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A Review on Di Methyl Thiazoldiphenyl-Tetrazoliumbromide (MTT) Assay in Cell Viability

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Abstract: Assessment of cell viability plays a fundamental role in various aspects of basic and biomedical research. It provides crucial initial data prior to performing in vivo and clinical studies. Various types of assay are available for the detection of cell viability which range from the routine trypan blue dye exclusion assay to highly complex analysis of individuals cells. One of the fundamental key to decide before choosing an assay format is the information desired to be obtained at the end of a treatment period. Over the years, di Methyl Thiazoldiphenyl-Tetrazoliumbromide (MTT) based assay is one of the most exploited approaches in cancer research for measuring cell viability, cell proliferation and drug cytotoxicity. MTT assay involves conversion of MTT tetrazolium salt to purple formazon products by viable proliferating cells. MTT assay is known for its rapid and sensitive assay, thus in this review, we focus specifically on MTT-based assay which assess the viability of cell. Besides, this review also focused on the discussion of a standard procedure using MTT assay for measuring viability of cell.

Key word: Cell viability, MTT assay, dimethylthiazoldiphenyl-tetrazoliumbromide, formazon, proliferating cells, exploited approaches

INTRODUCTION

Cell viability: Cell viability can be defined as ability of a maintained sample to show a specific function and expressed a proportion of the same function of an identical fresh untreated sample. In vitro assessment of cell viability is essential to numerous aspects of basic and biomedical research (Riss *et al*., 2003). Besides, it is also a fundamental tool for screening new drugs and chemicals and provides initial data prior to performing in vivo and clinical studies (Riss *et al*., 2003).

Cell viability assays can roughly be categorised into those which analyze whole populations and those which analyze individual cells. The population analysis generates faster result data. However, it gives a less detailed result data than analysis on the single cell level (Stoddart, 2011). Viability may be measured as an index on a scale of 0-100% where higher index data indicates higher number of viable cells. There are two main roles of viability assay, one of which is as experimental assay used to optimise variety parts of preservation procedure during its experimental development. The other is as predictive assay used to predict the quality of an individual sample in an established technique.

Development of cell viability assay: There are a wide range of assays available for the detection of cell viability. These assays are based on various cell functions such as cell membrane permeability, mitochondrial enzyme activity, ATP production and cellular uptake activity (Xu *et al*., 2015). One of the earliest cell viability assays in which is still currently used today is the trypan blue dye exclusion assay (Stoddart, 2011). Other commonly used dye indicators are neutral red and nigrosin (Nath *et al*., 2005). These dyes act as an indication of membrane integrity of living cells, the dyes will diffuse through the cell membrane and stain cellular targets or structures of dead cells (Nath *et al*., 2005). Viable cells do not take in the dye from the surrounding medium because of its intact functional cell membranes and thus appears unstained (Stoddart, 2011). However, nonviable cells do not have an intact functional membrane, thus they take up dye from

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their surroundings (Stoddart, 2011). The conventional method of performing analysis of trypan blue cell viability involves manual staining and use of a hemocytometer for cell counting. However, recent advances in instrumentation have led to a number of semi-or fully automated systems that can increase the throughput and accuracy of this technique (Stoddart, 2011).

Eventually, homogeneous assays for cell viability have been developed and are automated as a High Throughput Screening (HTS). The diverse cell viability assays using high-throughput fluorimetric, colorimetric and luminescence-based assays were developed and optimized to measure cell viability directly in 96-well plate format using a scanning spectrophotometer (Weyermann *et al*., 2005). Biochemical event and cellular ATP content that occurs in living cells are used as viability marker for HTS assays (Stoddart, 2011). Improvement of viability assays which rely on metabolic activity of cells and coupled with HTS method are being preferred to be used in now a days (Stoddart, 2011).

One of the first best known metabolic dyes is 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Stoddart, 2011). Since, the introduction of MTT by Mosmann many other similar dyes of tetrazolium salts such as XTT (2,3-bis-(2-methoxy-4nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), MTS (3-(4, 5-dimethyl thiazol-2-y l) 5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium 5-carboxanilide) (Riss and Moravec, 1992) and the derivatives of WST (Water-soluble Tetrazolium salts) are being used in colorimetric assays (Tan and Berridge, 2000). These assays are based on the ability of reductases and electron carriers of actively proliferating viable cells to transform the tetrazolium salt into coloured formazan derivatives (Riss and Moravec, 1992; Tan and Berridge, 2000; Nath *et al*., 2005; Stoddart, 2011). In contrast to MTT assay, XTT, MTS and WST derivatives assays are reported to be more sensitive and require no additional steps to dissolve the insoluble product (Scudiero *et al*., 1988; Stoddart, 2011). However, major advantage of MTT assay is it can be metabolised by most cell types as compared to other similar tetrazolium salts dyes (Stoddart, 2011). In addition, capacity of the assay had increased dramatically with the development of a 96-well assay format (Riss and Moravec, 1992).

