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An overview of the most common methods for assessing cell viability

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ABSTRACT

Background and Purpose: Measuring the proliferation and survival of cells is very important when studying the influence of various substances on cells. In this respect, several methods have been standardized to assess cell viability. These assays include common methods such as Trypan blue colorimetric method rather than the more complex ones such as MTT or XTT. Each of these methods has some merits and demerits compared to others. Some factors such as cost, speed, sensitivity and the required equipment are involved in selecting the suitable procedure. Despite the simplicity and cost-effectiveness of cell morphology evaluation, the sensitivity of this method is not very high and it is not adequate for short-term effects of materials. Trypan blue method is a common practice in the assessment of cell viability due to damage to cell membrane. This method, like other methods depends on the integrity of the membrane (Lactate Dehydrogenase release and fluorescent assays), is ineffective where there is cell damage without membrane damage. Despite being highly accurate, cologenic, fluorescent and flow cytometry assays are expensive and laborious. MTT assay as a simple, robust, rapid and cost-effective method is able to simultaneously evaluate a large number of samples. XTT assay is newer and more sensitive than MTT method. But contrary to MTT, it is not suitable for all kinds of cells. Generally, MTT assay is widely used as a reliable method. The current study aims to provide an overview of the most common methods used to evaluate cell viability and also weigh up the pros and cons of each method.

Keywords: cell viability; proliferation; survival; MTT; XTT; flow cytometry

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INTRODUCTION

Cell viability and proliferation is the foundation of assessing cells reaction to external factors. Assessment methods are essential for cells biology and drug discovery. In these methods, cell viability assessment is necessary in order to identify cell response to the external factors [1]. So far; various methods have been presented to evaluate cell viability, or in other words, cytotoxicity. These methods can be based on morphology changes or variations in the permeability of cell membranes or disruption of cell activities [2, 3]. Accordingly, in the present research, we deal with a review of the most common and widely applied cell viability assessment methods versus diverse compounds induced in cells (Table 1). Performing all of the methods require basic data about cell culture. Thus, a brief review on cell culture basics is presented.

Review on cell culture basics

Cell culture is used in many laboratory activities. One of its applications includes cell survival tests under the influence of external factors [4]. Cell culture covers the cells growth, mainly animal cells outside their natural environment [5].

Table 1: Various cell viability assessment methods

Morphology	
Membrane integrity	Colorimetric method(Trypan blue exclusion dye)
	LDH release
Cell function	Esterase enzyme activity evaluation
	Dehydrogenase enzymeactivity (MTT, XTT)
	Cell proliferation power Colonogenic assay
Cell Death Mechanism	Apoptosis and necrosis (flowcytometryassay)

Isolation cells of a living organism are done in different ways. These methods may include the separation of blood cells from the bloodstream, cells isolated from tissue by enzymes (collagenase, trypsin and proteinase) and isolation of cells from whole tissue culture [6].Cell conditions in vitro differ from those in vivo. The vital elements for cell maintenance in vitro include culture media, supplements, additives and incubation conditions. There are different types of culture media. The point deserving to notice is that each cell needs a specific medium. Typically, the majority of cells along with culture medium require some animal serum containing different bio-molecules and growth factors. Other additives used are antibiotics. These substances are applied to prevent cell contamination. The appropriate temperature varies for different cells. Mammalian cells require temperature range 34-38ºC for incubation. Also, cells need the right amount of O_2 and CO_2 [7].

The isolated cells are in two forms suspended and adherent that depending on cell type, passage conditions differ. The suspended cells are transferred from culture flasks into falcon tubes and after centrifugation, the surfactant is discarded and a new medium is added. But about the adherent cells, there are diverse methods such as enzymatic methods (using trypsin) and or psychical methods (applying scraper) [8].

Cell Morphology

One of the ways to analyze cytotoxicity is to monitor cell morphologic changes [2, 9, 10]. The transformed or swollen or opaque cells signal a problem in cell [10] (Fig. 1). Despite most of the cells morphologic changes are reversible, the non-reversible transformation in the cell morphology indicates severe damage to cell [2,10].The morphologic changes can encompass the nucleus density drop [12, 13], changes on cell surface (14), cell volume [15], and or cytoskeleton [16]. These transformations can be recorded by microscope (Invert) [9]. Each of the cell morphologic changes occur under certain circumstances. Therefore, this method isn't much efficient for cell viability assessment compared to other ones [2], and in case of observing the transformation; it requires more specialized analyses [10].

