Research Article / Araştırma Makalesi

Cellular Imaging Analysis of MTT Assay Based on Tetrazolium Reduction

Tetrazolyum İndirgemesine Dayalı MTT Testinin Hücresel Görsel Analizleri

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Abstract

Background: MTT assay is a colorimetric test to evaluate cell metabolic activity of living cells via reduction of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to insoluble formazan crystals by mitochondrial activities. The reduction of the tetrazolium dye is thought to occur by NADPH-dependent oxidoreductase enzymes in the cell cytosol. The MTT test is used to measure the cytotoxicity or cytostatic activity of generally plant and chemical compounds and toxic materials. In this study, it was aimed to monitor the uniformity of formazan formation at equal time intervals by visualizing the reduction of tetrazolium salts in cells.
Materials and Methods: In the study, K562 cells were used to observe the reduction of tetrazolium salts to MTT formazan crystals in cells. K562 cells were seeded in culture plate under sterile conditions. After adding 10 µL of 5 mg/mL MTT solution to the culture plate, the cells were incubated for 4 h at 37 °C in a humidified environment with 5% CO_2. During the culture process, the cells were imaged at 15 minute-intervals for 4 hours.
Results: The behavior of viable and non-viable cells against MTT and the process of converting MTT to MTT formazan crystal by living cells were clearly monitored.
Conclusions: Visual analysis of MTT reduction directly from the incubator with image recordings at equal time intervals showed the perfect homogeneity of MTT degradation of the cells over time. With our study, we can state that the MTT test is an ideal test method for cytotoxicity research.

Key Words: MTT assay, Tetrazolium salts, Formazan crystals, Reduction.

ÖZ.

Amaç: MTT testi, 3- (4,5-dimetiltiyazol-2-il) -2,5-difeniltetrazolyum bromürün, mitokondriyal aktivitelerle çözünmeyen formazan kristallerine indirgenmesi yoluyla canlı hücrelerin hücre metabolik aktivitesini değerlendirmek amacıyla kullanılan kolorimetrik bir testtir. Tetrazolyum boya indirgenmesinin, hücre sitozolünde NADPH'ye bağımlı oksidoredüktaz enzimleri tarafından meydana getirildiği düşünülmektedir. MTT testi genellikle bitkisel ve kimyasal kökenli bileşiklerin ve toksik materyallerin sitotoksisitesini veya sitostatik aktivitesini ölçmek için kullanılmaktadır. Çalışmada, hücrelerde tetrazolyum tuzlarının indirgenmesinin görselleştirilerek, eşit zaman aralıklarında formazan oluşumunun birörnekliğinin izlenmesi amaçlanmıştır.
Materyal ve Metod: Çalışmada, tetrazolyum tuzlarının hücrelerde MTT formazan kristallerine indirgenmesini gözlemlemek amacıyla K562 hücreleri kullanıldı. K562 hücreleri, steril şartlarda kültür plağına ekildi. Kültür plağına 10 µL 5 mg/mL MTT solüsyonu ekledikten sonra hücreler %5 CO_2'li nemli ortamda 37° C'de 4 saat inkübe edildi. Kültür işlemi sırasında hücreler, 15 dakika aralıklarla 4 saat süreyle görüntülendi.
Bulgular: Canlı ve cansız hücrelerin MTT'ye karşı davranışları ve canlı hücreler tarafından MTT'nin MTT formazan kristallerine dönüştürülmesi süreci net bir şekilde görüntülendi.
Sonuç: Eşit zaman aralıklarında görüntü kayıtları ile direkt inkübatörden MTT indirgemesinin görsel analizi, hücrelerin MTT degradasyonunun zaman içinde mükemmel homojenliğini gösterdi. Çalışmamızla, MTT testinin sitotoksisite araştırmaları için ideal bir test yöntemi olduğunu söyleyebiliriz.

Anahtar kelimeler: MTT testi, Tetrazolyum tuzları, Formazan kristalleri, İndirgeme.

