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Chapter

Cell Division, Cytotoxicity, and the Assays Used in the Detection of Cytotoxicity

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Abstract

Cell division is a phenomenon that is encountered in all cells in nature. While normal cell division results in proliferation in single-celled organisms, and development and repair in multicellular organisms, aberrant and untimely cell division results in tumor formation. Therefore, the understanding of the cell division is hidden in identifying the details of the molecular mechanisms that govern cellular division at the exact time and under right conditions. Sometimes these molecular mechanisms are distorted by both intrinsic and extracellular factors, and the division process halts or deviates to an abnormal pathway. At this point, it is essential that the abnormal cells are removed from the tissue by an appropriate mechanism. In this context, in this review, general and specific information about cell division and its molecular control mechanisms were discussed, and different types of cell death mechanisms were mentioned accordingly. In addition, chemical, biological, and physical cytotoxic agents that negatively affect cell division and their mechanisms of action are explained. Finally, a brief review of the principles of different cytotoxicity (cell viability and proliferation) test systems has been performed to provide a source of information for investigators who study cell viability, proliferation, or different types of cellular death pathways.

Keywords: cell division, DNA damage, cell cycle control, mutagens, cellular proliferation parameters, cytotoxicity assays

1. Introduction

Cell division is a common phenomenon that occurs in all living entities, except for cells that have completed somatic differentiation. When the cell reaches a certain volume, it performs division [1]. While the cell division leads to an increase in the number of individuals in single-celled organisms, in multicellular organisms, it ensures the growth of hair and nails, healing of wounds, cellular repair, somatic growth, and genesis of reproductive cells. Then, one might question that under what conditions do cells divide? Before moving on to the answer for this question, it is crucial to find the answer to this proposal: **Do cells divide because they grow? Or do they grow because they need to be divided?** The various assumptions about the view that advocates the premise that the cell divides because it grows are summarized below:

1. **Overall control attenuates as the cell grows**. Later in the process of cell growth, obstacles start to appear in the central management of the organelles in the cytoplasm. As a result, problems may arise in the way organelles perform their tasks and also in the control of the communication they perform with each other. In addition, the material exchange within the cell starts to become inextricable [2]. In summary, since the growth of the cell will cause a number of significant difficulties in performing intracellular coordination activities, the cell needs divisions to reduce its volume.

2. **As the cell grows, the "cytoplasm/nucleus" ratio varies in favor of the cytoplasm**. Since the increase in volume of the cytoplasm makes it difficult for the nucleus to maintain its control on the cell, it is crucial for cell to divide and restore the optimal cytoplasm/nucleus ratio [3].

3. **Since the cell membrane does not enlarge as fast as the cytoplasm, it becomes difficult for the cell to exchange material from the external environment during continuous cell growth**. To achieve sufficient material exchange from the membranes, the membrane-to-surface area must reach the optimum value [4]. As the cell continues to grow, difficulties in maintaining the surface-volume balance begin. Small or thin objects have a larger surface area than their volume. This gives them a large surface-to-volume ratio. However, large objects have a small surface area than their volume; thus they have a small surface-to-volume ratio [1].

This could be best explained by a balloon blowing event. As a balloon swells, the internal volume increases, but the membrane cannot simultaneously compensate for this volume increase and, thus, explodes after a certain time period. Cells need to divide to prevent this negative event (otherwise, cells would become lysed).

4. **Environmental factors and hormones can lead the cell to division**. In general, if any tissue has been damaged for any reason, cell division takes place and performs the repair process. In this context, growth hormone may be given as an example of the effect of hormones on cell division. These and similar hormones have mitogenic functions that trigger a cell division signal [5–7].

In fact, from a scientific point of view, the cell is divided not because it grows, but rather it requires division. In the process of cell division, the dividing cell is called a parental or host cell. The parental cell is divided into two daughter cells. Subsequent divisions are then repeated via the so-called process, the cell cycle.

Cells regulate the division process by communicating with each other through chemical signals generated by specific proteins called cyclin and cyclin-dependent kinases [8]. These signals play a key role in determining when cells will begin to divide and stop dividing. Cell division is important for the growth of the organism and wound healing. It is also important that the cells terminate dividing at the appropriate time [9]. Otherwise, cancer occurs because the cells do not stop the division at the required time.

Cells in the organism perform division for growth and/or repair; on the one hand, they undergo apoptosis (by cellular turnover) for various reasons to maintain homeostasis. Because some cells, such as epidermal cells, are constantly lost, new cells must be produced via cell division. For instance, 30,000–40,000 epidermal cells are killed per minute by apoptosis [10]. In other words, we lose about 50 million cells each day. Therefore, cell division is very important in tissues where cells are lost very rapidly. However, other cells such as the nerve cells are either not divided or very rarely divided [11].

