ATATURK UNIVERSITY PUBLICATIONS

Review

DOI: 10.5152/Pharmata.2023.1287964

In Vitro Cytotoxicity Test Methods: MTT and Neutral Red Uptake

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ABSTRACT

The use of in vitro cytotoxicity tests is increasing day by day with the increase in human exposure to chemicals because these tests involve less cost and less time than in vivo methods. The purpose of in vitro cytotoxicity tests is to detect cell viability. The toxicity of the xenobiotic applied to the cell should be determined. In vitro cytotoxicity tests are frequently used in analyses to detect cell viability, such as drug development and cancer research. This review focuses on MTT and Neutral Red Uptake analyses, which are the most commonly used in vitro cytotoxicity test methods. At the same time, how in vitro cytotoxicity tests are performed, where they are used, and what are the advantages and disadvantages of these tests are also reviewed in this article. The purpose of this article is to help researchers who will use the information about these frequently used tests by compiling them.

Keywords: Cytotoxicity, cell viability, in vitro tests, MTT, NRU

INTRODUCTION

Developing a new therapeutic agent is a long and costly process. Preclinical studies is an important step to eliminate unsuitable candidates and reduce costs before clinical research is undertaken.[1] Therefore, the use of in vitro tests by pharmaceutical companies has increased in recent years.[2] Several methods are needed to ensure the safety of new agents in drug development. For this purpose, in vitro cytotoxicity test methods have been developed.[3]

Cytotoxicity is the inhibition of the synthesis of certain macromolecules in the cell as a result of various events and damage to the structure of the cell. Cytotoxicity tests are in vitro test methods that determine the extent of this damage to cells.[2] In vitro cytotoxicity tests also measure the ability of a compound to cause cell damage.[4] In in vitro cytotoxicity tests, cells are cultured in one microtiter well plate. In direct proportion to the proliferation and growth rate of the cells, the viability of the cell is indirectly measured with the dye used in vitro cytotoxicity test methods. Such tests determine whether the investigated chemical has a cytostatic effect as well as a cytocidal effect that causes the death of cells.[5] In vitro cytotoxicity test methods are important for preclinical studies because the accuracy of the data obtained as a result of these methods will affect the success of the drug candidate to continue.[1,6] With in vitro cytotoxicity tests, the use of animals to find the LD50 values of xenobiotics has been reduced, and xenobiotics have been rapidly screened. At the same time, these tests are simple, repeatable, and economical.[7] Many methods are used to detect cell viability or cytotoxicity in vitro.[8] In this review article, MTT and Neutral Red Uptake (NRU) analyses, which are the most commonly used cytotoxicity test methods to measure cell viability, are reviewed.

MTT

This test method is a colorimetric method introduced by Mossman in 1983 to determine the viability of cells after chemical, physical, and biological processes are applied to cells.[9,10] This method is used to determine the growth and viability of adherent cells. Recently, it has also been used to determine antimicrobial activity and microbial growth.[11-13]

MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a yellow dye that forms positively charged, mono-tetrazolium salts surrounded by 4 nitrogen atoms.[14,15] Since the MTT reagent is positively charged, it can pass through the cell membrane.[16] Thus, tetrazolium salts are converted into purple-colored formazan crystals by the mitochondrial dehydrogenase enzyme in the cell. The amount of formazan crystal increases in direct proportion to the number of viable cells.[17] After the crystals formed are dissolved in a suitable solvent (DMSO or isopropyl alcohol), reading is made by spectrophotometric or microplate reader method.[18] Cell viability is calculated by the following equation:

Received: March 10, 2023
Accepted: April 28, 2023
Publication Date: April 29, 2023

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Cite this article as: Mazlumoglu BŞ. In vitro cytotoxicity test methods: MTT and neutral red uptake. *Pharmata* 2023;3(2):50-53.

Relative cell viability (%) = 100 × (*A*/*B*)

Here, *A* indicates the viable cells in the experimental well and *B* indicates the viable cells in the control.[18]

The MTT test is done as follows:

1. After applying drugs to the adherent cells and keeping them in the CO_2 incubator for a sufficient time, the cells are ready for MTT application.[19]
2. A stock solution of MTT tetrazolium salt is prepared at 5 mg/mL in PBS and stored at +4°C in the dark.[20]
3. Drugs applied at different concentrations to the wells of the 96-well plate and media in the control groups are removed before MTT application.[17,21]
4. 0.1 mL of medium containing MTT is added to each well of a 96-well plate.[22]
5. After the plates are wrapped with aluminum foil, they are incubated for 1-4 hours in a CO_2 incubator at 37°C.[23]
6. At the end of this period, the liquid parts in the wells are carefully removed and discarded.[24]
7. DMSO is added to dissolve the crystals in the wells. After waiting for 10-15 minutes and after a short shaking on the microtiter plate shaker, the reading is taken in the ELISA at 570 nm.[25]