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Introduction

The idea of turning the tetrazolium ring into MTT formazan by breaking it by active mitochondria, and the impression of living cells with the resulting color change, was proposed by Mossman in 1983 (1). Tetrazolium is known as heterocyclic compounds containing nitrogen with four atoms in the tetrazole ring as an organic salt group. MTT, a water-soluble yellow tetrazolium dye, is reduced to purple colored formazan crystals by mitochondrial dehydrogenases, and these crystals are analyzed spectrophotometrically after dissolving in Dimethyl sulfoxide [DMSO (Sigma-Aldrich)] (2). It measures cell viability in terms of reducing activity as the enzymatic conversion of the tetrazolium compound to water-insoluble formazan crystals by dehydrogenases occurring in the mitochondria of living cells, although reducing agents and enzymes found in other organelles such as the endoplasmic reticulum are also included (3). While rapidly dividing cells exhibit a high rate of MTT reduction, cells with low metabolism slightly reduce MTT (4).

MTT method is a method frequently used in cell culture studies. In vitro cytotoxicity tests are measurement methods performed in cell culture in order to evaluate substances that have drug characteristics or whose toxic profile has been investigated and with these tests; it is possible to analyze a large number of substances in a short time (5). For years, tetrazolium salts have been used to measure oxidoreductase activity, subcellular localization of oxidoreductases, and detect super oxide radicals (6).

The tetrazolium / formazan method is used to assess the inhibition of dehydrogenase activity by anticancer chemotherapeutic drugs in excised tissue sections. As a vital *in situ* staining process, this phenomenon has also been used to identify viable colonies of mammalian cells in soft agar culture and to facilitate *in vitro* drug susceptibility analyzes with human tumor cell populations in primary culture (7).

Although the MTT assay is designed for use in eukaryotic cell lines, it has recently been applied for bacteria and fungi. As the mechanism of MTT reduction has been studied in detail, mostly considering eukaryotic cells, it has led to the generation of a wide variety of MTT based protocols for bacterial enzymatic activity assessment (8).

In the study, it was aimed to monitor the functioning of the MTT test on a cellular basis depending on the time-period and to determine whether the formation of formazan in the cells during the test showed a uniform increase in equal time intervals.

Materials and Methods

Cell line

K562 cells are of erythroleukemia type and are round and non-adherent cells. In culture, they exhibit much less clumping than most other suspension lines, possibly due to down regulation of some surface adhesion molecules. Since our study was based on cell culture, ethics committee approval was not received.

Cytotoxicity induction

In order to monitor live and dead cells in the culture medium, etoposide, one of the chemotherapeutic agents known to have cytotoxic effects on cells, is added to the culture medium in dose 4 µM (IC_{50} of etoposide for K562 cells was 8.47 µM) (9). In this way, it was possible to examine morphologically that living cells reduce the MTT dye to formazan crystals and that the non-living cells are unresponsive to the dye.

MTT assay

K562 cells were allowed to proliferate in Iscove's Modified Dulbecco's Medium [IMDM (Sigma-Aldrich)] containing 10% Fetal Calf Serum [FCS (Sigma-Aldrich)] in culture flask at 37 ºC in a humid environment with 5% CO_2. After the cells started to float in culture medium in solitary groups, serial passages of the cells were continued with fresh medium changes. Before MTT analysis, the cells taken from the suspension culture were centrifuged, the supernatant was separated and the cells were transferred to the culture petri dish in the same medium at 10^4 cells / mL Etoposide (Lastet®, Spain) was added to the culture medium at a dose of 200 µg/mL to induce death in some cells after 48 h of culture period. At 72 h of culture, 5 mg/mL MTT (Sigma) solution was added to the culture medium in 10 µl/mL dose and incubation was continued for 4 h.

Cell imaging

The petri dish in which K562 cells were planted was placed on the inverted microscope (Juli BR) in the incubator and the culture process of the cells was started. The imaging process of the cells was started after the addition of the MTT solution, and the recording of the images continued during the 4 h culture period. The conversion of MTT into formazan crystals by the cells in culture medium was visualized and recorded. The time-dependent image records are black and white, depending on the characteristics of the microscope used inside the incubator. At the end of the 4 h incubation period with MTT, the culture petri dish was viewed in another inverted microscope to emphasize the color properties of the formazan crystals and the images were recorded in digital environment.

e study was performed in cell culture using a standard cell line and no human material or experimental animal were used. Therefore, ethics committee approval is not required.