Depending on the cell type, cell division has two mechanisms: mitosis and meiosis. Each of these cell division mechanisms has unique characteristics. In mitosis, the parent cell (diploid, 2n) is divided into two daughter cells with the identical number of chromosomes [12]. This type of cell division is essential for basic growth and repair. On the other hand, the parental cell in meiosis (diploid, 2n) is divided into four cells with two successive cleavages (meiosis I and meiosis II), and the number of chromosomes are half the main cell (haploid, n) [13]. The reduction of the diploid chromosome number to haploid is important for sexual reproduction, and recombination in meiosis I is the source of genetic diversity.

The cell, which has not yet started to divide, is in the interphase of the cell cycle. Cells have to be divided by certain periods, though each cell actually passes most of its time in the interphase. The interphase is the period when a cell is prepared to divide and initiate the cell cycle [14]. During this time, the cells have to obtain nutrients and energy. The host cell also synthesizes a copy of its DNA to share equally between the two daughter cells.

However, if cell division is not required for the functional integrity of the organism, negative regulation of the cell cycle is performed [15]. In contrast to the positive regulators, the negative regulators function in a direction that halts the cell cycle. In this process, a large number of molecular components and signaling are involved. The most well-studied negative regulatory molecules are the retinoblastoma protein (Rb), p53, and p21 [16]. These three proteins have been commonly referred as tumor suppressor proteins. Like the p53 and p21 proteins, Rb proteins are also a group of tumor suppressor proteins observed in many cell types. Most of the knowledge of cell cycle regulation have been obtained from studies using cells that have lost control of cell cycle regulation [17–19]. It has been discovered that cells that are uncontrolled (becoming cancerous) have these regulatory proteins damaged or nonfunctional [20, 21]. In each case, the major cause of uncontrolled progression through the cell cycle is errors in the abovementioned regulatory proteins. In this case, the possibility of uncontrolled cellular proliferation is raised. When DNA damage is detected, p53 protein halts the cell cycle and DNA repair enzymes are activated to repair the damage. However, if DNA damage cannot be repaired, the p53 protein may trigger apoptosis (programmed cell suicide) to prevent the replication of damaged chromosomes [22]. Different cellular death pathways including apoptosis, in the context of this chapter, are therefore summarized below.

2. General mechanisms of cell death

2.1 Apoptosis

Apoptosis, also known as programmed cell death, is a regulated cellular destruction program that facilitates the removal of damaged or excess cells. This process is critical for many physiological processes including embryonic development and tissue homeostasis in adulthood [23, 24].

Multicellular organisms have developed suppressive processes that prevent the proliferation of cells displaying aberrant proliferation or improper tissue infiltration. These processes function to block tissue hyperplasia, tumor formation, and metastatic distribution of tumors. Processes such as the cell cycle arrest, cellular aging, and apoptotic cell death, which remove malicious cells capable of initiating tumor growth, can also be included in this mechanism [25].

2.2 Autophagy

Autophagy (or autophagocytosis) (autóphagos in Ancient Greek) means "self-devouring." It is the natural regulating mechanism that extracts the nonfunctional (junk) components of the cell [26]. The term "autophagy" was first used in 1963 by the Belgian biochemist Christian de Duve [27]. In the 1990s, the Japanese autophagy researcher Yoshinori Ohsumi discovered the mechanisms of autophagy in yeast cell by identification of autophagy genes and received the 2016 Nobel Prize in Physiology and Medicine for his studies [28]. Autophagy allows the regular degradation and recycling of cellular components [29]. The cytoplasmic components targeted in this pathway are separated by a double-membrane vesicle named autophagosome from the rest of the cell [30, 31]. The autophagosome then fuses with the lysosome and performs the digestion of the cellular components in between its membranes. In general, three types of autophagy have been reported to date, including macro- and microautophagy and chaperone-mediated autophagy. Although the autophagic process, in the context of the disease, was observed to be an adaptive stress response that increases survival, it has been observed in other cases that it increased cell death and morbidity. In the case of excessive cellular starvation, disintegration of cellular components promotes survival by ensuring that cellular energy levels remain constant [32]. Autophagy, which acts as a protective response to biological stress in mammalian cells, removes damaged proteins and organelles from the cytoplasm and allows for the reconstitution of components in their structure using lysosomal content. In the case of moderate stress, autophagy may undertake the task of survival; however, in the event of excessive stress, it can activate the programmed cell death pathway [33]. Because of the dysregulation of autophagy in many diseases including cancer, it is crucial to understand how the transition from autophagy to apoptosis occurs. Cells that respond to exogenous stress were found to be consistent in their quantitative autophagy and apoptosis measurements [34]. On the other hand, defective apoptosis in immortalized epithelial cells renders cells substantially tumorigenic. In apoptosis-defective cells, activation of AKT (protein kinase B) or allelic degradation of Beclin1 inhibits a pathway of survival due to autophagy, thereby enhancing susceptibility to metabolic stress. Although autophagy acts as a buffer against metabolic stress, the simultaneous disruption of apoptosis and autophagy mechanism promotes necrotic cell death in vitro and in vivo. Therefore, the inhibition of autophagy by certain conditions, such as nutrient starvation, may render apoptosis-resistant tumors susceptible to apoptosis [35]. While apoptosis acts as a cellular quality control mechanism in the organism, autophagy acts as an intracellular quality control mechanism. Collectively, autophagy and apoptosis are not interchangeable metabolic pathways, and autophagy can be assumed as one of the components of apoptosis.

