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VISUAL EXPERIMENT

MTT assay to evaluate the cytotoxic potential of a drug

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ABSTRACT

Quantification of cell viability and proliferation form the fundamental for numerous *in vitro* assays in response to external factors. An MTT assay is a colorimetric assay based on assessing the cell metabolic activity. A549 Lung adenocarcinoma cell line was used to see the cytotoxic potential of a new drug for initial screening of apoptosis or necrosis. The biochemical mechanism behind the MTT assay involves NAD(P)H-dependent cellular oxidoreductase enzyme that converts the yellow tetrazolium MTT [3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] into insoluble (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan (formazan). The formed formazan can be dissolved with dimethyl sulfoxide (DMSO) to give a purple color with characteristic absorption at 540 nm. Intensity of purple color is directly proportional to the cell number and thus indicating the cell viability.

INTRODUCTION

When a new drug, either natural source or synthesize, is under investigation needs to examine its safety to the host cell or the cytotoxic effect in cancer cell. This is well-known as the cell viability test. This viability cell test may vary from the simple one to the completed one. For example, exposure of cell to trypan blue can be useful to indentify the viable cell (unstained) using a microscope. Dead cell is not stained with trypan blue (Strober, 2001). This method is based on the cell membrane permeability. However, trypan blue staining cannot be used to distinguish between the healthy cells and the cells that are alive but losing cell functions.

Another common method of examining the cell viability is the estimation of lactate dehydrogenase (LDH) level. LDH is present within the cytoplasm. When the integrity of a cell membrane is lost, then the cytoplasmic LDH comes out of the cell, the LDH concentration in the extracellular medium is increased. This method is less sensitive than the MTT assay (Fotakis and Timbrell, 2006).

Other methods used for the assessment of cell viability based on various cellular functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity. Among them tetrazolium (MTT) is one of the most frequently methods. This method uses colorimeter to determine the cell viability (Mosmann et al., 1983). The MTT reagent yields low background absorbance values in the absence of cells. In MTT assay, the linear relationship between metabolically active cells and the color produced is established, thus allowing an accurate quantification of changes in the rate of cell death or proliferation (van de Loosdrecht et al., 1994). MTT is the commonly applied method for evaluation of cell viability and cytotoxicity for screening the drugs. The MTT assay based on the reduction of MTT (yellow colored) and other tetrazolium dyes depends upon cellular metabolic activities due to NAD(P)H-dependent cellular oxidoreductase enzymes (Berridge et al., 2005) (Figure 1). The healthy and rapidly growing cells exhibit high rates of MTT reduction to formazan while the dead or inactive cells fail to do so. The final product of MTT reduction is a purple color formazan that can be easily dissolved in DMSO. Viability in the MTT assay is connected with the quantification of formazan at 540 nm which is linearly associated with the enzyme activity and indirectly the number of viable cells. High purple color intensity denotes higher cell viability while the decrease in purple color intensity signifies the reduced cell number and thus cytotoxicity of the given substance.

MATERIALS AND EQUIPMENTS

1. A549 Lung adenocarcinoma cell line (Sigma-Aldrich, USA)
2. Culture media (RPMI-1640): Contains 20 mM HEPES, L-glutamine and phenol red with pH >7.2 (Sigma-Aldrich, USA)
3. MTT powder (Sigma, USA)
4. Dimethyl sulfoxide
5. Microtiter plate reader (ELISA reader)
6. 96-well microtiter plate (flat-bottomed)
7. Inverted microscope
8. Sterile falcon tubes (15 mL)
9. Multi-channel pipettes
10. Serological pipettes
11. CO_2 incubator
12. Sterile pipette tips
13. Laminar flow hood
14. Hemocytometer
15. Benchtop centrifuge
16. Centrifuge tubes