Besides that resazurin-based (PrestoBlue and alamarBlue) methods are also one of the assays being used as it can be measured by either colorimetry or fluorimetry methods (Xu *et al*., 2015). Similar to the mechanism of reduction of tetrazolium salts, blue weakly fluorescent resazurin indicator dye is converted to highly fluorescent pink fluorescent resorufin by active mitochondria (Nath *et al*., 2005). In the viable cells, resazurin-based dye reduction occurs during cellular respiration process by accepting electrons from NADPH, NADH, FADH, FMNH and cytochromes. Thus, PrestoBlue and alamarBlue transform from non-fluorescent to strong fluorescent form. Number of metabolically active cells is propotional to the conversion to strong fluorescent forms (Xu *et al*., 2015). It can then be evaluated quantitatively using fluorescence or absorbance measurements (Xu *et al*., 2015).

Selection to MTT assay: Currently, numerous methods have been developed to assess cell viability. The selection of cell viability assay usually depends on the familiarity with the assay, availability of materials or equipment and convenience of the assay in terms of complexity and duration (Jakstys *et al*., 2015). Despite, methodological differences, these assays are considered to provide comparable cell viability results (Riss and Moravec, 1992). One of the fundamental key to decide before choosing an assay format is the information desired to be obtained at the end of a treatment period. If this fundamental purpose of study is not well understood, this causes misunderstanding of principles of the assays and improper application of the assays and eventually will lead to misinterpretation of the cell viability results data (Jakstys *et al*., 2015).

Over the years, MTT-based assay are one of the most widely exploited approaches in cancer research for measuring cell viability, proliferation and drug cytotoxicity (Stepanenko and Dmitrenko, 2015). The colometric MTT assay is commonly use as HTS and can be quantified by ELISA plate reader. It was a prototype tetrazolium salt introduced by Mosmann for cellular bioassays. Currently, it is used widely to detect viable cells (Tan *et al*., 2009; Teodoro *et al*., 2012; Wang *et al*., 2015; Zeng *et al*., 2016). This assay is versatile as it can also be used for proliferation and cytotoxicity assays (Zhang *et al*., 2016).

MTT is a yellow tetrazole which is reduced to purple formazan in viable cells. An increase in cell viable number will cause a proportionate increase in the amount of MTT formazan formed which will then resulting in an increase of absorbance. The use of the MTT assay method does have limitations where it can influence by either the physiological state of cells or variance in mitochondrial dehydrogenase activity. Nevertheless, the MTT assay method is useful in measurement of cell growth in response to mitogens, antigenic stimuli, growth factors and other cell growth promoting reagents, cytotoxicity studies and in the derivation of cell growth curves (Ittiyavirah *et al*., 2014).

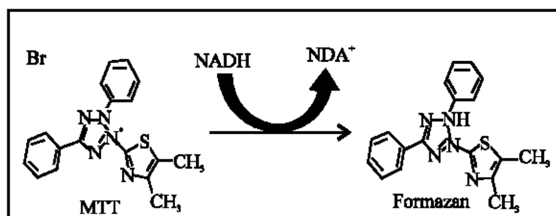

Fig. 1: Reduction process of MTT into formazon using enzyme reductase (Riss *et al*., 2013)

Principle of MTT assay: Principle of colometric MTT assay is related to mitochondrial activity of viable cells which primarily occurs in cytoplasm and rarely in the mitochondria and cell membrane (Meerloo *et al*., 2011). MTT mainly measures the mitochondrial dehydrogenate activity in the a viable cell. The mitochondrial dehydrogenate activity is highly dependent on the concentration of intracellular NADH. During the reduction process, NADH is reduced to NAD+, thus MTT is converted to formazon (Fig. 1). As a consequence of these metabolic processes, the formed insoluble purple formazan will crystalized and radiate from the cells for a few hours.

