viable cells and can be measured at OD 540 nm. However, non-viable cells are unable to take up this dye.

1.20. Colony Formation Assay

Colony formation assay is based on the identification of the number of cells forming colonies in cell cultures. It starts by seeding the cells at low densities, and then after 24 hours, the cells are treated with compounds. The colonies are counted after a certain growth period. This measures the ability of cells to proliferate in either in normal conditions or under stress conditions stemming from toxic compounds. It is one of the most consistent methods for evaluating cell viability, Nonetheless, it is a time-consuming assay and laborious when working with numerous samples (54).

2. CONCLUSION

The discovery of potent cancer drugs involves deciphering the broad range cell death mechanisms including changing in cell metabolisms, DNA fragmentations, cell morphology, caspase activation, and mitochondrial changes. There are a number of decisions to make when selecting the appropriate assay for measuring or detecting the changes that occur in the cells in response to pharmacological compounds. All the assays mentioned in this review have their distinct pros and cons where each needs to be optimized based on the cell types, as well as including a proper negative and positive control for your experiment. Thus, there are no standard cytotoxicity assays, but many factors should be considered such as sensitivity, accuracy, reagent stability, and ease of use. For example, metabolic assays (such as ATP and MTT) where the reduction of substrates is affected by changes in intracellular mitochondrial activity which might have no straight effect on cell viability. This interference sometimes may generate false-positive results. For that reason, the results are needed to be validated by another cell vitality assay.

Consequently, due to the existence of several pathways to quantify cell death levels and the complex relationships between these cellular modalities, most likely a single assay is not sufficient to define the type of cell death, and thus, it is advised to use different but also effective assays with the accurate mechanisms in order to identify and generate significant and clear-cut data assisting to evaluate the cell death.

REFERENCES

Back, S. A., Khan, R., Gan, X., Rosenberg, P. A., & Volpe, J. J. (1999). A new Alamar Blue viability assay to rapidly quantify oligodendrocyte death. Journal of neuroscience methods, 91(1-2), 47-54. doi:10.1016/S0165-0270(99)00062-X.

Berridge, M. V., Herst, P. M., & Tan, A. S. (2005). Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnology annual review*, *11*, 127-152.

Bommer, M., & Ward, J. M. (2016). Micromolar colorimetric detection of 2-hydroxy ketones with the water-soluble tetrazolium WST-1. Analytical biochemistry, 493, 8-10. doi:10.1016/j.ab.2015.09.012.

Boulares, A. H., Yakovlev, A. G., Ivanova, V., Stoica, B. A., Wang, G., Iyer, S., & Smulson, M. (1999). Role of poly (ADP-ribose) polymerase (PARP) cleavage in apoptosis Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. Journal of Biological Chemistry, 274(33),22932-22940. doi:10.1074/jbc.274.33.22932.

Brand, M. D., & Nicholls, D. G. (2011). Assessing mitochondrial dysfunction in cells. Biochemical Journal, 435(2), 297-312. doi:10.1042/BJ20110162.

Campos, C. B., Paim, B. A., Cosso, R. G., Castilho, R. F., Rottenberg, H., & Vercesi, A. E. (2006). Method for monitoring of mitochondrial cytochrome c release during cell death: Immunodetection of cytochrome c by flow cytometry after selective permeabilization of the plasma membrane. Cytometry Part A, 69(6), 515-523. doi:10.1002/cyto.a.20273.

Chan, F. K. M., Moriwaki, K., & De Rosa, M. J. (2013). Detection of necrosis by release of lactate dehydrogenase activity. In Immune Homeostasis (pp. 65-70). Humana Press, Totowa, NJ. doi:10.1007/978-1-62703-290-2_7.

Crawford, E. D., & Wells, J. A. (2011). Caspase substrates and cellular remodeling. Annual review of biochemistry, 80, 1055-1087. doi:10.1146/annurev-biochem-061809-121639.

Darzynkiewicz, Z., Galkowski, D., & Zhao, H. (2008). Analysis of apoptosis by cytometry using TUNEL assay. Methods, 44(3), 250-254. doi:10.1016/j.ymeth.2007.11.008.

de Moraes, G. N., Carvalho, É., Maia, R. C., & Sternberg, C. (2011). Immunodetection of caspase-3 by Western blot using glutaraldehyde. Analytical biochemistry, 415(2), 203-205. doi:10.1016/j.ab.2011.04.032.

Decker, T., & Lohmann-Matthes, M. L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. Journal of immunological methods, 115(1), 61-69. doi:10.1016/0022-1759(88)90310-9.

