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Use and comparison of MTT, XTT and iCELLigence methods in the evaluation of drug toxicity

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ABSTRACT

Toxicology tests are one of the fundamental methods used in biological and pharmaceutical research. These tests are used to evaluate the viability, proliferation and toxic responses of cells. The study of biological activities of cells plays a critical role in drug development, cancer research, toxicology and various biotechnological applications. Drug toxicology is an important field of research to determine the harmful effects of drugs on cellular and biological systems. In vitro tests are widely used for accurate evaluation of drugs and their toxic effects. These tests examine the effects of drugs on cell cultures, rapidly revealing their potential harmful effects in terms of time and resources. This review discusses the advantages of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and XTT (sodium 3'-[1-(phenylamino)-carbonyl]-3,4-tetrazolium]-bis (4 methoxy-6-nitro) benzene-sulfonic acid hydrate) tests, which are widely used as cytotoxicity tests, as well as the newer method iCELLigence. MTT and XTT are widely used and reliable tests that measure cell metabolism; both methods are very effective in assessing cell viability, but provide limited dynamic data. In contrast, iCELLigence is a newer technology and provides more in-depth data by monitoring the real-time responses of cells. iCELLigence continuously monitors the growth rate and morphological changes of cells, allowing for more comprehensive and sensitive results compared to traditional methods. Comparison of these methods allows determining which methodology provides more appropriate results according to different research needs.These tests are also used to define the concentration range over which more comprehensive and detailed in vitro testing can be performed to obtain meaningful data on parameters such as genotoxicity, mutation induction or programmed cell death. This review aims to compare these three methods and discuss their advantages and limitations in the assessment of drug toxicity.

Keywords: iCELLigence, MTT, XTT, drug toxicity, cytotoxicity

INTRODUCTION

Toxicology tests are important scientific studies conducted to determine the harmful effects of a chemical or biological agent on organisms. The use of these tests is widespread in many areas, especially drug development, environmental protection, food safety and control of industrial chemicals.[1,2] In the pharmaceutical industry, these tests are vital for testing the safety of new drugs, while in environmental sciences they are used to determine the effects of chemical waste on nature.[3,4] Additionally, toxicological testing of food additives is a critical tool for determining whether these substances pose harm to human health.[5] These tests are generally conducted to detect substances that may cause genetic mutations or have carcinogenic effects.[6] These tests measure the response of cancer cells to treatment and play an important role in the development of new drugs. Cytotoxicity tests determine the ability of drugs to target and kill cancer cells and their potential to harm healthy cells. They also help monitor the development of resistance to treatment and enable the development of more selective and effective treatment strategies.[2,6]

Cytotoxicity is defined as the potential of a compound to induce cell death.[7] Detailed studies on the dose and time dependent changes in toxic effects on cells can provide valuable information about necrosis, apoptosis and other mechanisms, together with observation of the effects on the cell cycle and the reversibility of these effects.[8,9] Therefore, in vitro cytotoxicity tests are important and necessary to determine, for example, the potential of a compound to cause cell death by damaging essential cellular functions. Determining the dose at which 50% of the cells are affected (IC_{50}) allows quantitative comparison of the effects of a single compound in different systems or of several compounds in different systems.[10]

MTT test, one of the common methods used in toxicity measurement, measures cell viability based on the metabolic activities of cells. MTT is reduced in living cells to form purple formazan crystals. The density of these crystals varies according to the level of viability of the cells and is usually measured spectrophotometrically.[11] Although the MTT assay is a widely used and relatively simple method in many

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cell types, it has limitations such as resolution issues and the possibility of missing transient toxic effects.[12]

Similarly, the XTT test is another tetrazolium-based method used to measure the metabolic activity of cells. XTT, similar to MTT, is reduced to form soluble formazan products, which makes the measurements more reliable and sensitive. The XTT test has advantages over the MTT test, especially in cases where cell density is high.[13] The accuracy of this test is increased because it does not require the use of solvents, thus providing a clearer measurement of cell death and viability.[14,15]