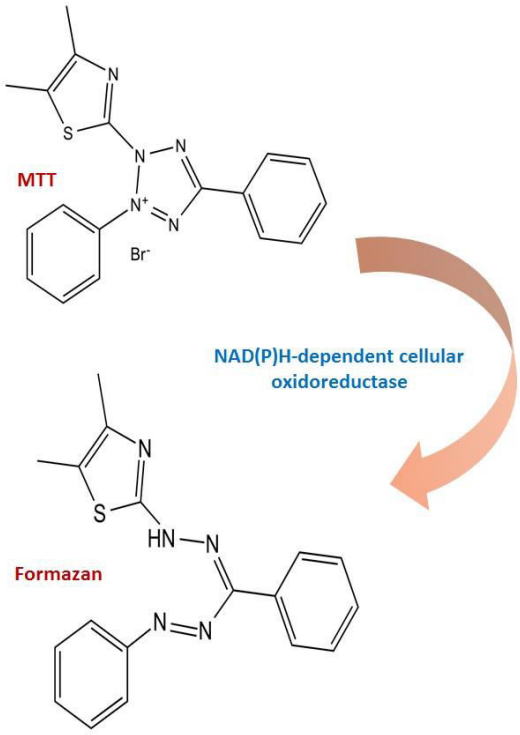

Figure 1: Flow diagram representing the systematic conversion of MTT to formazan

PREPARATION OF REAGENTS

MTT stock solution: Dissolve 500 mg MTT powder in 10 mL phosphate buffer solution. Stir the solution with a magnetic stirrer for about 1 hour in the dark. Filter the sterilized solution with a 0.22 mm filter (Millipore, Ireland) and then store it in 10-mL aliquots (50 mg/mL) at -20°C (van Meerloo et al., 2011). The working solution (5 mg/mL) will be prepared on the day of experiment by dilution.

VIDEO CLIP

MTT assay: 3 min 56 sec

PROTOCOL

1. Cells were seeded in a 96-well flat-bottom microtiter plate at a density of 1 × 10^4 cells/well and allowed to adhere for 24 hours at 37°C in a CO_2 incubator.
2. After 24 hours of incubation, culture medium was replaced with a fresh medium.
3. Cells were then treated with various concentrations of the desired compound for 24 hours at 37°C in a CO_2 incubator.
4. After 24 hours of incubation, culture medium was replaced with a fresh medium.
5. Subsequently, 10 µL of MTT working solution (5 mg/mL in phosphate buffer solution) was added to each well and the plate was incubated for 4 hours at 37°C in a CO_2 incubator.
6. The medium was then aspirated, and the formed formazan crystals were solubilized by adding 50 µL of DMSO per well for 30 min at 37°C in a CO_2 incubator.
7. Finally, the intensity of the dissolved formazan crystals (purple color) was quantified using the ELISA plate reader at 540 nm. Schematic presentation of assay format has been given in Figure 2.