2.3 Necrosis

Another pathway of cell death is necrotic cell death. Necrosis (Greek: death) is a form of cell injury that results in premature death of cells in living tissues through the mechanism of autolysis [36]. Necrosis is caused by factors other than cells or tissues, such as infection, toxins, or trauma, which cause irregular digestion of cell components. Unlike apoptosis, it is not a controlled or programmed type of death. Apoptosis is often beneficial to the organism, while necrosis is almost always disastrous and may be fatal [37]. Cell death from necrosis does not follow the path of apoptotic signaling, but more diverse receptors are activated, resulting in loss of cell membrane integrity and uncontrolled release of cell death products into the extracellular domain [36]. This initiates an inflammatory response in the surrounding tissue that activates leukocytes and nearby phagocytes and eliminates dead cells by phagocytosis. However, microbial

damaging agents released by leukocytes can cause irreparable damage to the surrounding tissues from the lateral side [38]. There are six different models of necrosis recognized morphologically. These include coagulative necrosis, liquefactive necrosis, gangrenous necrosis, caseous necrosis, fat necrosis, and fibrinoid necrosis [39].

2.4 Necroptosis

Necroptosis is a programmed necrosis or inflammatory cell death pattern. Conventionally, necroptosis, unlike regularly programmed cell death by apoptosis, is associated with non-programmed cell death resulting from cellular damage or infiltration of pathogens. The discovery of necroptosis has shown that cells are capable of performing necrosis in a programmed manner and that apoptosis is not always the only preferred form of cell death [40].

3. Cytotoxicity

The effect of being toxic to cells caused by toxic agents is called cytotoxicity. Exposing cells to a cytotoxic compound may result in various outcomes in the cell. At this point, the cells may actively progress into the death phase. Furthermore, the cells may activate the controlled cell death (apoptosis) program, or necrosis may occur where the membrane integrity is lost and uncontrolled death is being executed due to cell lysis. Cells undergoing the process of necrosis commonly swell rapidly, lose membrane integrity, stop metabolism, and secrete their contents into the extracellular space. Furthermore, cells with rapid necrosis in vitro do not have enough time or energy to initiate apoptotic mechanisms and therefore will not express apoptotic indicators. Apoptosis is characterized by well-defined cytological and molecular events involving cytoplasmic shrinkage, nuclear condensation, and controlled cleavage of DNA by the endonucleases. Cells in culture undergoing apoptosis eventually undergo secondary necrosis. At this time, the cell stops metabolism and loses the integrity of its membrane [41].

Cytotoxic agents are known as all the elements that are toxic to the cells, which include the factors that prevent their growth and sometimes cause death, and are also used to treat certain disorders. Chemical and biological substances or physical agents can cause cytotoxicity by affecting the cells in varying degrees. These agents include chemical agents that act by inhibiting synthesis (such as nucleic acid and protein synthesis) in the cell, by affecting cellular energy production pathways (mitochondrial effect), or by attenuating the integrity of the membrane in the cell (plasma membrane or intracellular organelles that have membranes).

3.1 Chemical cytotoxic agents (cytostatics)

- Inhibitors of dihydrofolate reductase responsible for purine and pyrimidine biosynthesis.
- Inhibitors of DNA biosynthesis (cytarabine).
- DNA intercalators (anthracyclines and anthracenediones).
- Agents inducing DNA strand break formation (bleomycin).
- DNA topoisomerase inhibitors (camptothecin, anthracyclines, anthracenediones, anthrapyrazole, and etoposide).

- Cytotoxic agents that cause formation of DNA adducts (cyclophosphamide, melphalan, chlorambucil, hexamethylmelamine, busulfan, dacarbazine, mitomycin C, and cisplatin).

- RNA degradation (inhibition of RNA biosynthesis by anthracyclines).

