Chapter

Colorimetric Cytotoxicity Assays

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Abstract

Cytotoxicity experiments are carried out to evaluate whether a chemical has cytotoxic potential. Because of its ease of use and compatibility with data collected from *in vivo* investigations, cell-based cytotoxicity studies have emerged as a viable alternative to animal trials in research. Cell-damaging events such as apoptosis, autophagy, and necrosis may occur after exposure to cytotoxic substances. Thanks to the cell-based cytotoxicity studies, basic information is obtained about the cytotoxic effects of the tested substance. To measure cell viability, a variety of techniques are used. Regardless of the sort of cytotoxicity investigation that was carried out, the crucial thing is to figure out how much metabolic activity there is in the cells at the end of the experiment. Cytotoxicity detection methods are generally colorimetric, luminescent, and enzymatic methods. In colorimetric methods, measurement is based on color change using tetrazolium salts, such as MTT, MTS, XTT, WST. Three main steps are followed in tetrazolium compound toxicity tests. Toxic compounds are introduced to cells in the initial stage. The poisonous chemical is eliminated in the second phase and followed by the addition of the tetrazolium compound. The metabolically active cells are determined in the last stage by using a spectrophotometric approach to measure color change.

Keywords: cytotoxicity, colorimetric assay, formazan, *in vitro*, metabolic activity

1. Introduction

Cytotoxicity assays measure the destructive capacity of substances on living cells or tissue systems. These assays play a key role in the biomedical field as they provide information on the therapeutic potential of the biochemical molecules. Cytotoxicity assays can be applied to both *in vivo* and *in vitro* systems where each method has some advantages and disadvantages. In this chapter, we present and discuss conventional *in vitro* colorimetric cytotoxicity assays that were optimized for spectrophotometric analysis.

There are many cytotoxicity tests with different mechanisms and sensitivities. However, cytotoxicity determination methods are generally examined in three groups. These are colorimetric, luminescence, and enzymatic methods. Luminometric methods are divided into fluorescence and bioluminescence. Fluorometric assays utilize fluorescent substances such as resazurin and are performed with a fluorometer or fluorescence microplate reader. On the other hand,

cytotoxicity detection in bioluminescent methods is made by an enzyme called luciferase. In addition, in the real-time bioluminescence method, the exposure of cells to cytotoxic substances has become possible to be examined during the exposure. Enzymes leaking into the medium following cell damage or death have also been considered markers of dead cell counts. Among these enzymes, lactate dehydrogenase (LDH), which stands out with its stability, has taken its place among viability tests as a marker of cell death.

Colorimetric methods are techniques based on color change using tetrazolium salts or specific staining of cells using crystal violet and neutral red dyes. The first colorimetric method was described by Mossman et al., in 1983, which was developed as an alternative to the labor-intensive, time-consuming, and costly radioactive methods of measuring surviving and/or proliferating mammalian cells [1]. Furthermore, they can measure large numbers of samples with a high degree of precision directly in the plate by using a spectrophotometer or plate reader [2, 3]. There are also methods used especially in routine cell culture processes, such as microscopy (staining with trypan blue-Thoma slide) or an automatic cell counter that can be used in cytotoxicity studies. However, these methods are very time-consuming and are not suitable for studies with a large number of samples.

The basic principle of colorimetric assays lies in the color development as a result of metabolic activity rate of the cells. When a reagent meets with the viable cells, it is converted to a colored product by the metabolic activity of the cells. Such color change can easily be detected, measured, and quantified via spectrophotometric measurements as absorbance at a specific wavelength (**Figure 1**). Collected signal reflects the viable cell population or metabolic activity of the cells. As dying cells lose their function to convert substrate into a detectable colored product, the observed signal would be less than the active population.