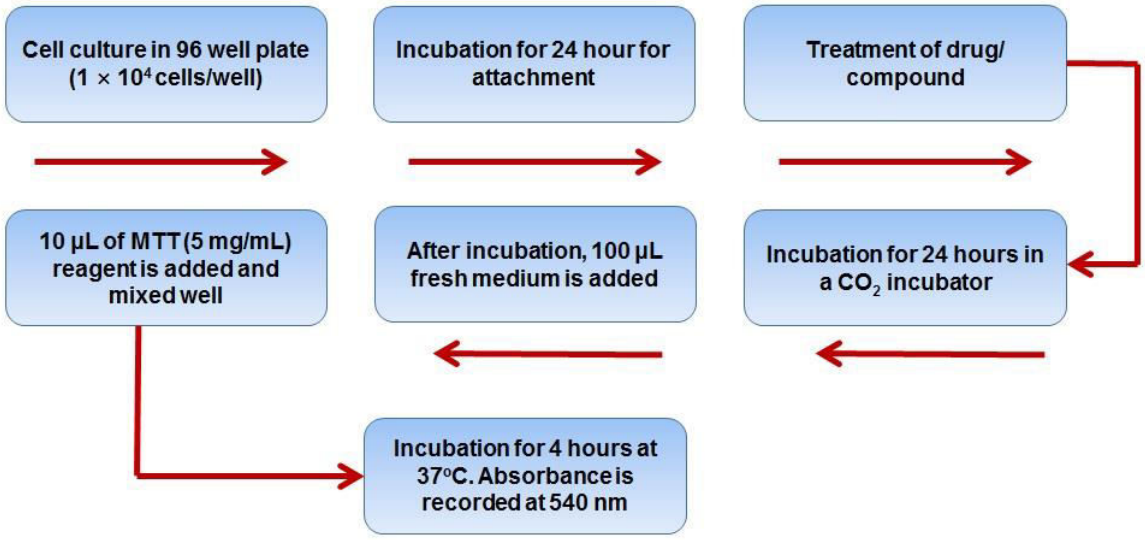

Figure 2: Schematic representation of MTT assay protocol

DISCUSSION

In the present study, the systematic experimental steps in order to determine the potential cytotoxicity of drug at different concentrations by MTT assay are presented in video form. It is shown that a decreasing absorbance at 540 nm in the cells treated with increasing concentration of the drug in comparison to the control cells without any treatment. A decreased absorbance in the cells treated with drug suggesting cytotoxicity. MTT assay significantly helps the researchers to determine whether any of the test compounds has cell toxicity or proliferative activity (Alley et al., 1988, Mosmann et al., 1983).

In this study, attached cells are used in the microtiter plate. That is why, flat-bottomed 96-well microtiter plate is preferred. But in case of suspension cells, either round bottom wells or flat bottom wells are used.

The number of cells in the microplate is not unique for different cell lines and primary cells. The number of cells in the microtiter plate must be optimum to get good result. The number of cells is influenced the level of mitochondrial activity and the rate of proliferation. To get optimum result, several concentrations of cells should be plated in 5-7 plates. Then measure the optical density using colorimeter daily to determine the growth curve of the cell line to prevent overgrowth, which will influence the experiment. The starting optical density value of day 0 should not exceed 0.125 (van Meerloo et al., 2011). The optimal

concentration of plating is established when cells have no lag phase. Then the assay should not proceed further after the log phase.

In this study, the number of cells in the microtiter plate is 1×10^4 cells/well. In case of leukemic cell lines, lower number of cells ($3.0\text{-}4.0 \times 10^3$/well) is used, whereas in primary acute myoloblastic leukemia cells, it should be in the range of $0.08\text{-}0.12 \times 10^6$ cells/well. For primary acute lymphoblastic leukemia cells, it should be 0.16×10^6 cells/well. In case of lung adenocarcinoma cell line, the number of cells will be 1×10^4 cells/well.

MTT is water-soluble that is taken up by the viable cell. The reduction product of MTT is a water-insoluble blue formazan, that must be dissolved for calorimetric measurement. Ethanol, propanol, acid-isopropanol, acid-isopropanol plus 10% Triton X-100, mineral oil (unspecified), or DMSO have all been suggested. DMSO is found to be the most satisfactory (Morgan, 1998).

There are many advantages of MTT assay in particularly its simplicity and effectiveness, which make it more suitable to assess the anti-inflammatory and anti-cancer activities of any test samples at preliminary levels.

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PRECAUTION

The initial cell number must not be seeded more than 1×10^4 cells per well.

Removal of culture media should be done carefully to avoid cell detachment. Culture medium containing high concentration of protein (such as serum, albumin) may cause precipitation when MTT solution is added

Incubation times should be consistent when making comparisons

MTT is toxic and harmful. So, it should be handled carefully. The solution is light sensitive. Care should be taken to protect it from light. Reconstituted MTT solution is stable when stored frozen.

Microbial contamination may contribute to the cleavage of MTT and formation of MTT formazan yielding erroneous results.

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