Finally, iCELLigence technology monitors toxicity and cell viability in real time using the electrical impedance of cells. This technology continuously measures the morphological changes and proliferation rates of cells and detects their response to cell death through changes in electrical conductivity. In this way, electrical changes that occur when the integrity of the cell membrane is disrupted can indicate cell toxicity.[16] iCELLigence is an important tool, especially for long-term and dynamic toxicity monitoring studies.[17]

MTT ASSAY

The MTT test is based on the determination of the metabolic activities of cells, especially by the reduction of mitochondrial dehydrogenase enzymes. Mitochondrial dehydrogenases are located in the cell's energy production center and are involved in basic biochemical processes of the cell, such as oxidative phosphorylation. These enzymes are key components in cell metabolism and are necessary for maintaining cell viability.[18] Mitochondrial activity in living cells is critical for cellular energy production and metabolism. Cells produce ATP using glucose and other substrates for energy production, and some metabolic byproducts are released during this process. The MTT assay provides a measure that reflects these processes and indicates metabolic activity.[19]

The MTT compound is reduced in living cells to form a purple compound. This colorchange is considered an indicator of cellular metabolism. The MTT compound is a compound with a tetrazolium structure. Mitochondrial dehydrogenase enzymes in living cells reduce this compound. Reduced MTT changes from a yellow solution to insoluble formazan crystals with a purple-blue color. Formazan is a product of the mitochondrial activities of cells and this reaction occurs only in metabolically active cells (**Figure 1**).[20] When formazan accumulates, its absorbance value is usually measured using a spectrophotometer. This measurement serves as a biomarker of the metabolic activity of cells. High formazan concentration indicates high cellular metabolism and viability, while low concentration indicates cellular death or low metabolic activity.[21]

The results of the MTT test are usually measured in optical density (OD) values. The purple color obtained is proportional to the cells' metabolic activity; this value is used to understand whether a cell culture is healthy. In order to interpret the test correctly, appropriate control groups and standards must be used.[22]

The protocol uses a standard 96-well plate. This can be scaled up, however, to suit a different plate format. The absorbance

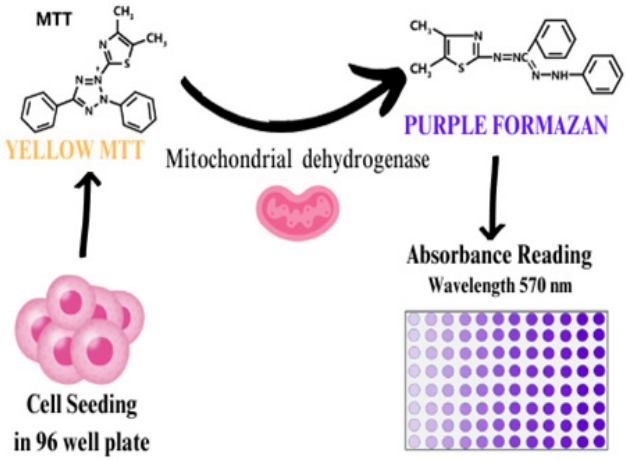

Figure 1. Conversion of MTT to formazan by mitochondrial dehydrogenase activity

of the samples at 570 nm is read using a microplate reader to determine the "half-maximum inhibitory concentrations" (IC_{50}). IC_{50} are calculated using appropriate statistical calculation methods.[11]

The cheapness of the chemical components used in the MTT test and the ease of application providesa great advantage in being a fast and economical test. In addition, the ability to work with a 96-well plate offers the opportunity to apply wide-range doses. With these aspects, it can be used on different cell types and is widely preferred in many drug researches.