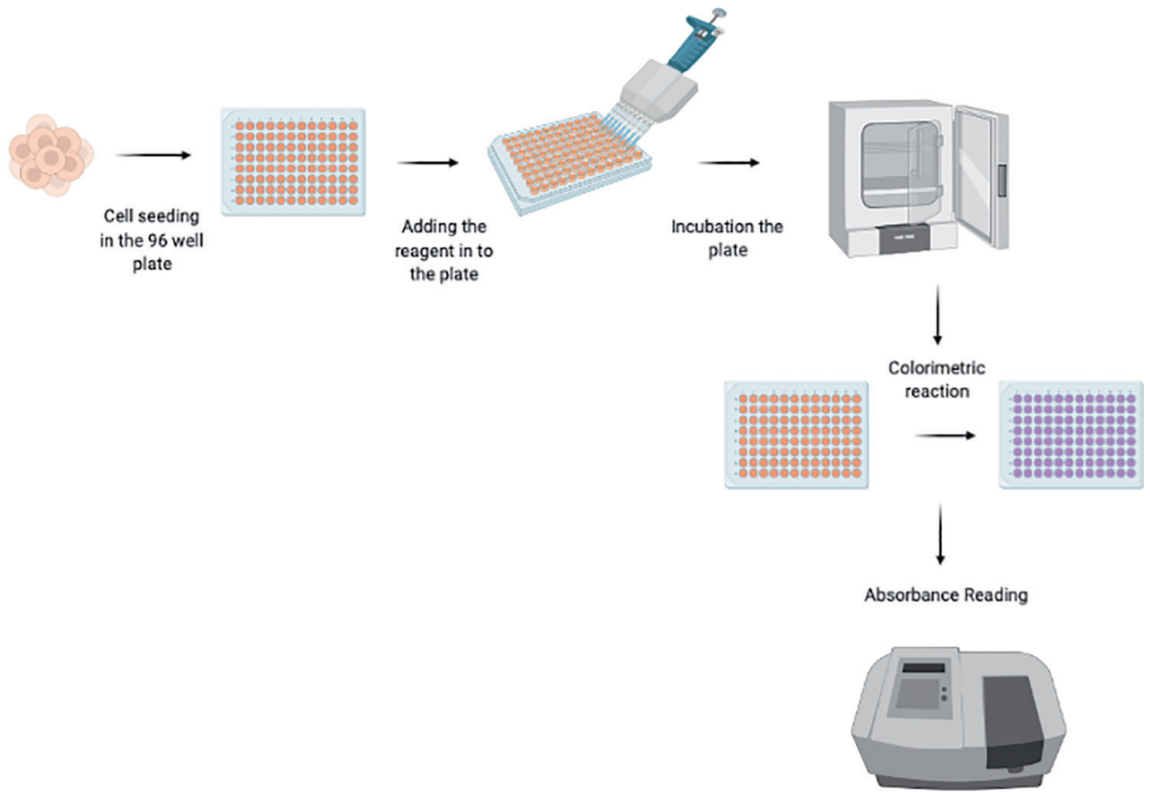

Figure 1.
A simple representation of colorimetric detection assays.

2. Colorimetric assays with tetrazolium salts

In colorimetric cytotoxicity methods, measurements are made based on color change upon addition of tetrazolium salts. Tetrazolium salts are heterocyclic organic compounds. The reduction of tetrazolium salts by gaining electrons enables them to transform into a formazan structure and leads to a color change. The tetrazolium ring can be broken by active mitochondria, and therefore, color change can only occur in metabolically active cells. Taking advantage of these properties, many tetrazolium compounds have been developed [4]. However, only a few of the tetrazolium salts have been accepted and adapted to biological systems. The most widely used tetrazolium salts are listed below:

- MTT: 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl-2tl tetrazolium bromide
- XTT: 2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide
- MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2-(4-sulfophenyl) -2H-tetrazolium
- WST-1: 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H tetrazolium
- WST-8: 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H tetrazolium

These compounds can be divided into two groups based on their ability to enter the cell and dissolve in the medium. MTT sits in one of these groups that the tetrazolium salt that has the ability to enter the cell. This salt is a positively charged compound and can be reduced within the cell by conveniently passing the membrane of eukaryotic cells. However, the formazan from reduction is insoluble in water and therefore precipitates in the medium as crystals. Unlike MTT compounds, MTS, XTT, and WST compounds are naturally negatively charged and cannot fully penetrate the cell membrane. Therefore, they use an electron-accepting molecule with themselves. The electron acceptor molecule enters the cell, takes electrons from the cytoplasm or plasma membrane, and returns to the medium to reduce the tetrazolium compound. Formazan, which is formed as a result of the reduction of these compounds, is soluble in water and the medium. Phenazine methyl sulfate and phenazine ethyl sulfate are generally used as electron acceptors in these reactions [5].

3. Spectrophotometry-based colorimetric assays

Tetrazolium salts and their formazan products have been popular candidates for spectrophotometric methods. In this section, the most commonly used salts and their usage methods will be explained.

3.1 MTT assay

Tetrazolium salts are a large group of heterocyclic organic compounds that form highly colored and generally insoluble formazan upon reduction. These compounds,

which were first prepared and used in 1894, have been widely used in tests for both biological redox systems and indicators of vitality. MTT is one of the first tetrazolium salts introduced by Mossman in 1983 [1, 6].

MTT molecule is a mono tetrazolium salt and its reagent is 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl-2tl tetrazolium bromide, consisting of a positively charged quaternary tetraazide ring nucleus containing four nitrogen atoms surrounded by three aromatic rings containing two phenyl moieties and a thiazyl ring (**Figure 2**) [7, 8].

MTT reduction is one of the most commonly used methods to measure cell proliferation and cytotoxicity. It is an important substance in ongoing tests to determine cytotoxic responses to mitogens, antigenic stimuli, and growth factors. The MTT test is used not only for cell samples, but also for testing tissue cultures. It is used in skin irritation, skin corrosion, and eye irritation tests [9].