- Nucleoprotein (inhibition of nucleoprotein synthesis by L-asparaginase) and microtubule biosynthesis inhibitors (antitubulin, colchicine, dolastatin, taxol, tritlisin, vinblastine, and vincristine).

- Agents that cause cytotoxicity by modulating the mitochondrial permeability transition pores and increasing the mitochondrial membrane potential and affecting the energy transmission pathways in neoplastic cells. These agents include staurosporine, poly (ADP-ribose) polymerase, 6-aminonicotinamide, 6-methyl-mercaptopurine ribid, 6-mercaptopurinoside, 6-aminonicotinamide and 6-methyl-mercaptopurinoside, and N- (phosphonacetyl)-L-aspartic acid [42].

3.2 Biological cytotoxic agents

In this group, toxic molecules derived from viruses, bacteria, fungi, plant, and animal origin are generally included. Bacterial endo-/exotoxins and antibiotics in this group are the most widely recognized molecules. Biological agents such as lipid hydrolyzing enzymes, sphingomyelinases C and D, cholesterol oxidase, helianthus toxin, streptolysin, and saponin damaged cultured human skin fibroblasts and erythrocytes with different cholesterol levels. However, erythrocytes with high cholesterol levels were found to be more sensitive to toxins [43]. Cytotoxic agents used by invertebrates include oxygen and nitrogen reactive intermediates, antimicrobial peptides, lectins, cytokines, and quinoid intermediates of melanin [44]. It has also been found that bacterial cytotoxins act by targeting the actin components of the cell skeleton of eukaryotic cells [45].

3.3 Physical cytotoxic agents

Physical agents such as heat, ultrasonic vibrations, and radiation have cytotoxic effects. The toxicity induced by "lethal heat shock" in *Saccharomyces cerevisiae* (yeast) cells was found to be primarily due to oxidative stress. The possibility that mitochondrial membrane disruption in aerobic cells exposed to heat stress is hundreds of times higher than cells in anaerobic conditions reinforcing this possibility [46]. An in vitro study of Chinese hamster ovarian (CHO) cells revealed that the cytotoxicity of drugs affecting the plasma membrane was synergistically increased by ultrasound application [47]. It has also been found that the use of ultrasonic microbubble increases the cytotoxic effects of chemotherapeutic drugs on tumor cells [48]. In addition, many studies in the literature on the cytotoxic effect of radiation can be found.

4. Universal cytotoxicity parameters

Assays to measure the reduction in the cell viability (inhibition of growth/division or death by apoptotic-necrotic pathways) are called "cytotoxicity" tests. These experiments can be performed by in vitro and/or in vivo test systems in different cell types using various techniques. While some of these parameters (mitotic index, replication index, nuclear division index, etc.) contribute to the indirect demonstration of cell division dynamics, some of them directly contribute to the

demonstration of cell viability (MTT, MTS, XTT, WST, etc.) [49]. A brief summary of these methods can be found below; however, before explaining these test methods, brief information about some of the most commonly used cytotoxicity parameters that provide us quantitative information about the different cellular processes will be discussed.

4.1 Mitotic index

The mitotic index of a cell population is expressed as a proportion of the population at any mitotic stage (e.g., per mille = 1/1000). Mitotic index is an important criterion for the growth and multiplication of tissues. Because it is calculated in fixed and stained cell preparations at a particular divisional phase, the mitotic index reflects only the division stages of cells at the time of fixation [50]. Furthermore, since cell division is a process that follows DNA replication under normal conditions, the mitotic index is indirectly a parameter that is also associated with DNA replication. One can ask: why has mitotic index attracted considerable interest as a viability parameter for decades in genotoxicity tests? The answer to this question clearly lies in cell division. A successful cell division requires coordination between different cell cycle checkpoints, especially G1/S and G2/M transitions [51]. These cell cycle control points, which regulate the sequence and timing of sub-phase transitions, are essential in maintaining genomic integrity and in realizing a healthy cell division [52]. Therefore, no matter what type of cell is being examined under the microscope, the MI is a universal parameter capable of giving information indirectly about all the subcomponents and control points of the cell cycle and is used to measure cytotoxicity in living organisms. The calculation of MI shows minor differences between plant and animal cells. In plant and animal cells, the MI is simply calculated as follows and is given in percentage:

Plant cells:

$$MI = \frac{Prophase + Metaphase + Anaphase + Telophase}{Total\ number\ of\ cells} \times 100 \quad (1)$$

Animal cells:

$$MI = \frac{Metaphase\ cells}{Total\ number\ of\ cells} \times 100 \quad (2)$$

For the calculation of MI in animal cells, the reason for counting only the cells in the metaphase stage is the use of special chemicals (e.g., colchicine, vincristine, vinblastine) that inhibit microtubule polymerization in the metaphase stage during mitosis. Therefore, the cell cannot proceed further than the metaphase stage.