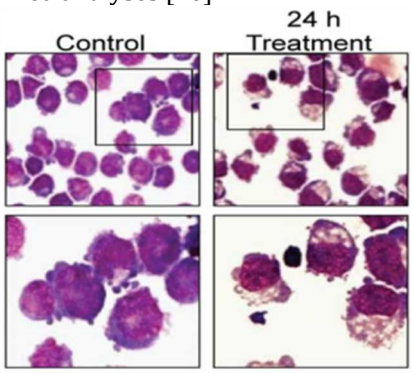

Figure 1: The morphologic changes in the cells exposed to external substance (K562 cells exposure to imatinib for 24 h) (11).

It is worth mentioning that examining the morphologic transformation is easier and more applicable in plant cells [17, 18].

Membrane Integrity Assessment Methods

Trypan Blue Exclusion Dye Colorimetry

One of the colors commonly applied for cell viability assessment is trypan blue (19, 20). The basis of this method is that trypan blue isn't capable to penetrate live cells' membrane while passing dead cells (due to not being able to control substance transfer) and accordingly, live cells and dead cells are seen as transparent and dark blue respectively [20, 21] (Fig.2).

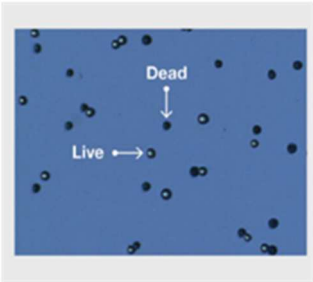

Figure 2: live /dead cells under the influence of trypan blue

In this method, first, the cells are cultured in a 24-well plate and then incubated for 18-24 h (37°C and $Co_2$5%) (for the cells adhering to plate bottom and being put under suitable growth conditions [21]). After this period, various concentrations of the question compound are added and again the plates are transferred to the incubator for the required time (22). After the due time, cell suspension is prepared from each of the plates separately and in 1:1 ratio of the cell suspension and trypan blue (4% w/v in PBS) is mixed and using a hemocytometer slide, live cells (transparent) and dead ones (dark blue) are counted [22]. In this method, it should be noticed that if staining time exceeds more than 5 mins, live cells can be stainable [19, 21]. With the following formula, we can determine the cells viability % [21]:

Viability %= (counted live cells number/ total number of counted cells) ×100
Cytotoxicity =1-viability

It is worth mentioning that trypan blue method is applied as an adequate method for counting live cells before any subsequent use of cells [10].

LDH Release

Another method expressing cell membrane integrity is LDH release. Increasing plasma membrane's permeability following cell death excretes cell content outside the cell [18, 23, and 24]. This method has its basis on catalytic activity of the enzyme LDH of the following reaction [24].

NADH + pyruvate ←LDH→ NAD^++lactate

During this method regarding NADH absorbance can be measured at wavelength 340nm, LDH levels excreted from cell can be measured [2, 3, and 24].For performing this method after exposure to cells with external compound and exposure time passing, the cell container is slightly shaken so that the released LDH concentration gets homogenous in them [24].Then the supernatant is collected and centrifuged for 5 m at 3000rpm .No cell should be transferred [23,24].In order to assess LDH activity, the compound NADH (25mg/ml) and sodium pyruvate (1mg/ml) in Phosphate buffer solution (PBS) is applied [25].This compound and the collected culture media are mixed in a 96-well plate and their absorbance is read by spectrophotometer at wavelength 340 nm [12].The data analysis is expressed as a percentage of control absorbance [12].The most eye-catching shortcoming of the analysis methods for membrane integrity is that maybe the affecting location of toxic substances is somewhere other than cell membrane .This means that until the time the damage hasn't spread to membrane, the mentioned methods are inapplicable .Thus , it is recommended to employ other cell viability methods accompanied with these ones [26-28].

Cell Function Analysis Methods

Fluorescent Probes (Esterase Enzyme Activity Evaluation)

Evaluating cell viability by Fluorescence microscopy has got widespread in recent years. Fluorescein diacetate (FDA) and 5-(6)-carboxyfluorescein diacetate (CFDA) are fluorescent markers that due to their hydrophobic property can pass live cells' membrane. These two substances are degraded into green fluorescent products under the effect of intracellular esterase that cannot exit the cell because of being hydrophilic [18, 28].

If these markers are used accompanied with Hoechst33342 (a fluorescent marker able to stain all live and dead cells), it is possible to estimate the live cells % [10].