The decrease, that is, reduction, of MTT, which passes through the eukaryotic cell membrane very easily, causes the nuclear tetrazole ring to deteriorate and the formation of a violet-colored water-insoluble formazan (**Figure 3**).

As MTT is a positively charged molecule, it can easily pass through the cell and the mitochondrial inner membrane of metabolically active cells. Such viable cells reduce MTT into formazan in their mitochondria as they keep their metabolism in regular activity. The intensity of intracellular formazan produced by this redox reaction is measured via colorimetric-based system in spectrophotometry *in vitro*.

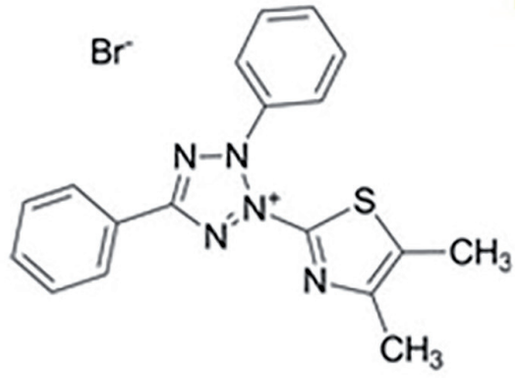

Figure 2.
The molecular structure of MTT tetrazolium salt.

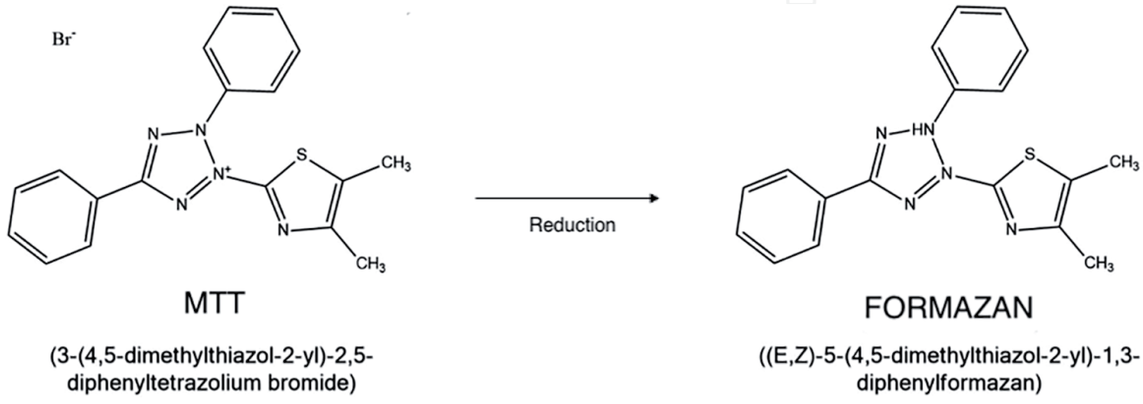

Figure 3.
Redox reaction principle of MTT assay.

The MTT test takes place as a three-stage process. In the first step, the cells or tissue samples to be tested are exposed to the toxic substance. Tetrazolium salt is added to the sample obtained after the toxic substance is removed. In this process, MTT is used as the tetrazolium salt [8].

MTT is originally a yellow-colored substance. During cell proliferation, a reduction leads to an increase in the mitochondrial dehydrogenase enzyme activity and forms a purple crystal structure, formazan (**Figure 4**). The MTT assay is typically performed in several hours (1–4 hours) after incubation of cells with MTT. The formazan crystals that are formed after reaction are insoluble in water. In order to measure the absorbance, they must be dissolved in a suitable solvent, which solubilizes the product. Therefore, the formazan is usually dissolved with a solvent such as dimethyl sulfoxide (DSMO) or isopropanol before the measurement recording [10, 11].

The concentration of formazan dissolved in the appropriate solvent is determined by optical density at 570 nm. As a final step, the color change in the sample should be measured by the spectrophotometric method (**Figure 5**). The reason for this step is to measure cell viability. The measured OD values are considered to be a representation of the intracellular reduction of formazan concentration and thus of MTT. The cellular viability rate of the untreated control group is taken as 100% and proportional calculations are made according to this control group.

One of the reasons why the MTT assay is preferred is that it is a reliable and fast method. The fact that the MTT assay has fewer steps compared with other tests increases the reproducibility. Among the positive aspects of the MTT assays are that more than one sample can be examined with a single test mechanism and the result is sensitive [12].

As with every assay, MTT assays also have disadvantages. If these disadvantages are well understood and necessary precautions are taken, it does not show a negative effect during the application of the test.

As stated earlier, MTT formazan is insoluble in water. After the reaction, it forms needle-shaped crystal structures in the cells. Therefore, these formed crystals must be dissolved before proceeding to the measurement phase. If this dissolution step is not performed well, there may be a difference in absorbance between the wells [13].