Although not always, a significant decrease in MI correlates with genotoxicity. A reduction of MI by 50% or less indicates a lethal effect on the cells, and this is called lethal dose of 50 [53]. The decline in mitotic index may be related to inhibition of DNA synthesis or delay/stop in cell cycle phases (G1, S, or G2). Occasionally, there may be also significant increases in MI compared to control. This may be due to a reduction in the duration devoted to DNA repair [54] or the acceleration of the transition between the phases of the cell cycle [55]. Both events can result in an uncontrolled progression of cell proliferation.

In recent years, a new formula of mitotic index has been developed which takes account of only actively dividing cells. According to this hypothesis, only actively dividing cells, i.e., metaphase and anaphase cells, are included in the calculation. This formula (active mitotic index) provides additional information on the percentage of actively dividing cells [56, 57]:

$$AMI = \frac{Metaphase + Anaphase}{Total\ number\ of\ cells\ observed} \quad (3)$$

Another characteristic of the MI is that it provides critical information about the progression of the disease and the course of treatment in certain diseases such as cancer. Since one of the hallmarks of cancer is a high rate of cell proliferation, MI can therefore be used as an important predictor in the prognosis of various types of cancer. In this context, high levels of MI were associated with hepatocellular carcinoma (HCC) and invasive breast carcinoma, while low MI values were associated with negative node status, diploid DNA content, low S-phase fraction, and positive estrogen (ER) and progesterone (PgR) receptor status in breast cancer [58–60].

4.2 Nuclear division index (NDI)

Such as the mitotic index, nuclear division index is a parameter that provides information about the numerical value of cell proliferation. The difference of NDI from MI is that NDI is calculated based on the number of nuclei in divided (or interphase) cells. Thus, NDI is a parameter that is directly related with the DNA replication. NDI may be used to gain quantitative information on cell cycle progression of the lymphocytes after phytohaemagglutinin (PHA) stimulation. This index is frequently employed as a useful research tool for understanding the kinetics of cell cycling in lymphocyte cultures. However, although it is simply a tool to measure the rate of division in viable cells, an increase in cell death via necrosis or apoptosis does not always cause a reduction of the NDI in surviving cells [61]. On the other hand, it has been experimentally shown that NDI was associated with various malignancies and could be used as a screening strategy as a cytogenetic biomarker in cancer such as the MI. It has been reported that the mean NDI values were significantly lower in patients with colorectal cancer (CRC) or polyps than in patients with normal colonoscopy. Therefore, NDI was proven to be useful in screening strategies for CRC [62]. The NDI is calculated as in the following formula:

$$NDI = \frac{(M1) + (2 \times M2) + (3 \times M3) + (4 \times M4)}{N} \quad (4)$$

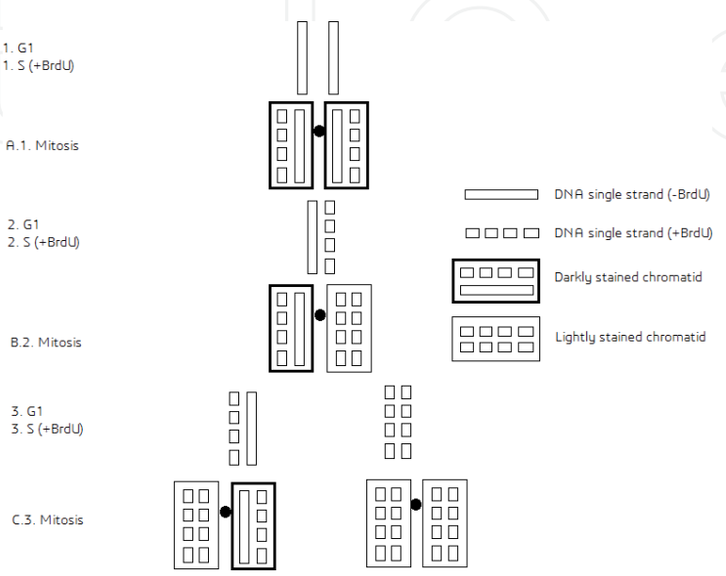

Figure 1.
Schematic representation of the differentiation of cells undergoing the first, second, and third mitotic division with the introduction of BrdU into DNA molecule.

where M1–M4 represent the number of cells with one to four nuclei and N is the total number of the cells scored [63].