In addition to all this, the MTT test only measures cellular metabolic activity, which may be insufficient to examine all aspects of cellular death or toxic effects. Additional studies may be needed for more detailed research outside of cellular metabolism. The MTT test is also limited to rapid observation of cellular responses. As a result, it lacks the ability to monitor dynamic changes.[23]

XTT ASSAY

The XTT assay is based on cellular metabolism, similar to the MTT assay, but provides more sensitive and stable results. XTT is reduced by mitochondrial dehydrogenases in living cells, creating a more stable colored compound.[24]

XTT Compound is a yellow, water-soluble tetrazolium compound. Mitochondrial dehydrogenase enzymes in living cells reduce this compound. Reduced XTT forms a orange colored insoluble formazan product in connection with the metabolic activities of living cells. Cells produce ATP in energy production, especially by using substrates such as glucose and oxygen. In this process, mitochondrial dehydrogenases of cells (e.g. NADH dehydrogenase and succinate dehydrogenase) reduce XTT compound. This reaction only occurs in metabolically active cells, because it is necessary for the continuation of mitochondrial functions and ATP production. Reduced XTT forms formazan crystals in an insoluble form. The density of these formazan crystals is proportional to cellular metabolism. A orange formazan solution is formed by the reduction of XTT. This color change is considered an indicator reflecting the cell's metabolic activity. The density of the orange formazan deposit increases

depending on the energy-producing capacity of the cells and their mitochondrial functions (Figure 2).[25,26]

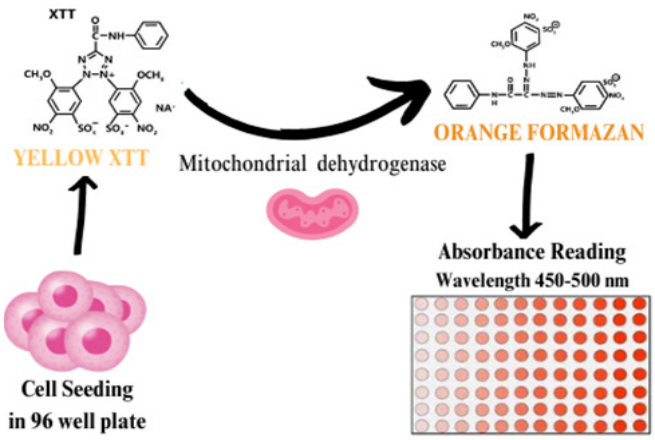

Figure 2. Conversion of XTT to formazan by mitochondrial dehydrogenase activity

The formazan formed can be separated from the solution because it is insoluble and particulate. As a result, the color intensity can be measured. Usually, the absorbance value is measured at a specific wavelength (usually 450-500 nm) using a spectrophotometer. High absorbance indicates that the cells have high metabolic activity and are alive, while low absorbance indicates that the cells have low metabolic activity or are dead.[27] As in the MTT test, IC_{50} values are calculated with the appropriate program and the results are analyzed.[28]

The XTT test provides reliable results even at low cell densities. This allows us to obtain more sensitive results. Since the XTT compound is more stable, the color change is more reliable and increases accuracy. In addition, the XTT test provides results more quickly than the MTT test. However, the XTT test is a more costly method compared to the MTT test because it requires more expensive chemical compounds and devices. In addition, just like the MTT test, the XTT test only measures metabolic activity and does not provide information about the detailed mechanisms of cell death.[29] It also limits our studies on cellular mechanisms such as MTT.[14]

iCELLigence REAL-TIME CELL ANALYSIS SYSTEM

The iCELLigence™ system is a technology that measures the electrical responses of cells. This approach analyzes the electrical properties of cells, especially changes in the electrical resistance of the cell membrane. Biological activities of cells, such as growth, proliferation, differentiation, and apoptosis, are associated with electrical changes in the cell membrane. This method is based on the principles of impedance spectroscopy and electrical impedance, which are commonly used in microelectronics. Electrical impedance is the resistance that cells exhibit to electrical current. Electrical impedance changes as the growth and metabolic activities of cells change the ion flows in their membranes, the membrane potential, and the physical properties of the cells. These changes provide information about the state of the cells. For example, as the cell proliferates, the cell membrane becomes thicker, resulting in increased electrical resistance. On the other hand, external factors that lead to cell death can cause a decrease in electrical resistance.[16,30]

iCELLigence™ continuously measures these electrical changes using a biosensor surface onto which cell cultures are placed. As the cells adhere to these surfaces, they establish an electrical connection with the surface, and the biological activities of the cells cause the electrical responses to change over time (Figure 3). These responses are then analyzed through data processing software to obtain many parameters such as cell viability, growth rate, drug responses, and toxicity levels.[31]