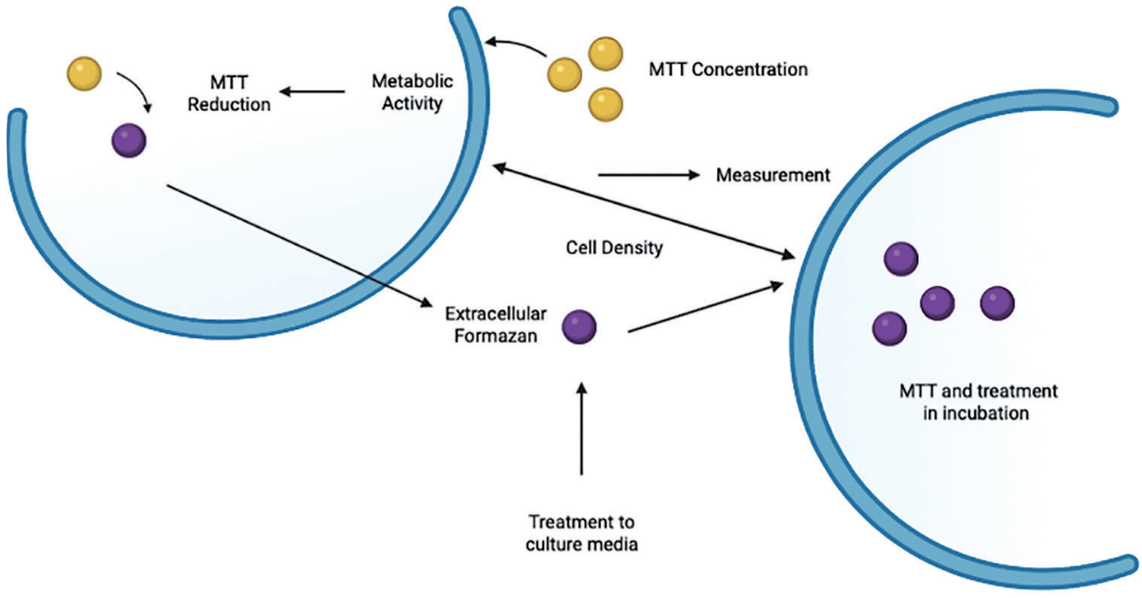

Figure 4.
The illustration of the mechanism of MTT.

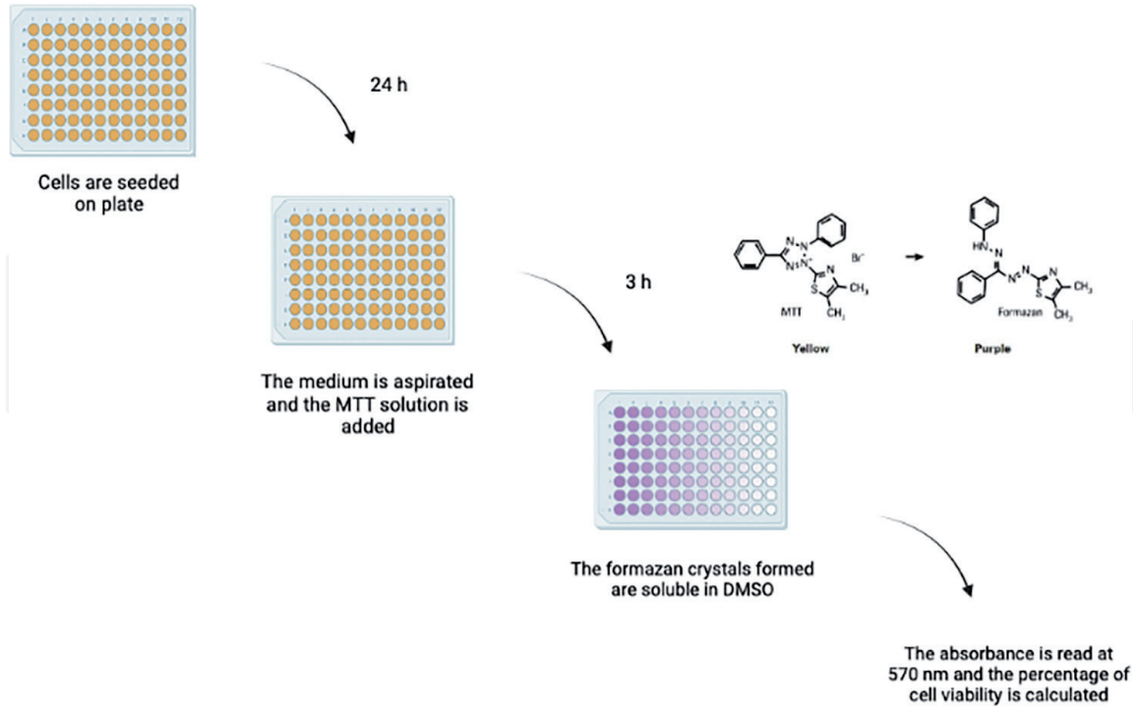

Figure 5.
General steps of MTT assay.