4.3 Proliferation index (replication index, BrdU incorporation)

Proliferation index (PI) or replication index (RI) is a parameter that is used to investigate the rate of DNA replication. It is basically a method based on the principle of integration of BrdU into the strands of DNA. BrdU, deoxythymidine (dT), and deoxyuridine (dU) are molecules which are analogues of each other. The difference between these molecules is due to the fact that the chemical groups bound to the fifth C atom in the heterocyclic benzene ring are different (**Figure 1**).

5. In vitro cytotoxicity (cell viability) assays

Before proceeding with the widely used in vitro cytotoxicity tests, it is worth mentioning why these tests are more preferred than animal tests:

1. Although animal tests can provide pathological information, these tests have ethical concerns.

2. In vitro tests conducted in a test tube using cells grown from an organ can be used to test the toxic effects of substances on specific tissues.

3. In vitro tests enable us to screen minimal quantities of chemicals.

4. It could be also possible to study specific subcellular pathways such as signaling pathways and oxidative stress.

However, despite these advantages offered, in vitro cytotoxicity assays cannot fully substitute in vivo assays because:

1. The chemicals may be metabolized inside the whole body rather than specific organs.

2. In vivo tests can be conducted lifelong.

3. In vivo tests enable us to determine the presence of chemicals in multiple organs and their distribution in the body as a whole.

4. Specific systems such as the reproductive system and respiratory system can be examined in vivo.

5. Different routes of entry such as the skin, inhalation, and gut can be tested in vivo.

6. In vivo tests enable us to calculate and model toxicokinetic effects, in terms of uptake and removal, and the half-life of the chemical in the body.

Cytotoxicity tests are among the first in vitro bioassays used to predict toxicity of various substances in different tissues. The need for safety assessment of drugs, cosmetics, food additives, pesticides, and industrial chemicals increases year by year. **Figure 2** demonstrates an overview of the compartments which are targeted by the cytotoxicity test systems within the cell.

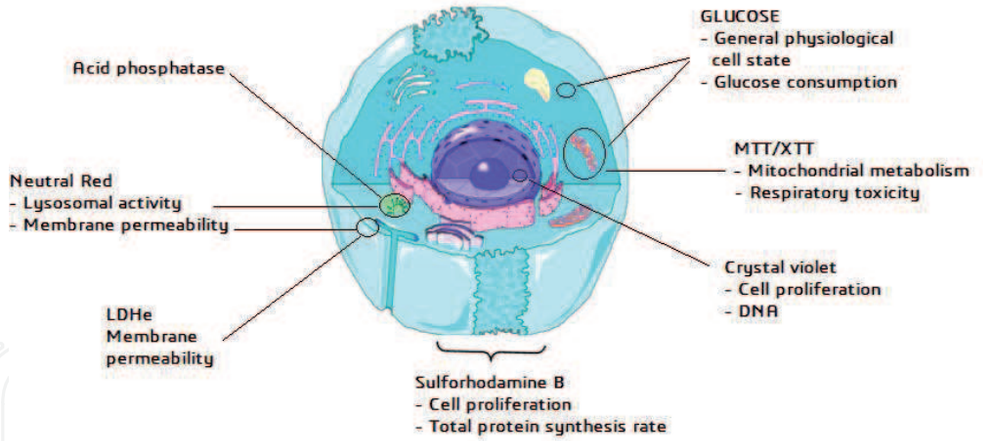

Figure 2.
Overview of general targets of cytotoxicity test systems in the cell.

According to their targeted compartments in the cell, in vitro cytotoxicity assay methods could measure viability or toxicity basically in four different ways: (I) proliferation (direct viable cell count), (II) cell division (DNA synthesis by ^{3}H thymidine uptake), (III) metabolism (MTT, alamar blue, ATP production), and (IV) membrane (leakage of lactate dehydrogenase from dead cells). However, many cytotoxicity tests, although they differ from each other in practice, allow testing of relatively similar cellular processes and endpoints. Therefore, these tests, below, are summarized without dividing into different categories.

5.1 Apoptosis assay

Apoptotic cells are recognized by reduced DNA content and morphological changes such as nuclear condensation that can be detectable by flow cytometry, trypan blue, or Hoechst staining. Changes in the structure and function of the plasma membrane are determined by the appearance of phosphatidylserine on the plasma membrane that reacts with the Annexin V-fluorochrome conjugates. Propidium iodide (PI) staining allows distinction between early and late apoptotic events [64].

5.2 ATP assay

The assessment of metabolic functions by the cellular ATP content is an established method of measuring cytotoxicity that is essential for screening drugs and determining toxicological safety. As ATP plays a central role in cellular metabolism, the intracellular level of ATP is strictly regulated in normal cells. Furthermore, cell injury results in not only reduced ATP synthesis but also immediate diminution of endogenous ATP levels which is caused by the escape of ATP-converting enzymes (e.g., ATPase) [65]. Therefore, quantification of the intracellular ATP content is crucial for the assessment of the degree of cellular toxicity [66].