Advantages and Disadvantages of MTT Analysis

Advantages

I. When compared with other in vitro cytotoxicity test methods, MTT analysis is a sensitive test that detects the dose-dependent degradation of xenobiotics in cell function.[26] In addition, MTT offers significant advantages over other viability experiments in terms of speed, simplicity, and precise quantitation.
II. During MTT analysis, the removal of the medium before formazan crystals are formed is the only process. Other than that, it does not require any washing process. This provides an advantage for the use of MTT analysis in non-adherent cell lines.[27]
III. MTT analysis analyzes more samples in less time without exposure to radiation.[28,29]

Disadvantages

I. DMSO or isopropyl alcohol used in MTT analysis can affect the structure and physicochemical properties of the bacterial cell membrane. Therefore, when used in bacterial growth analysis, it may cause erroneous results.[11]
II. In MTT analysis, crystals are formed in the cell.[30] Since these are insoluble in water, these crystals must be well dissolved before measuring because there are differences in absorbance between the wells.[31]
III. The sensitivity of MTT analysis may differ according to cell types.[24]
IV. MTT analysis is based on the conversion of tetrazolium dye to formazan. The rate of conversion to formazan is dependent on metabolic activity and the number of mitochondria. This can cause many interactions.[1]

Neutral Red Uptake Test

Neutral Red Uptake, one of the in vitro cytotoxicity test methods, is used to evaluate the cytotoxicity of various chemical agents such as pharmaceuticals and cosmetics.[32] It is also used to detect the toxicity and phototoxicity of physical agents.[33] Borenfreund and Puerner[34] established the standard protocol for the NRU test in 1984. Then, the In Vitro 3T3 NRU Phototoxicity Test, which was conducted to evaluate phototoxicity in 2000, was accepted in EU member states. It was accepted as an OECD test guideline in 2004.[35] Neutral red (3-amino-7-dimethyl-amino -2-methylphenazine hydrochloride) is a weak cationic dye that dissolves in water and gives a deep red color at slightly acidic pH.[34] The NRU cytotoxicity test procedure is a viability test based on the capacity of living cells to bind neutral red weak cationic dye in their lysosomes.[36,37] It is known that the amount of neutral red dye taken up by the cells is directly proportional to the total number of viable cells.[37]

The construction of the NRU test is as follows:

1. Adherent cells are kept in a CO_2 incubator for 24 or 72 hours after drug administration.[1,33]
2. About 0.04 mg/mL of neutral red working solution is prepared. It is incubated overnight at the same temperature as the cells.[33,38]
3. At the end of the waiting period in the incubator, the condition of the cells to which the drug was applied is checked with a microscope.[33]
4. Neutral red medium was centrifuged for 10 minutes at 1800 rpm to dissolve the precipitated dye crystals.[33]
5. The medium containing the cells is aspirated.[33]
6. 0.1 mL of neutral red medium is added to each well of the 96-well plate.[33]
7. The 96-well plate is left in the incubator for 2-4 hours.[33]
8. At the end of the period, the neutral red medium is removed.[33]
9. The wells are washed with 0.15 mL of PBS.[33]
10. 0.15 mL of neutral red destain solution per well is added.[33]
11. The plate is left on the microtiter plate shaker for approximately 10 minutes until the solution is homogeneous.[33]
12. The plate is measured at 540 nm in the spectrophotometer.[38]

Advantages and Disadvantages of NRU Analysis

Advantages

I. NRU analysis is easy. It provides fast and reliable analysis of large amounts of chemicals in a short time.[32,39]
II. NRU analysis is inexpensive, sensitive, and offers less interference than other tests.[33,34] It also does not use the labile reagents required for cell viability tests using tetrazolium salts (MTT, MTS, XTS, etc.).[33]

Disadvantages

I. NRU analysis is generally not affected by temperature; however, this analysis is affected by contaminants.[40]

DISCUSSION

Today, cytotoxicity test methods are used to determine the toxicological properties of any chemical agent.[31] The use of in vitro cytotoxicity tests has increased in recent years due to the simplicity of their methods and correlation with in vivo cytotoxicity test data,[41] as an alternative to animal testing.[2] In vitro cytotoxicity tests provide important tools for improving human in vitro to in vivo extrapolation.[4] In cell culture, several methods have been developed to study cell viability and cell proliferation. Thus, it is ensured that many samples can be analyzed quickly at the same time.[42] MTT analysis is the most widely used cytotoxicity test among tetrazolium salts.[13] MTT analysis is read using a plate

reader or by spectrophotometric measurement to quantify the number of metabolically active cells.[42,43] NRU analysis is another method. The basis of this method is based on the principle that the neutral red dye, which passes through the cell membrane by diffusion, accumulates in the lysosomes and measures the viability of the cell by spectrophotometric method. At the same time, this method can be used to detect whether there is damage to the cell membrane.[44]

In summary, the advantages and disadvantages of MTT and NRU analyses are mentioned in this review article. The above-mentioned in vitro cytotoxicity tests are colorimetric methods that measure cell viability, which are still used effectively today, despite their disadvantages.

Peer-review: Externally peer-reviewed.

Declaration of Interests: The author declare that they have no competing interest.

Funding: The author declared that this study has received no financial support.

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