Derick, S., Gironde, C., Perio, P., Reybier, K., Nepveu, F., Jauneau, A., & Furger, C. (2017). LUCS (Light-Up Cell System), a universal high throughput assay for homeostasis evaluation in live cells. Scientific reports, 7(1), 18069. doi:10.1007/978-1-4939-6346-1_2.

Dunigan, D. D., Waters, S. B., & Owen, T. C. (1995). Aqueous soluble tetrazolium/formazan MTS as an indicator of NADH-and NADPH-dependent dehydrogenase activity. Biotechniques, 19(4), 640-649.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic pathology*, *35*(4), 495-516.

Franken, N. A., Rodermond, H. M., Stap, J., Haveman, J., & Van Bree, C. (2006). Clonogenic assay of cells in vitro. Nature protocols, 1(5), 2315. doi:10.1038/nprot.2006.339.

Goodwin, C. J., Holt, S. J., Downes, S., & Marshall, N. J. (1995). Microculture tetrazolium assays: a comparison between two new tetrazolium salts, XTT and MTS. Journal of immunological methods, 179(1), 95-103. doi:10.1016/0022-1759(94)00277-4.

Gross, A., McDonnell, J. M., & Korsmeyer, S. J. (1999). BCL-2 family members and the

mitochondria in apoptosis. Genes & development, 13(15), 1899-1911. doi:10.1101/gad.13.15.1899.

Gurtu, V., Kain, S. R., & Zhang, G. (1997). Fluorometric and colorimetric detection of caspase activity associated with apoptosis. Analytical biochemistry, 251(1), 98-102. doi:10.1006/abio.1997.2220.

Hardwick, J. M., Chen, Y. B., & Jonas, E. A. (2012). Multipolar functions of BCL-2 proteins link energetics to apoptosis. Trends in cell biology, 22(6), 318-328. doi:10.1016/j.tcb.2012.03.005.

Hollville, E., & Martin, S. J. (2016). Measuring apoptosis by microscopy and flow cytometry. Current protocols in immunology, 14-38. doi:10.1002/0471142735.im1438s112.

Howell, B. F., McCune, S., & Schaffer, R. (1979). Lactate-to-pyruvate or pyruvate-to-lactate assay for lactate dehydrogenase: a re-examination. Clinical Chemistry, 25(2), 269-272. doi:10.1093/jac/dkr570.

Jost, L. M., Kirkwood, J. M., & Whiteside, T. L. (1992). Improved short-and long-term XTT-based colorimetric cellular cytotoxicity assay for melanoma and other tumor cells. Journal of immunological methods, 147(2), 153-165. doi:10.1016/S0022-1759(12)80003-2.

Keepers, Y. P., Pizao, P. E., Peters, G. J., van Ark-Otte, J., Winograd, B., & Pinedo, H. M. (1991). Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for in vitro chemosensitivity testing. European Journal of Cancer and Clinical Oncology, 27(7), 897-900. doi:10.1016/0277-5379(91)90142-Z.

Kepp, O., Galluzzi, L., Lipinski, M., Yuan, J., & Kroemer, G. (2011). Cell death assays for drug discovery. *Nature reviews Drug discovery*, *10*(3), 221.

Lee, P. Y., Costumbrado, J., Hsu, C. Y., & Kim, Y. H. (2012). Agarose gel electrophoresis for the separation of DNA fragments. Journal of visualized experiments: JoVE, (62). doi:10.3791/3923.

Liu, J., Bhalgat, M., Zhang, C., Diwu, Z., Hoyland, B., & Klaubert, D. H. (1999). Fluorescent molecular probes V: a sensitive caspase-3 substrate for fluorometric assays. Bioorganic & medicinal chemistry letters, 9(22), 3231-3236. doi:S0960894X99005661 [pii].

Méry, B., Guy, J. B., Vallard, A., Espenel, S., Ardail, D., Rodriguez-Lafrasse, C., ... & Magné, N. (2017). In vitro cell death determination for drug discovery: A landscape review of real issues. *Journal of cell death*, *10*, 1179670717691251.

Neri, S., Mariani, E., Meneghetti, A., Cattini, L., & Facchini, A. (2001). Calcein-acetyoxymethyl cytotoxicity assay: standardization of a method allowing additional analyses on recovered effector cells and supernatants. Clinical and diagnostic laboratory immunology, 8(6), 1131-1135. doi:10.1128/CDLI.8.6.1131-1135.2001.

O'brien, J., Wilson, I., Orton, T., & Pognan, F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. The FEBS Journal, 267(17), 5421-5426. doi:10.1046/j.1432-1327.2000.01606.x.