5.3 Autophagy assay

Autophagy is a process which is characterized by the formation of double-membraned vesicles called autophagosomes, which isolate the cellular components targeted for devastation and fuse with lysosomes to deliver their content for degradation. Autophagy can be determined by two different methods as direct and indirect. Direct tests are based on the turnover of long-lived proteins and lactate

dehydrogenase (LDH) sequestration, while indirect tests include western blot-based assays, fluorescence microscopy-based methods, electron microscopy, and flow cytometry and imaging flow cytometry [67].

5.4 BrdU assay

BrdU is a thymidine analogue used in cell proliferation studies. BrdU in the cell culture medium is incorporated into DNA during DNA synthesis. Once the membrane permeabilization is performed, the cellular incorporation of BrdU can be detected by anti-BrdU-specific antibodies. For this purpose, flow cytometry or immunohistochemistry techniques can be used [68].

5.5 Cell tracking

This is a procedure used to monitor cell movement and location by the implementation of "tracking" probes that pass through the membrane into the cytoplasm and then become membrane impermeable. The "tracking" dyes to be used in this type of experiments should be passed to the daughter cells during multiple generations, but not transferred to the neighboring cells they are in contact [69].

5.6 Gram staining

Although traditionally named "Gram staining," commercial kits for testing cell viability and proliferation now include dyes such as CF®488A WGA, DAPI, Ethidium Homodimer III (EthD-III), etc. in addition to the conventional Gram stain; CF-488A (WGA) stains N-acetylglucosamine structure in the peptidoglycan layer of Gram-positive bacteria with green fluorescence. EthD-III, which is a nucleic acid binding dye, is membrane impermeable and selectively stains the compromised plasma membranes of bacteria with red fluorescence. DAPI, which can pass through the membrane and bind to DNA, stains all bacterial cells in blue [21].

5.7 LDH assay

The cytoplasmic enzyme lactate dehydrogenase is a stable ubiquitous enzyme found in all cells. A key feature of apoptosis, necrosis, and other forms of cellular damage is that the LDH is released into the cell culture, while the plasma membrane is damaged. By using the NADH which is produced in a coupled reaction to reduce a second compound during the conversion of lactate to pyruvate, LDH activity can be quantified. In this protocol, the reduction by NADH of a yellow tetrazolium salt (INT) into a red water-soluble formazan-class dye is quantified at 492 nm absorbance. The amount of formazan occurring in culture is directly proportional to the amount of LDH and, indirectly, to the number of dead or damaged cells in the culture media [70].

5.8 Live/dead staining

Live/dead assay is used for quantification of cell viability using flow cytometry or fluorescence microscopy. This assay utilizes fluorescent dyes to label live and dead cells with a one-step protocol. "Live cell" dye stain intact and viable cells into green. It can pass through the membrane and does not fluoresce until the ester groups in the dye molecule are removed by the intracellular esterases. The excitation (max) and emission (max) are 494 nm and 515 nm, respectively. On the other hand, "dead cell" dye labels cells with damaged plasma membrane into red. It does

not have the ability to pass through the plasma membrane and binds to DNA with a high affinity. The degree of the fluorescence emitted by this dye increases up to >30-fold when it is bound to DNA. The excitation (max) and emission (max) are 528 nm and 617 nm, respectively [71, 72].

5.9 Lysosomal staining

These dyes are fluorescent dyes such as "CytoPainter green" or "acridine orange" (AO), which are used to measure lysosomal integrity in proliferating and nonproliferating cells. These lysotropic dyes preferentially accumulate in lysosomes via the lysosome pH gradient. Their fluorescence is significantly increased after they become trapped in lysosomes. These dyes are also useful in cell adhesion, drug resistance, chemotaxis, apoptosis, cell viability, and adherent cell studies [73].

5.10 Membrane potential assay

Membrane potential or membrane voltage refers to the difference of electric charges across a cell membrane. Electrical potential difference across the cell membrane is a novel method to monitor cell death (apoptosis) in single cells. It has been shown that the depolarization of plasma membrane in response to microinjection of cytochrome c into the cytosol is a reliable indicator of apoptotic cell death [74]. The dye used for this assay is a lipophilic and anionic dye that can move across the cytoplasmic membrane of healthy cells, dependent on the membrane potential across the plasma membrane. The fluorescence intensity of the dye increases when the dye is bound to proteins in the cytosol. Following depolarization of the cells, more dye enters the cells, and the increased cellular concentration of dye binding to lipids and proteins results in an increase in fluorescence signal. On the other hand, following hyperpolarization, dye exits the cells, and the decreased intracellular concentration of dye results in a decrease of fluorescence signal. The excitation wavelength of the dye is 488 nm of the argon ion laser [75].