The DSMO substance used during the dissolution process of MTT formazans can have a toxic effect. Care should also be taken when adding DSMO to the wells. The pipette used during the application may damage the formazan crystals. This can create undesirable deviations in the results.

MTT formazan can be a toxic substance due to its structure. Therefore, a control group should be established for cell death observed due to MTT formazan toxicity. In this way, false-negative or -positive results can be avoided.

3.2 XTT assay

After the MTT test developed by Mossman (1983), other tetrazolium compounds such as XTT (2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) were described by Scudiero et al. in 1988 for the colorimetric method [14]. The XTT procedure is easy to measure proliferation, so it is an acceptable solution for quantifying cells and determining their viability. XTT is a method that is used to determine how cells respond to different growth factors, foreign chemicals, drugs, etc.

XTT testing is a fast, responsive, simple, and safe strategy for using the determination of cytotoxicity. It also offers a high level of sensitivity and precision yet the XTT assay's performance is highly dependent on the mitochondrial dehydrogenase activity of living cells. Therefore, some factors can affect the final absorbance reading. These factors are changes in the reducing capacity of living cells resulting from enzymatic regulation, pH, cellular ion concentration, cell cycle variation, and other environmental factors [15].

Having negatively charged ions on the structure, XTT cannot penetrate the cellular and mitochondrial membrane. Therefore, it is required to incorporate electron acceptor molecules such as phenazine methyl sulfate, phenazine ethyl sulfate, etc.

Phenazine methosulfate (PMS) is an electron acceptor often used in XTT tests. The oxidized (cationic) form of PMS has a yellow color, whereas the reduced derivative shows no color. The reduced PMS is utilized in this assay as an electron carrier since it is rapidly oxidized by oxygen. PMS can also be reduced non-enzymatically by NADH and NADPH. The reduction at the cellular level is gained by PMS at the plasma membrane level and reduces XTT outside the cells, thereby increasing the water solubility of the dye and formazan [5].

As illustrated in **Figure 6**, the tetrazolium salt XTT is reduced to orange-colored formazan by metabolically active cells. The electron acceptor molecules receive electrons from the cell and initiate the redox reaction to reduce the tetrazolium compound. The orange-colored formazan is dissolvable in water, thus the color intensity can be measured with a spectrophotometer. The number of metabolically active cells is proportional to the intensity of the color detected [3].

XTT testing is a fast, responsive, easy to use, and safe method for using the determination of cytotoxicity. It also has high sensitivity and accuracy. In contrast to some other salts, the formazan dye is soluble in aqueous solutions and can directly be quantified using a scanning multiplate spectrophotometer (ELISA-based, **Figure** 7). This enables a high degree of accuracy, allows online data processing by computers, and thus allows a high number of samples to be handled quickly and conveniently (4).

3.3 MTS assay

The MTS assay, which is produced as an alternative to the MTT assay, is an MTT analog that is generally formed by adding sulfonate, methyl, or similar groups to the MTT tetrazolium salt. The structure of the MTS tetrazolium salt is (3- (4,5-dimethylthiazol -2-yl) -5-(3-carboxymethoxyphenyl) 2-(4-sulfophenyl) -2H-tetrazolium) (**Figure 8**). It is also called one-step MTT assay because it is an analog developed to facilitate the MTT assay [16].

MTS tetrazolium salt is negatively charged by nature. As this situation does not allow passage through the cell membrane, the help of intermediate electron acceptor (IEA) molecules is needed. Electron acceptor molecules such as phenazine methyl sulfate (PMS) or phenazine ethyl sulfate (PES) enter the cell, and electron acquisition occurs from the cytoplasm or plasma membrane, and the reduction reaction of the tetrazolium salt takes place. The formazan (dark pink/red color), which is produced as a product after the reduction reaction, is easily soluble in water. Unlike the MTT assay, since the formazan formed after the reduction reaction is water-soluble, there is no need for a second procedure during the test (**Figure 9**) [17–19].

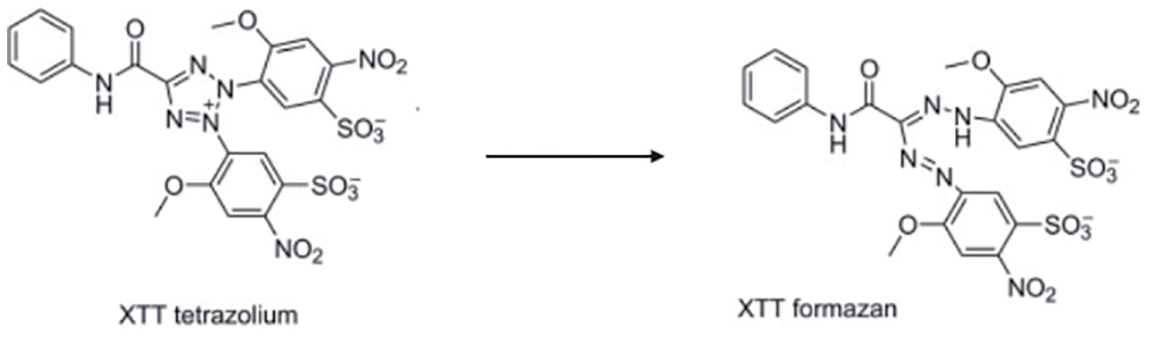

Figure 6.
Conversion of XTT to formazan by mitochondrial dehydrogenase.