Colors adhered to nucleic acid like EtHD (Ethidium homodimer) can penetrate dead cells following membrane damage and via adhering to nucleic acids, they emit red-orange light [30, 31].

The new substance recently used is CAM (Calceinacetoxy methyl ester) [19]. The advantage of CAM, compared with other esterase reagents, is its high fluorescent capability, low speed of fluorescence erasure, reduced exit of cell and its survival against PH variations [32-34]. CAM (live cells indicator) is used in the novel methods along with EtHD (dead cells indicator) [35, 36].

In this method, cells are exposed to the external substance for a certain period and incubated for 24 h at room temperature [35]. CAM 5 Mm solution in DMSO is used and diluted to 80 µM by PBS. Similarly, EtHD solution is prepared at 150µM concentration in PBS. These two solutions are added to the cells (the cells affected by external substance and control cells) so that a layer covers the cells' surface [35].

To evaluate cells viability by fluorescent microscope, cells are photographed. Calcein results in homogenous intense green fluorescent (about530^{nm}) in living cells and via adhering to

dead cells' DNA, EtHD leads to light red fluorescent (<600nm<) [36]. Calcein induced green color in whole cytoplasm and nucleus and EtHD induced red color are constrained to nucleus [34] (Fig.3).

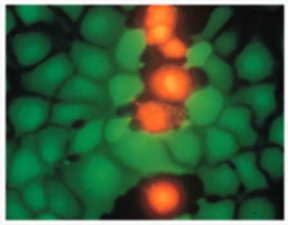

Figure 3: living/dead cells by fluorescent microscope. Green color is due to CAM and is live cells indicator and orange is due to EtHD and is dead cells indicator

MTT Method

MTT: 2-(4, 5-dimethyl-2-thiazolyl)-3, 5-diphenyl-2H tetrazolium bromide

One of the methods for evaluating cell viability is colorimetry, a simple and yet highly precise method. Among the applied colors, MTT and XTT are mostly applied [37-39]. Tetrazolium as an electron acceptor reduces to formazan by the enzyme succinate dehydrogenase (2, 20). In MTT assay, yellow tetrazolium salt in the live cells reduces to purple insoluble crystals of formazan[9].Thus the higher the live cells, the more formazan are produced [2]. Formazan crystals are dissolved by a detergent and measured in a 96-well plate by Elisa Reader [41]. This method was first introduced by Mosmann in 1982 [42] (Fig.4).

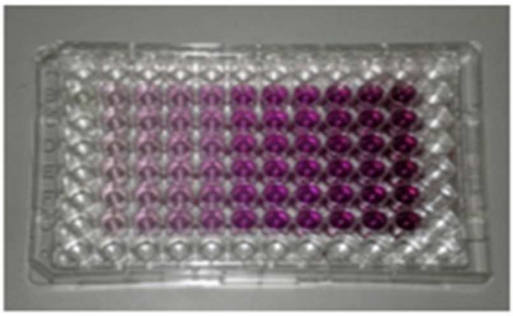

Figure 4: A profile of 96-well plate after MTT test. Purple signifies formazan

In order to produce MTT solution,5 mg of MTT powder is dissolved in PBS solution (5mg/ml) [21,41,43]. For this purpose, first 10^4cells and 100µl of culture medium are added to each well of 96-well plate [44]. Three replicates were consider for each concentration (3 wells as control (containing culture medium and cell) and 3 wells as blank (containing only the medium) [41]. Then it is incubated for 18-24 h in order for the cells to adhere to the plate bottom ($CO_2$5% and 37 ° C) [21,41,40]. After this period, various concentrations of the desired compound is added to the wells and incubated for 24,48 and 72 h. After the desired intervals for the test, 10µl of MTT solution is added to each of the wells for each 100µl of the culture medium and the plates are incubated for 3-5 h [21, 45]. Then the supernatant is removed and to dissolve the yielded formazan, the suitable detergent (Isopropanol (21, 45) or DMSO [41, 46]) is added to the wells. It is preferred to slowly shake the plates for 10-15 mins [21,41]. Subsequently, the plates' light absorption is read at wavelength570nm [21, 41, 45]. The cell viability % is calculated by the following formula: the data are reported in the form of cell survival diagram (Fig.5).