5.11 Mitochondrial staining

Although mitochondrial staining is performed in different compartments of mitochondria, it is usually a test performed on the mitochondrial membrane or mitochondrial matrix. Mitochondrial membrane dyes are cell permeant and accumulate in active mitochondria that have intact membrane potentials. The signal will be bright if the cells under examination are healthy and have functional mitochondria; however, if the mitochondrial membrane potential is lost, the signal will be dimmer or will disappear [76]. In addition to staining the mitochondrial membrane, some dyes stain specific molecules in the mitochondrial matrix. One of them is the "fluorescent mitochondrial hydrogen peroxide indicator" stain, which serves to visualize hydrogen peroxide in the mitochondrial matrix of living cells. This dye gives strong emissions at 528 nm in the presence of H_2O_2. In an in vitro model of Parkinson's disease, it is used to detect the local increases in H_2O_2 [77].

5.12 MTT, XTT, MTS, and WSTs assays

5.12.1 MTT assay

In living cells, MTT (a yellow tetrazole) is reduced to formazan (purple). To convert (dissolve) the insoluble formazan into a colored solution, a solubilization solution (DMSO, acidic ethanol solution, or a solution of sodium dodecyl sulfate in

diluted hydrochloric acid) is added. This colored solution can be quantified by measuring its absorbance using a spectrophotometer usually between 500 and 600 nm wavelength. The absorption degree of light depends on the solvent [78].

5.12.2 XTT assay

Due to its higher sensitivity and higher dynamic range, XTT has replaced MTT. Since the formed formazan dye is water-soluble, it eliminates the final solubilization step [79].

5.12.3 MTS assay

MTS, in the presence of phenazine methosulfate (PMS), forms a formazan product which has a max. Absorbance of 490 nm in PBS. The MTS assay is often regarded as a "one-step" MTT assay, since it offers the advantage of adding the reagent directly to the cell culture without any intermediary steps necessary in the MTT assay. Since the intermediary steps remove remnants of colored intermediates in the MTT assay, this advantage, however, makes the MTS assay sensitive to colorimetric interference as these intermediates remain in the one-step MTS assay [80].

5.12.4 WSTs

WSTs (water-soluble tetrazolium salts), a series of other dyes for MTT assays, are designed to give different absorption spectra for the formed formazans. WST-1, particularly WST-8, have advantages over MTT in that they are reduced outside the cells and form a water-soluble formazan. Finally, unlike MTT, WST assays (1) can be read directly, (2) provide stronger signal than MTT, and (3) are less toxic to cells (unlike membrane-permeable MTT, and the resulting insoluble formazan piles up inside the cells) [81].

5.13 In silico prediction of chemical toxicity

A relatively newer discipline, and highly hot topic, which has become increasingly important in recent years, is the computer-aided in silico toxicity prediction of drugs, food additives, or various industrial chemicals for which a previous wet-lab toxicity outcome is available. For this purpose, QSAR, the CAESAR project, and other virtual screening methods such as docking (i.e., AutoDock, Surflex) software have found wide use in the last decade [82–84]. While these software use special parameters (e.g., genetic algorithms), the details of these parameters do not fall into the scope of the main subject of this review article.

6. Conclusion

A thorough understanding of the molecular mechanisms of cell division makes it easy to understand cell death. Cells perform division at the right time according to the chemical signals from their internal and external environment. Aberrations in cell division often induce uncontrolled cell division and result in tumor formation. Here, the importance of cytotoxicity parameters and assays in experimentally normal or abnormally divided cells emerges. Many diseases show a direct correlation with the parameters used to measure the division behavior of cells. Thus, a detailed understanding of the molecular mechanisms of cell division and cell death by cell proliferation and cytotoxicity tests is critical in distinguishing between normal

and healthy cells. Another important aspect is the selection of the right cytotoxicity assay when working on different cell death mechanisms such as apoptosis, necrosis, necroptosis, or autophagy. Although they appear to reveal very different death pathways, in fact, cytotoxicity assays mainly target a certain number of cellular compartments (lysosome, membrane, nucleus, cytoplasm, mitochondria). In recent years, with the development of in silico toxicity assessment software, cytotoxicity experiments have been transferred from the laboratory to the computer environment. However, the fact that the details of the intracellular milieu are not completely understood yet still keeps the accuracy of the in silico toxicity estimates at a certain level, and therefore it is logical to anticipate that the wet-lab applications (especially in vitro cytotoxicity tests) to measure cell proliferation and cytotoxicity will continue to be the first choice.

Conflict of interest

The authors declare that they have no competing interests.

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