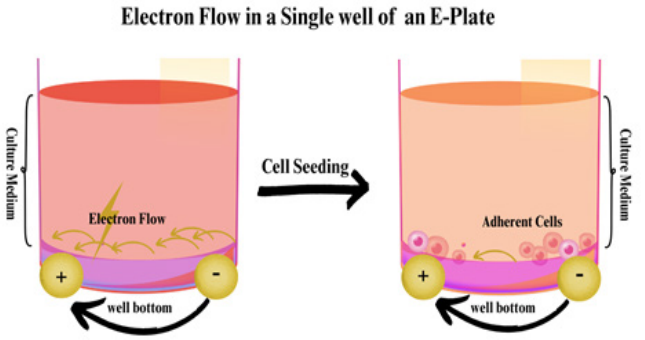

Figure 3. In the iCELLigence system, electron flow from the negative terminal to the positive terminal and the decrease of this electrical effect with cell proliferation

Cells create an electrical barrier between the cell membrane and intracellular fluids.[32] This barrier changes depending on the activity of ion channels, pumps and transport systems in the cell membrane.[33] As cells grow, the physical and electrical properties of this barrier change. iCELLigence™ analyzes cellular activities by measuring these changes.[34]

iCELLigence™ is a much newer system than the other 2 methods. The main feature that sets iCELLigence apart from the others is its real-time monitoring feature. This allows you to watch how cellular responses change over time, which gives you access to dynamic data. While its high-volume wells allow you to use more cell media, iCELLigence offers effective results with fewer cells than other tests. In addition to all this, it provides both electrical and morphological data, providing more detailed information about exactly how cellular responses occur. The ability to easily monitor cellular changes over time through the system allows us to see the onset and development of drug toxicity, which in turn allows us to understand the dynamics of drug toxicity over time. Moreover, this system not only allows us to see the data simultaneously, but can also create graphics. In Figure 4, we can see our graphic example created with the icelligence system in our previous work.[2]

In addition to all these, the system uses gold-plated plates, which are high-volume plastic or glass alternatives. However, these plates have a small sample area compared to other methods, so it may be necessary to plan the study by setting up the system multiple times to try different dose ranges. In addition, the iCELLigence system requires more expensive equipment and technologies. The system's installation and operation may be more complex than other tests.[33,34]

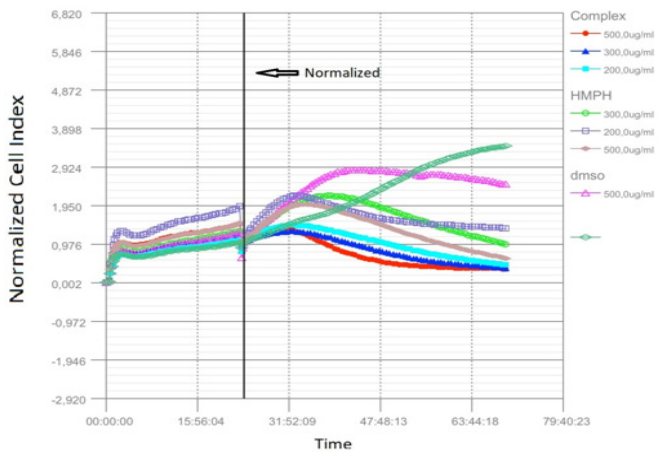

Figure 4. Example graph from iCELLigence system showing normalized cell index

DISCUSSION

Schröterová and colleagues[35] who tried different cytotoxicity assays and compared them when looking at the antiproliferative effects of selenium compounds in colon cancer cells, could not detect a cytotoxic effect except at the highest concentration of the selenium compound. Their MTT assay was more sensitive and gave mutually comparable results with a significant decrease in the measured parameters in a concentration-dependent manner.[35]

In the study conducted by Atmaca et al.[36] in which resveratrol cytotoxicity in human cancer cell lines was calculated by comparing different methods, it is clearly seen that the IC_{50} values were lower as a result of simultaneous monitoring compared to the XTT method. This shows us that the sensitivity of the real-time test is higher.