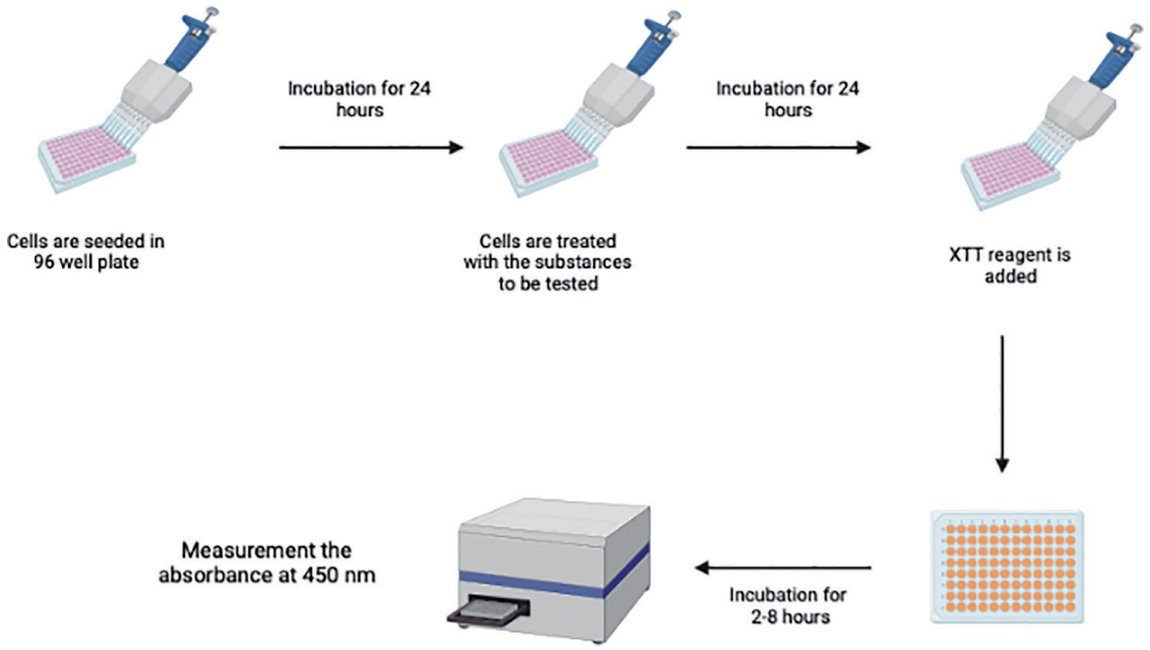

Figure 7.
Illustration of XTT assay principle.

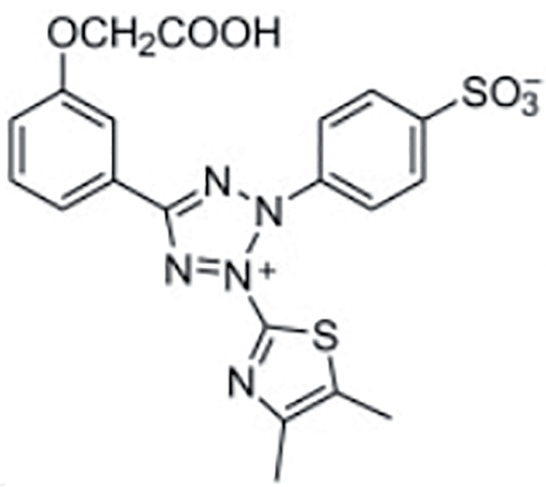

Figure 8.
Chemical structure of MTS tetrazolium salt.