Organic solvent is required to dissolve the formed formazon crystals before measuring the absorbance. The original published method of the MTT assay describes the use of acidified isopropanol as an organic solvent to solubilize the formazan crystals. Eventually, a variety of methods have been used to solubilize formazan crystal, including the use of acidified isopropanol, detergents (10% Sodium-Dodecyl-Sulfate, SDS), Triton X-100, Nonidet P-40) dimethyl sulfoxide and dimethylformamide. Absorbance of the solubilised formazon crystal can be measured in a broad peak wavelength between 570 and 590 nm. MTT assay conditions can alter metabolic activity and MTT reduction without affecting cell viability.

MATERIALS AND METHODS

Methodology of MTT assay: The MTT compound is soluble in culture medium and cell permeability. The reagent powder needs to be prepared in solution form by dissolving with phosphate buffered saline. There are three main steps in reagent preparation; MTT solution; Solubilization Solution (SDS) and MTT assay protocol.

For a routine assay, MTT solution was prepared as 5 mg/mL of 1 tetrazole in phosphate buffered saline (PBS, 10 mM, pH = 7.2) and sterilized by filtration. The solution was refrigerated for a maximum of 2 weeks. If microbiological contaminations occur during storage period it could be easily detected as purple formazan will precipitate.

MTT assay is prepared by using two reagents: 40% (vol./vol.) dimethylformamide (DMF) glacial acetic acid and 16% (wt./vol.) Sodium Dodecyl Sulfate (SDS). After MTT reagent is prepared and adjusted to pH = 4.7, the assay plate must be incubated at a temperature of 37°C for 1-4 h for signal development (Stockert *et al*., 2012). The SDS is then added and the absorbance is recorded by colorimeter.

Cell viability is determined by the colorimetric MTT metabolic activity assay. Cultured cells (1×104 cells/well) were seeded in a flat bottom 96-well plate at a temperature of 37°C and exposed to varying concentrations for 24 h. Cells treated with medium will served as a negative control group. After removing the supernatant of each well and washed twice by PBS, 20 μL of MTT solution (5 mg/mL in PBS) and 100 μL of medium were then added. After 4 h incubation, the resultant formazan crystals were dissolved in dimethyl sulfoxide (100 μL) and the absorbance of intensity were measured by a microplate reader at 570 nm with a standard reference wavelength (~680 nm) (Zhang *et al*., 2015). All experiments were performed in triplicate and the results were expressed as the percentage of viable cells with respect to the untreated control cells. Cell viability (%) was calculated as ((mean absorbance of the sample-reference absorbance)/reference absorbance of the control)×100.

RESULTS AND DICUSSION

Advantages and limitation of MTT assay: MTT assay is a rapid and sensitive spectrophotometric assay for determining viability in cultured cell lines. It is use for certain applications in normal and drug resistant cell line determinations. The advantage of MTT assay as a high-throughput assay had successfully made an inventory in cell technology by replacing the radioactive isotope based 3H-thymidine incorporation assay.

Initially, the method by Mosmann involved a time-consuming procedure as it requires the solubilisation of formazan crystals in acid-isopropanol solution. However, several modifications had been made to improve the sensitivity of this assay. One of these includes the addition of Dimethylformamide (DMF) to solubilise formazan crystal in aqueous medium, followed by washing step with PBS and solubilising formazan crystals using DMSO (Rensburg *et al*., 1997). However, the drawback of using DMSO is not only does it complicates the assay but also involves exposure to potentially hazardous solution (Ishiyama *et al*., 1996). Besides that increasing the

Table 1: Summary of alternatives application in MTT assay

Applications	References
Assessment of hormone and growth factor	Steenbrugge *et al.* (1991)
Cytostatic and cytotoxic effects	Romijn *et al.* (1988) and Ting *et al.* (2007)
Chemosensivity testing	Loosdrecht *et al.* (1991), Hayon *et al.* (2003) and Sargent (2003)
Measuring radiosensitivity	Price and McMillan (1990)
Clonogenicity	Schweitzer *et al.* (1993)
Growth factors in fluids	Nargi and Yang
Microbial activity	Thom *et al.* (1993)
Three-dimensional histocultures	Singh *et al.* (2002)
Sensitivity testing new drugs	Klumper *et al.* (1995), Kaspers *et al.* (2005) and Oerlemans *et al.* (2008)
Drug screening on cell lines	Sargent (2003)
Measuring drug sensitivity ex vivo to predict clinical outcome	Klumper *et al.* (1995)
Testing drug combination on cell lines	Sargent (2003) and Hubeek *et al.* (2006)

incubation time will also results in increased assay sensitivity as increase in accumulation of formazan colour. But the weakness of this prolong incubation time causes cytotoxicity towards the detection reagents (NADH) that will affect luminescent signal generated by the cell (Riss *et al*., 2003).