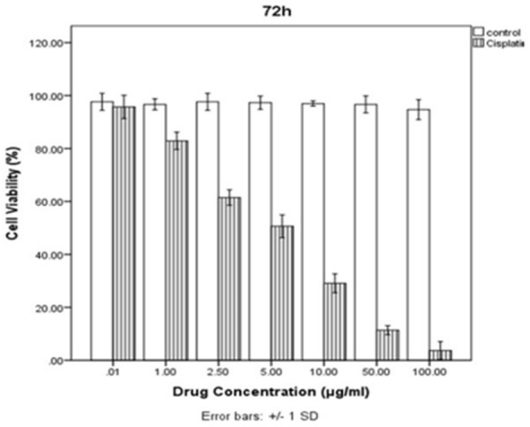

Figure 5: the measurement of cell viability (%) A549 line when exposed to Cisplatin during 72 h incubation [44]

Viability %= (extract affected Wells OD –blank OD/control OD-blank OD) ×100

XTT Assay

XTT: Sodium 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt. The requirement of formazan solution preparation resulting from MTT before analyzing by spectrophotometry assays, makes the study cells inapplicable for more use. This issue brings about using another Tetrazolium salt known as XTT. This compound is reduced to a water-soluble formazan reaching equilibrium with the culture medium [47].

XTT assay was first introduced by Scudiero in 1988 [48]. XTT assay is similar to MTT [49]. XTT reduces to light orange formazan in living cells (Fig.6). XTT is used as a compound with intermediary electron receptor helping reduction .PMS (Phenazine methosoulfate) as an

electron carrier boosts XTT reduction and the production of its formazan product [47].In this assay, 10^4 cells with 150µl culture medium are added to each well of 96-wellplate. For each concentration, several replicates are considered.

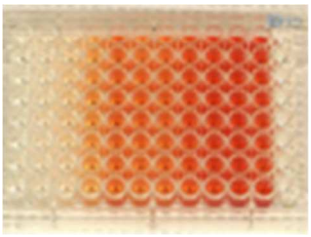

Figure 6: A profile of post-XXT test 96-well plate, orange signifies the presence of formazan

Then the plates are incubated for 24 h ($CO_2$5% and 37° C). When this period is over, various concentrations of the question compound are added to each well. After that, 1000:15 ratio of XTT reagent and activation reagent are mixed so that 45µl of this mixture is added to each well. The plates are incubated for 4 h and then are read directly by spectrophotometer at wavelength 450nm (reference wavelength 630nm) [49].

Colonogenic Assay

One of the methods quantitatively evaluating cytotoxicity is cologenic assay [50]. Assuming that each colony is derived from one cell, the number of the living cells can be approximated [20]. This method was introduced by Puck for the first time [51]. Colonization is based on cell's health and proliferation potential. Therefore, the major merit of this method is evaluating the overall effect of an external substance on cell survival regardless of its effect site and mechanism. That means, the result of this method indicates the toxicity or lack of toxicity of a substance [50].

During this method, 500-1000 cells along with4ml medium are added to each well of 6-well plate. Then it is incubated for 18-24 h ($CO_2$5% and 37° C). After this period passes, diverse concentrations of the external substance are added to the wells and incubated for 1-2 h. When this period is over, the supernatant is removed and the cells in the wells are washed with PBS or NS (normal saline 0.09%) and 4cc fresh medium is added to the wells. Three wells are taken as control (containing cell and medium without external compound). After that, the plates are put in the incubator for 7-14 d. After this period, the colonies number of each well is counted [50, 52]. In the colonies measurement, the cell aggregations including more than 50 cells are counted as a colony [50]. In order to count the colonies number, first, fixing and painting operations are conducted. . To do this, the plate contents are removed and eluted with PBS. Then the cells are exposed to violet crystal color 5% and methanol 50%for 30 mins [53]. For cells fixation, formalin 9% and for coloring trypan blue are applied (54, 55). And when this time is over, the plated are washed with water and dried at room temperature. Colonies counting is done in the following day [53]. To determine cells survival, the percentage of each well colony to control well colonies is calculated [50].