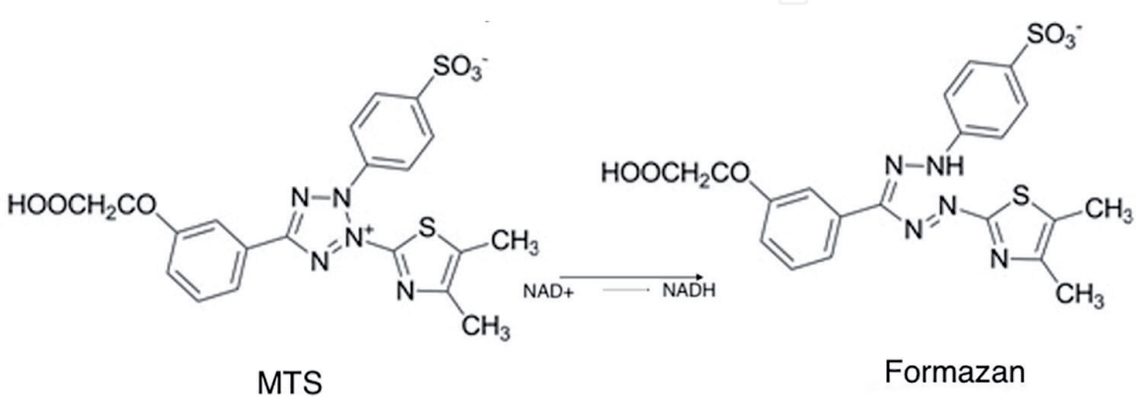

Figure 9.
Reduction of MTS to an aqueous soluble formazan through the transfer of electrons from NADH in the cytoplasm.

The test procedure performed is the same as the MTT assay. MTS salt added to the cells in the culture medium is measured by spectrophotometer at 492 nm after the determined incubation period (30 min–4 hours). MTS assay, which has taken its place among *in vitro* cytotoxicity tests due to its advantages, is sensitive, fast, and easy to apply. Although the MTS assay provides ideal properties for cytotoxicity measurements, the level of absorbance at 492 nm depends on the incubation time applied, the cell type, and the number of cells tested. Considering all these substances, it is a suitable and prone test for use in toxicological test evaluations in the right places [10, 19].

3.4 WST assay

The WST method is another colorimetric method based on the principle of tetrazolium salts that produces a water-soluble formazan product. Among the water-soluble tetrazolium salts, the most frequently used one today is WST-1 in the form of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H tetrazolium and another frequently used WST compound is WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H tetrazolium) (**Figure 10**) [20].

After the formed formazan crystals dissolve, they can be quantified quickly and easily at an absorbance value of 450 nm in a conventional microplate reader. The absorbance value measured spectrophotometrically in the WST-1 method is related to the number of viable cells (**Figure 11**). As the proliferation increases, the absorbance value increases due to the formation of formazan salt. Since proliferating cells

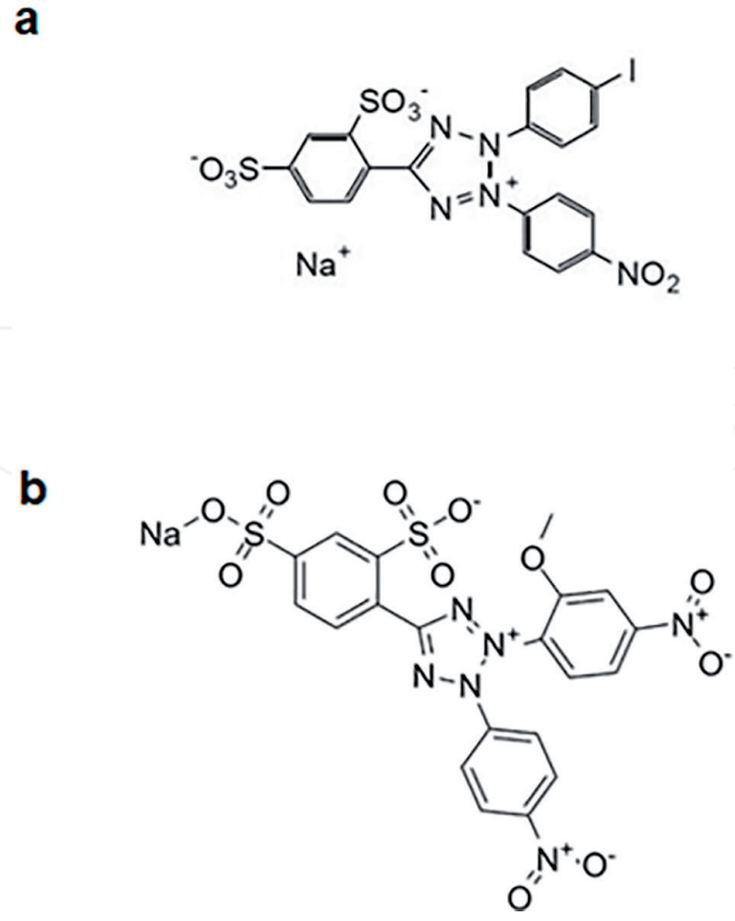

Figure 10.
Chemical structure of a) WST-1 tetrazolium salt and b) WST-8 tetrazolium salt.