Garcia and colleagues[37] who investigated the real-time cellular analysis method as a new approach, summarized the benefits of using this method in their study as follows: Daily assessment of cell viability is possible without disturbing the cell culture and without using dyes that could negatively affect the results. In addition, an automated test can be set up to produce results covering the period after the addition of test samples and left unattended for the duration of the experiment. This saves valuable time.[37]

iCELLigence real-time cell analysis system, in another study conducted to examine the cytotoxicity of drugs on cancer cell lines, it was emphasized that this system has a lower risk of contamination.[38]

MTT, XTT and iCELLigence tests are methods that offer different advantages in the in vitro assessment of drug toxicity. These differences are outlined in Table 1. MTT and XTT tests focus on cellular metabolism, providing fast and reliable results, but they cannot fully observe dynamic changes in cellular responses over time. iCELLigence, on the other hand, allows for real-time monitoring of cellular responses, providing more dynamic and comprehensive data. Each of these methods can help evaluate different aspects of drug toxicity and contribute to more comprehensive results throughout the drug development process.

The consumables used in the cell culture phase are common for all 3 methods. However, while the MTT and XTT reagents differ in the subsequent stages, there is no need for a separate reagent for the iCELLigence system. Similarly, while DMSO (dimethyl sulfoxide) is used as a solvent for MTT, DMSO and XTT solution are used for XTT, there is no need for a solvent in the iCELLigence system. Direct electrical response measurement without the need for a spectrophotometer is one of the most important advantages of iCELLigence. In addition to all these, the gold plate plate of the iCELLigence system and the 96 well plate that can be used in other methods are seen in Figure 5. In addition to all these, the iCELLigence method requires a main device that measures electrical impedance and a tablet system that will transfer the data it receives from there. This makes it a more expensive and complex method compared to other methods.

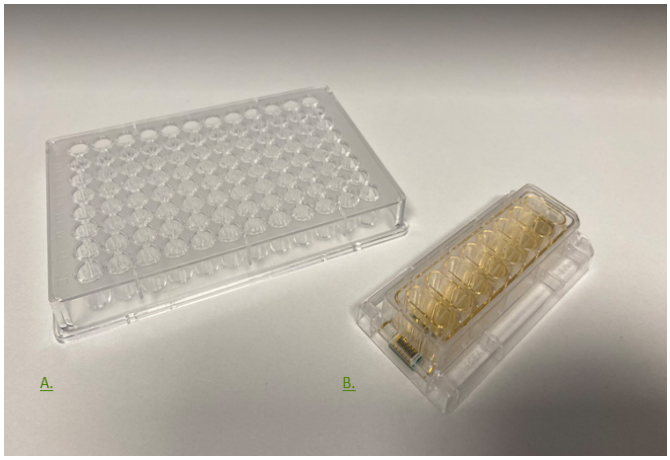

Figure 5. A) 96 well plate **B)** 8 well E-plate

MTT and XTT assays are widely used in cytotoxicity studies to screen new anticancer compounds due to their accuracy and relative simplicity. However, they cannot provide any information about the molecular mechanism of the drug's cytotoxic activity.