Other advantage of this assay is the requirement of fewer cells used if compare with standard cytotoxicity assays (Tonder *et al*., 2015). In addition, it allows for multiple sample concentrations measurement on a single 96-well plate as it rapidly quantitated using an automated spectrophotometric microplate reader. Thus, it is very suitable for determining viability and drug sensitivity. Conventional MTT assay usually involves tedious counting in a haemocytometer which can now be adapted to a microplate format.

The formazan product of the MTT tetrazolium accumulates as an insoluble precipitate inside the cells culture medium. Thus, acidification of the solubilizing solution has the benefit of the color change that may have reducing interference with absorbance readings (Riss *et al*., 2003). In addition, the pH of the solubilization solution can be adjusted to provide maximum absorbance (Plumb *et al*., 1989). Then, there are also several parameters which affected the signal development of MTT, the length of the incubation period, the number of viable cells and their metabolic activity (Riss *et al*., 2003). All of these parameters should be considered when optimizing the assay conditions to generate a sufficient amount of signal that can be detected above the background.

Alternatives application of MTT assay: MTT assay has been utilized with fresh human tumour cells lines and established cell lines of different origins (Jordan *et al*., 1992; Yamashoji *et al*., 1992) for the assessment of hormone and growth factor (Steenbrugge, 1991; Yabushita *et al*., 2003; Thangapazham *et al*., 2007) cytostatic and cytotoxic effects (Romijn *et al*., 1988; Ting *et al*., 2007) and three-dimensional histocultures (Singh *et al*., 2002). Other than that MTT had also been used for analysis of radiosensitivity (Price and McMillan, 1990), clonogenicity (Schweitzer *et al*., 1993) and microbial activity (Thom *et al*., 1993).

MTT-based assays plays a big role in cancer research studies where it has been applied to predict cancer drug chemosensitivity and the resistance (Loosdrecht *et al*., 1991; Hayon *et al*., 2003; Sargent, 2003). Hayon *et al*. (2003) concluded that pre-treatment chemosensitivity assays on leukaemic cells from individual patients could be helpful in selecting the most effective drug treatment options. The MTT assay is also suitable for measuring drug sensitivity in established cell lines and primary cells (Klumper *et al*., 1995; Kaspers *et al*., 2005; Oerlemans *et al*., 2008). The MTT assay is also suitable for screening for modulation of drug resistance (Sargent, 2003).

Besides that the *in vitro* MTT assay has also been extensively applied to predict drug sensitivity *in vivo* for primary pediatric acute lymphoblastic leukemia cells. Previous studies done by Hubeek *et al*. (2006) had observed a relatively good correlation between *in vitro* sensitivity and clinical outcome. Besides that, other studies also identified novel drug combinations which demonstrate significant synergism using MTT assay. It is also used to study the emergence of drug resistance in cell line models with a view to its prevention (Sargent, 2003) (Table 1).

Commercially available kits: A number of kits containing solutions of MTT, solubilization reagent as well as MTT reagent powder are now commercially available as shown below; commercially available kits:

- Cell Titer 96® non-radioactive cell proliferation assay. Promega corporation Cat.# G4000
- Cell Growth Determination kit, MTT based Sigma-aldrich cat.# CGD1-1KT
- MTT Cell Growth Assay kit. Millipore cat. # CT02
- Thiazolyl blue tetrazolium bromide (MTT Powder). Sigma-aldrich Cat. # M2128

The concentration of MTT solution and the nature of the solubilization reagent vary among various vendors. The amount of formazan signal generated is also depending on optimization of parameters (Riss *et al*., 2003). This parameters includes the cell type, number of cells per well, culture medium and others (Riss *et al*., 2003). Although, the commercially available kits are broadly applicable to a large number of cell types and assay conditions but the concentration of the MTT and the type of solubilization solution may need to be adjusted for optimal performance. For this reason, good understanding of reagent used and how it work is crucial in obtaining accurate data.

CONCLUSION

In conclusion, MTT assay as high throughput screening method serves as one of the most contemporary assay which can be use to analyze viability of cells. Besides that, not only does MTT assay benefited in cell viability assay but it can also be applied in other applications such as assessment of cell cytotoxicity and cell proliferation. This proves that MTT assay can provide a wider range of data set which can shorten the research time. The addition of DMSO provides benefit towards the sensitivity of this assay as it generates a more highly accurate data results. However, for this method, there are several restrictions and important factors that should be considered further.

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