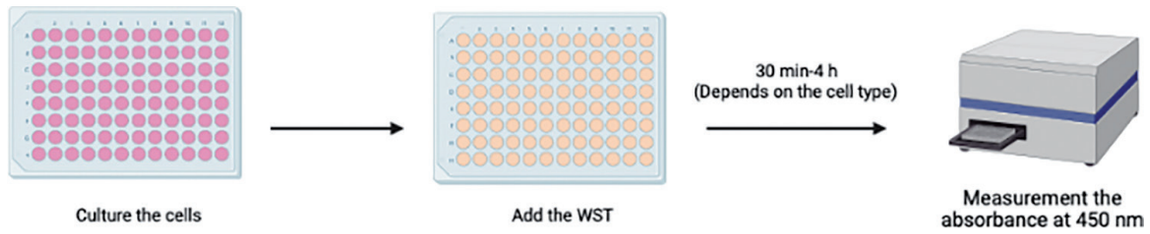

Figure 11.
Principle of WST assay.

show more metabolic activity than non-proliferating cells, this method determines not only cell viability and cytotoxicity, but also determines cell activation and proliferation. In this method, measurements can be made even at low cell concentrations without the use of additional agents or cell washing processes. However, it is a perfect solution for the quantification of cells and the determination of their viability without using radioactive isotopes. This method is based on the principle of measuring cell proliferation concerning various growth factors and nutritional components [21].

4. Discussion and conclusion

Evaluating cellular cytotoxicity is one of the most essential parts of studying cellular functions in biology. These assays are used to test a substance's effects on *in vitro* systems in the fields of including but not limited to oncology, biotechnology, drug discovery, pharmacology, product development, and medical device biocompatibility testing [17, 22]. When exposed to cytotoxic compounds, cells can undergo necrosis, apoptosis, and autophagy, or they could stop proliferating. Detecting this dynamic event is crucial for evaluating the mechanisms in action of cellular actions and pathways involved in cell death after exposure to toxic agents.

There are a variety of assays that can be used in general, such as dye exclusion assays, colorimetric, fluorometric, and luminometric assays [23]. Here, in this chapter, we reported the uses of colorimetric cytotoxicity assays where data are recorded using a multiplate reader. The idea for all is to use a compound to treat the cell and addition of a dye that changes its absorption spectra upon cellular reduction, which is directly proportional to the number of metabolically active cells. Thus, the principles of cytotoxicity assays are different from cellular viability assays, which typically measure viable cells rather than the metabolic activity. As cytotoxicity and viability assays can be utilized separately, additional tests may also be required based on the research aim. As such, they could be used together as complementary methods to get a better understanding of a cell's metabolic reaction.

It should be noted that most of the cytotoxic measurement assays actively affect cellular integrity, protein production, cellular trafficking and alter the cell fate by activating programmed cell death [24]. This makes them irreversible assays where the cells could not be used after the assay. Nonetheless, they provide rapid, robust, sensitive, and cost-effective means to determine whether a material contains potentially biologically harmful activity or substances.

One of the key factors in selecting the assay/dye type in cytotoxicity assays is the biological endpoint. As some experiments require certain types of cells, cellular sources should be carefully selected depending on the endpoint used in the cytotoxicity test.

Colorimetric assay	Mechanism	Advantages	Disadvantages
MTT	Determination of metabolic activity from mitochondrial dehydrogenase enzyme	Cost effective, robust	Needs additional step to solubilize. Less sensitive. Toxic to cells
MTS		Produced formazan is soluble. More efficient, more accurate. Less toxic to cells.	More expensive technique. Absorbance at 492 nm could be easily affected by incubation time.
XTT		Highly sensitive, accurate, safe to cells	Performance may be affected by environmental factors
WST		Easy to use, safe. Can be used in phenol red media.	More time consuming. Expensive. Performance may be affected by environmental factors

Table 1.
Summary, advantages, and disadvantages of different colorimetric biological assays used in vitro.

For example, in cytokine release studies, monocytes and fibroblasts should be the cell of choice [25, 26]. Similarly, cardiac-regeneration-related studies could be conducted with cardiac fibroblasts or cardiomyocytes for better results [27, 28].

Overall, colorimetric assays are simple, inexpensive, accurate, rapid, and sensitive methods in determining cellular toxicity. They are also applicable to both cell suspensions and adherent cells that make them attractive molecules in optimization studies. Although their main principle is based on metabolic activity determination, they all show some unique properties. We summarized their advantages and disadvantages in **Table 1**.

Main difference between the assays is that the MTT is not soluble in water, which requires an additional formazan dissolution step. In contrast, MTS, XTT, and WST assays use a different kind of tetrazolium salt, which produces a soluble formazan, reducing one step in the MTT assay procedure. Therefore, such assays are more efficient and less time-consuming when compared with the MTT assay.

It should be noted that environmental factors such as enzymatic regulation, pH, incubation time, temperature, cellular ion concentration, and variations in cell cycle could affect the performance of the colorimetric assays [29]. As such, an ideal cytotoxicity assay could differ from study to study depending on the aim, action mechanism, and environmental factors.

Conflict of interest

The authors declare no conflict of interest.

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