Results

In the study we examined and monitored the MTT reduction reaction of K562 cells on a cellular basis. The response of the cells to the MTT solution and the formation of MTT formazan crystals in the culture process were monitored in a time-dependent manner. After the addition of MTT solution to the culture medium, the cells were recorded in

the culture incubator at 15 minute-intervals and automatic digitally recorded.
K562 cells were monitored in the culture medium before the culture period, and their morphology was visualized before the culture process (Figure 1). When the images were examined, depending on the time period, MTT solution was increasingly converted into MTT formazan crystals in living cells, while no changes were observed in inanimate cells (Figure 2).

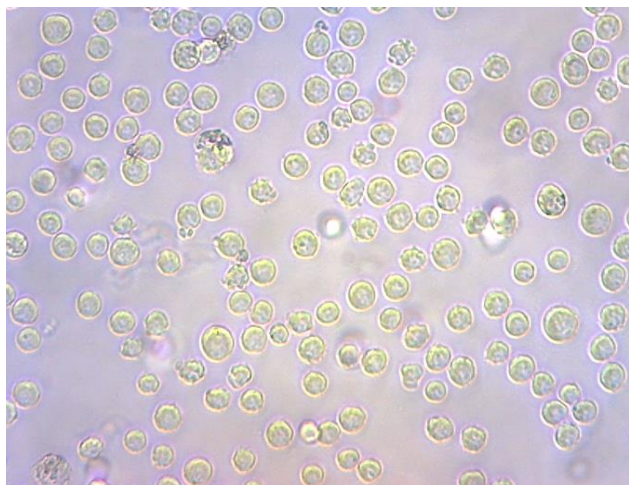

Fig. 1. Suspension culture image of K562 cells in culture flask before MTT test.
K562 cells grow as solitary cells in suspension culture, clustering of cells is observed in culture flask (mag. x100).

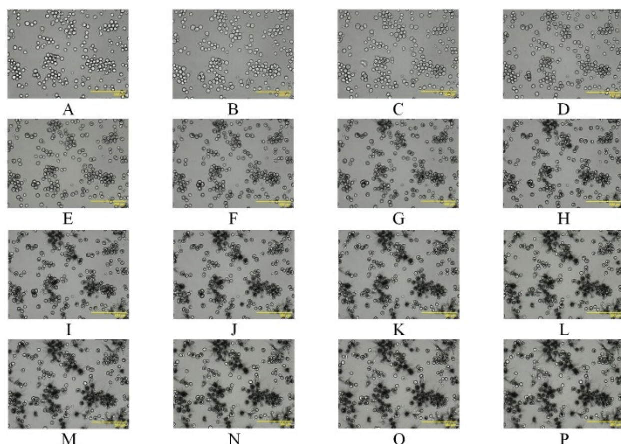

Fig. 2. The conversion of tetrazolium salt to MTT formazan in the culture process of K562 cells.
In order to visualize the MTT reduction of K562 cells, cells were imaged at 15 minute-intervals using the imaging system placed in the incubator. The capturing of the images started with the addition of MTT to the culture medium and continued throughout the 4 h incubation. During the culture process, the formation of MTT formazan crystals in the cells can be clearly observed (The magnification is shown as a scale on the images).

At the end of the incubation period with MTT, it was observed that purple colored formazan crystals were formed, and these crystals were located radially in the cell cytoplasm and partially spilled out of the cytoplasm of the cell (Figure 3).

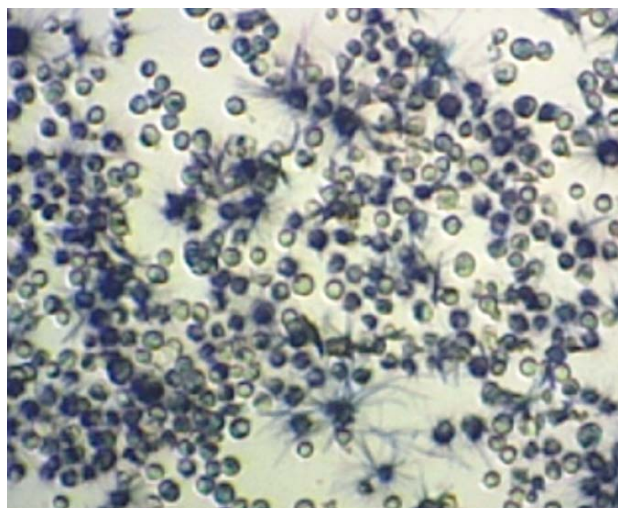

Fig. 3. Color imaging of MTT-formazan crystals formed in cells at the end of the culture process (mag. x100).