The fact that XTT is faster and less toxic than MTT makes it a preferred option in modern laboratories. On the other hand, the iCELLigence method is suitable for studies that require more sophisticated and advanced analyses. Although this

Table 1. Comparison of MTT, XTT and iCELLigence methods[24,28,34]

Method	Advantages	Limitations	Target
MTT assay	It is simple, economical and widely used. It gives fast results.	Measures only metabolic activity. Cannot observe all aspects of cell death.	Cellular metabolic activity
XTT assay	It provides more sensitive, effective at low cell densities and more stable results.	More expensive. Measures limited cellular mechanisms and only metabolic activity.	Cellular metabolic activity
ICELLigence system	It provides real-time monitoring and reliable results with fewer cells. It can perform in-depth analysis of cellular behavior.	It involves higher cost, technical difficulties, and more complex installation and operation.	Dynamic cellular behavior, real-time response monitoring

Table 2. Cellular response types and comparison by methods[39-41]

Response type	MTT assay	XTT assay	iCELLigence system
Metabolic activity (energy production)	High: MTT measures the metabolic activities of cells, specifically mitochondrial degradation.	High: XTT also measures metabolic activity, but is more sensitive and produces soluble formazan.	Medium-High: iCELLigence monitors cell behavior with electrical impedance measurements, an indirect indicator.
Cell proliferation	Medium: The MTT assay indirectly measures cell invasion, but is not specific.	Middle: Although XTT does not directly measure cell number, it monitors metabolic activity based on cell proliferation.	High: iCELLigence can monitor the spread and movement of cells in real time.
Apoptosis	Medium: MTT indirectly measures apoptosis but cannot recognize early stages.	High: The XTT assay can monitor metabolic changes due to apoptosis.	High: iCELLigence can monitor cell death and apoptosis processes based on electrical impedance changes.
Cell membrane integrity	Medium: Indirect monitoring for changes in cell membranes can be done with the MTT assay.	High: Soluble formazan formation in the XTT test provides information about the integrity of the cell membrane.	High: iCELLigence provides very precise, real-time data on cell membrane integrity.
Toxicity	Moderate: MTT indirectly measures toxicity, but early effects before cell membrane disruption cannot be detected.	High: XTT can more precisely measure the effects of toxic compounds on the cell.	High: iCELLigence detects toxic responses with real-time monitoring and can recognize effects faster.
Morphological changes	Low: The morphological status of cells is not directly observed in the MTT assay.	Low: XTT also does not focus on morphological changes, it monitors metabolic activity.	High: iCELLigence continuously monitors the physical morphology and behavior of cells.

method is ideal for monitoring biomarkers and observing the behaviour of cells in much more detail in real time, it is used in more limited areas due to high costs and the need for expertise.

If your experimental goal is to quickly assess the overall health and viability of cells, the XTT assay may provide more reliable and rapid results. Or if more in-depth biological analyses, complex tests such as apoptosis or biomarker monitoring are required, you can choose the iCELLigence method. Although the MTT assay is still economical and widely applicable, XTT or more advanced methods can be used in cases where biological factors other than cellular metabolism must be considered. Considering the advantages and disadvantages of each method according to your experimental goals will allow you to obtain more accurate and reliable results.

Most in vitro cytotoxicity tests focus on measuring cell necrosis. However, apoptosis, another important mechanism of cell death, is a process that needs to be evaluated with different methods. Inhibition of apoptosis is also an important parameter in terms of toxicology.

In addition, detailed studies on the changes in toxic effects on cells depending on dose and time provide important data by observing the reversibility of these effects as well as the effects on the cell cycle. Such studies provide valuable information on the mechanisms and types of toxicity, including necrosis, apoptosis and other cellular events. Comparisons according to the types of cellular responses are given in Table 2.[39-41]

RESULTS

As a result, in vitro cytotoxicity tests are required to determine cell death as a result of damage to basic cellular functions caused by a compound. These tests also provide the basis for understanding more complex parameters such as genotoxicity, mutation induction, and programmed cell death. Determining the dose at which 50% of the cells are affected (IC_{50}) allows comparison of the effects of a single compound in different systems or of several compounds in different systems. This review compares three different methods in many ways to help us understand the role of each method in the assessment of drug toxicity. It is possible to say that these data will be useful for decision makers and researchers, especially in drug development processes.

ETHICAL DECLARATIONS

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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