Cell death mechanism

Flow Cytometry

In response to threatening conditions or toxins, the cells undergo planned death or Apoptosis [58].One of the methods to assess apoptosis is to measure Phosphatidylcholine serine(PS) molecules level emerging on the surface of the cells under apoptosis by flow cytometry [59].Flow cytometry is a laser based technology widely applied in biophysics, used in the tasks including cell counting, sorting, indicator detection and protein engineering via cells suspension in fluid and passing through an electronic detector [60]. In this method, analyzing various physical and chemical parameters of thousands of particles occurs simultaneously [61]. To evaluate apoptosis mechanism in the study cells, double staining Annexin-V and Propidium iodide (PI) is used. In the presence of calcium ions, Annexin-Vmolecule binds to phospholipids, in particular to PS [59]. Concurrently, PI passes from the damaged cells' membrane and adheres to DNA. Therefore, the cells with planned death contain Annexin indicator and the cells with damaged membrane or necrosis contain PI indicator [62]. In this method, first 1×10^6cells are cultured in 6-well plates. Then they are exposed to the external substance and incubated for the desired period (CO_2 5% and 37ºC). After that all of the adhered and suspended cells are collected and a cell plaque is formed via centrifugation. Cell plaque is suspended in equal Annexin-V and PI and 1mL buffer and put at room temperature for 10-15 mins [60-63].

The results are illustrated in 4 forms. If the cells only have Annexin indicator, it indicates primary apoptosis. In case of merely having PI, it denotes necrosis and if they are positive to the both indicators, it shows advanced apoptosis or

necrosis. Normal cells have none of the two indicators and are negative to both [5, 8] (Fig 7).

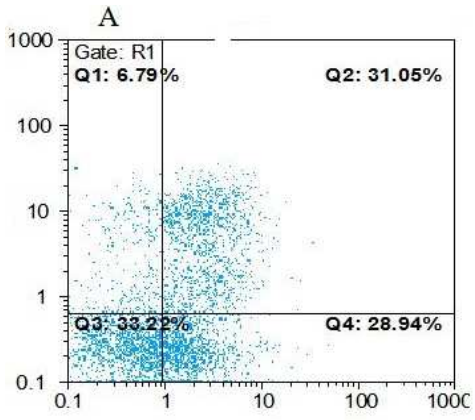

Figure 7: PTOX treatment induced5637 cells' apoptosis Annexin-V

DISCUSSION

In the present research, the differences between various methods on cell viability are discussed. Each of the aforementioned methods has its own Advantages and Disadvantages. One of the limits of cell morphology evaluation methods is the unstable morphological transformation. Generally speaking, observing the irreversible changes in the cell morphology suggests cell severe damage and thus, they may not be appropriate for studying short-term effect of the substances [2].

Trypan blue is a simple method to evaluate cell proliferation or death through their membrane integration examination (21). Of the major shortcomings of this method declining its sensitivity refers to the potential to absorb color by live cells in case of lengthening staining time more than 5 mins [56]. Nevertheless, the carcinogenic properties of trypan blue have led to its being used cautiously [21].

On the whole, in case the toxic substance effect site isn't cell membrane, the membrane integration evaluation methods (the release of lactate dehydrogenase and trypan blue colorimetric method) are not effective; besides, the sensitivity of these methods has been less raised compared to cell function screening assays such as MTT [1, 21].

One of the benefits of cell function screening assays (using fluorescent probes, MTT, XTT and colonogenic) is concurrently assessing high sample size. Despite the high accuracy [2], fluorescent assays are costly and require facilities like fluorescent microscope. Though of the main limitations this method has, similar to other membrane integration screening assays, is that in the cases where there is cell damage without membrane damage (e.g., in the initial stages of apoptosis induced cell death), this method doesn't work [1]. Flow cytometry as a highly accurate and sensitive method requiring high costs and specialized facilities has turned into an appropriate assay for investigating cell death mechanism and not just cell viability [61]. Colonogenic assay is a sensitive and highly efficient method. But concerning its high cost and troublesome nature, it isn't suitable for a large number of external substances [2]. Due to their ease at use and sufficient precision of the results, colorimetric assays are widely applied [37-39). MTT is a very rapid, sensitive and precise method to measure the activity of all cell lines. XTT being newer than MTT and has been reported more sensitive [48]. The major advantage of this method to MTT is fewer stages for performing it (omitting reaction product dissolving stage) [57]. Yet, MTT is a highly powerful test and metabolized by the majority of cells types while the new substances aren't known to all of the desired cells [57]. To sum up, the convenience behind utilizing MTT and its high accuracy, mention it as a reliable method [21]. For this reason, this method is extensively applied by the researchers in cell studies worldwide [2].

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Conflict of Interest
No conflict of interest has been confirmed by the authors.

Authors' Contribution
Mohammad Shokrzadeh developed the study concept and design and drafting of the manuscript. Mona Modanloo managed the literature review and drafting of the manuscript and act as corresponding author.

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