Discussion

MTT is one of the most widely used investigation method of *in vitro* cell viability, proliferation, cytotoxicity, and chemical and radiation sensitivity studies (10-15). The idea of visualizing MTT reduction on a cellular basis originated from the purpose of enlightening researchers on how to use visual analysis methods in extraordinary situations that they may encounter. In the study, MTT reduction was visualized step by step in a certain time period, and it was possible to analyze whether our method had worked effectively either during the application or at the end of the application.
In the first studies in this field, it has been shown that MTT is taken up into the cell by endocytosis and the decreased MTT formazan accumulates in the endosomal / lysosomal compartment. MTT formazan occurs in crystal form, coming out of the cell with a needle-like appearance (16).
Some conditions that affect the conversion of MTT to MTT formazan may affect the accuracy of the results obtained from this test. Amyloid β peptide (Aβ), a peptide molecule, can inhibit cellular MTT degradation. The Aβ peptide MTT significantly increases formazan exocytosis, resulting in inhibition of cellular MTT reduction (17).
When the MTT experiment is used to test the cytotoxic potential of methanol extracts of plants, interference may occur, resulting in false positive viability results. Some plant components may have reducing activity as dehydrogenase activity that converts the MTT compound into purple colored formazan. The use of the MTT assay in cytotoxicity tests is not suitable for some plant extracts and requires great care in studies with plants (18).
In a study conducted with *Neozygites parvispora*, this problem was tried to be overcome by using the colorimetric MTT method to determine the cell density while the hemocytometer was difficult to analyze due to the irregular cell shapes and sizes of this fungus. While underlining that the MTT method is an accurate and rapid method for

determining cell densities in small culture volumes, measurement of OD works well if cell shapes are regular, such as in yeasts, but is problematic due to the irregular cell shapes and sizes of *N. parvispora* (19).

In a study performed on 32D cells, a bone marrow-derived cell line, succinate, NADH and NADPH were used as substrates and subcellular localization where MTT reduction occurred was investigated. When succinate was used, it was observed that the MTT reduction activity was in the particle fractions of the cell and in the mitochondrial and light mitochondrial / lysosomal fractions, and when NADH and NADPH were used, increasing amounts of MTT reduction activity were associated with the soluble fractions of the cell and were less related to mitochondrial fractions. When examining the MTT reduction by mitochondrial fraction and the role of electron transport in MTT reduction, succinate-dependent mitochondrial MTT reduction was highly inhibited when respiratory chain inhibitors were used. Unlike succinate, NADPH-induced mitochondrial MTT reduction was observed to be unaffected by any of the respiratory inhibitors tested. Finally, it is underlined that most of the cellular depletion of MTT occurs extramitochondrially and in this event the pyridine nucleotide cofactors NADH and NADPH may play a role (20).

With our study, we believe that by performing a visual and time-dependent review of the MTT test, which is based on the MTT reduction reaction, it will help to shed light on the problems that may occur with the MTT test and help researchers better understand the events that occur in the MTT test.

The study was performed in cell culture using a standard cell line and no human material or experimental animal were used. Therefore, ethics committee approval is not required.

__Ethical Approval:__ The study was performed in cell culture using a standard cell line and no human material or experimental animal were used. Therefore, ethics committee approval is not required.

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Concept: M.Ü.B.; B.S.;F.S.
Literature Review: M.Ü.B.; B.S.;F.S.
Design : M.Ü.B.; B.S.;F.S.
Data acquisition: M.Ü.B.; B.S.;F.S.
Analysis and interpretation: M.Ü.B.; F.S.
Writing manuscript: M.Ü.B.; F.S.
Critical revision of manuscript: M.Ü.B.; F.S.

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