Ola, M. S., Nawaz, M., & Ahsan, H. (2011). Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. Molecular and cellular biochemistry, 351(1-2), 41-58. doi:10.1007/s11010-010-0709-x.

Ott, M., Robertson, J. D., Gogvadze, V., Zhivotovsky, B., & Orrenius, S. (2002). Cytochrome c release from mitochondria proceeds by a two-step process. Proceedings of the National Academy of Sciences, 99(3), 1259-1263. doi:10.1073/pnas.241655498.

Patel, H. D., Zaveri, A. D., Zaveri, D. N., SHAH, S., & SOLANKI, A. (2013). Comparison of the MTT and Alamar Blue assay for in vitro anti-cancer activity by testing of various chalcone and thiosemicarbazone derivatives. International Journal of Pharma & Bio Sciences, 4(2).

Peskin, A. V., & Winterbourn, C. C. (2000). A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). Clinica chimica acta, 293(1-2), 157-166. doi:10.1016/S0009-8981(99)00246-6.

Poręba, M., Stróżyk, A., Salvesen, G. S., & Drąg, M. (2013). Caspase substrates and inhibitors. Cold Spring Harbor perspectives in biology, 5(8), a008680. doi:10.1101/cshperspect.a008680.

Repetto, G., Del Peso, A., & Zurita, J. L. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nature protocols, 3(7), 1125. doi:10.1038/nprot.2008.75.

Riccardi, C., & Nicoletti, I. (2006). Analysis of apoptosis by propidium iodide staining and flow cytometry. Nature protocols, 1(3), 1458. doi:10.1038/nprot.2006.238.

Rieger, A. M., Nelson, K. L., Konowalchuk, J. D., & Barreda, D. R. (2011). Modified annexin V/propidium iodide apoptosis assay for accurate assessment of cell death. Journal of visualized experiments: JoVE, (50). Exp. 2011;(50). doi:10.3791/2597.

Sabine, V. S., Faratian, D., Kirkegaard- Clausen, T., & Bartlett, J. (2012). Validation of activated caspase- 3 antibody staining as a marker of apoptosis in breast cancer. Histopathology, 60(2), 369-371.doi:10.1111/j.1365 2559.2011.04024.x.

Saunders, P. A., Cooper, J. A., Roodell, M. M., Schroeder, D. A., Borchert, C. J., Isaacson, A. L., ... & Loegering, R. T. (2000). Quantification of active caspase 3 in apoptotic cells. Analytical biochemistry, 284(1), 114-124. doi:10.1006/abio.2000.4690.

Sawai, H., & Domae, N. (2011). Discrimination between primary necrosis and apoptosis by necrostatin-1 in Annexin V-positive/propidium iodide-negative cells. Biochemical and biophysical research communications, 411(3), 569-573. doi:10.1016/j.bbrc.2011.06.186.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., ... & Boyd, M. R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. JNCI: Journal of the National Cancer Institute, 82(13), 1107-1112. doi:10.1093/jnci/82.13.1107.

Strober, W. (2001). Trypan blue exclusion test of cell viability. Current protocols in immunology, A3-B. doi:10.1002/0471142735.ima03bs111.

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: controlled demolition at the cellular level. Nature reviews Molecular cell biology, 9(3), 231.

Twentyman, P. R., & Luscombe, M. (1987). A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. British journal of cancer, 56(3), 279. doi:10.1038/bjc.1987.190.

Ulukya, E., Ozdikicioglu, F., Oral, A. Y., & Demrci, M. (2008). The MTT assay yields a relatively lower result of growth inhibition than the ATP assay depending on the chemotherapeutic drugs tested. Toxicology in vitro, 22(1), 232-239. doi:10.1016/j.tiv.2007.08.006.

van Meerloo, J., Kaspers, G. J., & Cloos, J. (2011). Cell sensitivity assays: the MTT assay. In Cancer cell culture (pp. 237-245). Humana Press. doi:10.1007/978-1-61779-080-5_20.

Vives- Bauza, C., Yang, L., & Manfredi, G. (2007). Assay of mitochondrial ATP synthesis in animal cells and tissues. Methods in cell biology, 80, 155-171. doi:10.1016/S0091-679X(06)80007-5.

Yi CH, Yuan J. The Jekyll and Hyde functions of caspases. Dev Cell. 2009;16:21–3

Ziegler, D. V., Wiley, C. D., & Velarde, M. C. (2015). Mitochondrial effectors of cellular senescence: beyond the free radical theory of aging. Aging cell, 14(1), 1-7. doi:10.1111/acel.12287.