Cytotoxicity assay:Drugs detection

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Abstract:

The property of drugs nanoparticle to penetrate or permeate through various biological barriers are widely used for testing therapeutic efficacy. This testing however provides an opportunity to hamper the normal biological functioning. A constant effort is currently in progress to develop alternatives to the use of the live animal for the toxicity test of these xenobiotics e.g. drugs, chemicals, micro-organisms. Cytotoxicity depends on various factors like doses, exposure time and route of administration. Number of assays is in use to screen the effects on cells or tissue. These assays directly or indirectly measure cell viability which mainly based on alterations of plasma membrane permeability and consequent release of cellular components (e.g. enzymes) into the supernatant or the uptake of dye by non-viable cells and excluded by viable cells, ATP production, nucleotide uptake, protein synthesis etc.

Keywords:

cytotoxicity, LDH, MTT, Neutral red, Nanoparticles, Sulforhodamine, Trypan blue

Introduction:

Exposure of nanoparticles to humans is unavoidable as nanoparicle are widely in use. So a newly discovered drug undergoes testing prior to entering a human clinical trial or receiving regulatory approval to ensure safety and effective use. The first step in understanding how the drugs /agent react in body involves the studies of cell culture. Compared to animal use cellular testing is less ethical, easy to handle, less expensive. In case of cytotoxicity, it is important to take care that cell culture is sensitive to temperature, pH, osmotic pressure, oxygen, nutrients and waste concentration, in addition to these the concentration of potential agent that is being tested. Therefore, controlling the experimental condition is crucial for toxicity test (Lewinski et al., 2008).

The toxic agents that are being used can cause alteration in function such as ATP production, protein synthesis, enzyme function or cell death by apoptosis, autophagy and necrosis. Cell viability and cytotoxicity assay that are used for screening drugs are mainly based on cell membrane permeability, enzyme activity, cell adherence, ATP production, radiolabeled thymine uptake etc.

Apoptosis: It is a well sequenced event that is characterized by chromatin condensation and fragmentation, cell shrinkage, blebbing of plasma membrane and formation of apoptoic bodies which are recognized and eaten by phagocytes or neighbouring cells (Fink et al., 2005).

Autophagy: It is highly regulated lysosomal proteolytic degradation process which can engulf damaged cells by the formation of autophagosomes that upon fusion with lysosomes forms autophagolysosomes that degrade cellular component (Rabinowitz JD et al., 2010).

Necrosis: It is defined as negative form of cell death; it lacks sign of apoptosis or autophagy. Characterized by dilation of organelles, cytoplasmic swelling and vacuolation, bursting of plasma membrane leads to the spill up of cytoplasmic content and elicit a inflammation response (Fink et al., 2005).

Cytotoxicity assays

Cytotoxic assay based on plasma membrane integrity:

Exposure to certain cytotoxic agents can compromise the cell membrane which allows the release of cellula contents. Viability test based on this include neutral red, toluylene red, trypan blue exclusion assay, LDH assay.

Neutral red

The neutral red uptake cytotoxicity assay was developed at the Rockefeller University as a cell viability chemosensitivity assay (Borenfreund et al., 1984). It is based on the ability of viable cells to take up dye and bind to it. This is weakly cationic dye which penetrates the cell membrane by nonionic passive diffusion and concentrates in lysosome since, the pH is maintained lower than the cytoplasm. Here the dye become charged and

bind by electrostatic hydrophobic bonds to anionic and/or phosphate groups of the lysosomal matrix and retained inside the lysosome (Winckler et al., 1974., Nemel et al., 1979). The dye then can be extracted from the viable cells using an acidified ethanol solution which alter the cell membrane environment result in the release of dye from the cell. The absorbance of the solubiliized dye is quantified using a spectrophotometer, provides estimated number of viable cells in culture. For neutral red assay lysosome integrity is a highly sensitive indicator for cell viability. (Repetto et al., 2008)

The uptake of neutral red dye depends on the cell's capacity to maintain pH, through the production of ATP. Inside the lysosome there is a proton gradient to maintain pH, while in dead or dying cells pH is reduced so the dye that presents a net charge close to zero, enter the cell but are not able to retain. Consequently, the amount of dye retained is proportional to the number of viable cells (Filman et al., 1975).

Trypan blue

It is hemocytometer based assay which stains the dead cells with blue color while it unable to stain live cells as it is not permeable to plasma membrane. It is the most commonly utilized assay for cell viability (Mishell and Shiigi, 1980). Usefulness of this procedure is limited since the number of blue staining cells increases following the addition of the dye, requiring the cells to be counted within 3-5 min (Hudson and Hay, 1980). It is reported that trypan blue generally overestimated cell viability (Smith and Smith, 1989), gives inaccurate results in using EDTA treatment, trypsinization or scraping of anchored cells (Tennant, 1964) and is excluded from both metabolically active and nonviable cells when used in media containing serum proteins (Black and Berenbaum, 1964).

LDH assay

When plasma membrane is damaged cellular enzyme such as lactate dehydrogenase, adenylate kinase and glucose-6- phosphate dehydrogenase which is present in all cells released in culture media, they can be used as a cell death marker. However, adenylate kinase and glucose-6-phosphate are not stable and only lactate dehydrogenase doesn't its activity during assay. Therefore, it is more reliable than other enzyme based cell death assays.

In this assay pyruvate is oxidized from lactate by cytosolic enzyme LDH from dead and leaky cells with the reduction of NAD^+ to NADH, then 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT) react with pyruvate and give water soluble red color formazan product(Decker and Lohmann-Matthes, 1988; Lappalainen et al., 1994; Nachlas et al., 1960) that is measured by spectrophotometer. The amount of formazan is directly related to quantity of LDH in the culture which in turn is inversely related to the viable cells.

MTT(3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay based on mmetabolic activity

MTT is the first assay to be developed for a 96- well format that gives high throughput screening cell viability (Berridge et al., 2005., Corey et al., 1991., Mosmann, 1983). It is the colorimetric assay based on the metabolic activity of viable cells. Tetrazolium salts have positive charge on core of quaternary tetrazole ring which facilitate cellular uptake via plasma membrane potential (Ψ_{PM} -30 to -60 mV). Yellow color tetrazolium salt is reduced to blue color water insoluble formazan crystal by falvin-containing enzyme, succinate dehydrogenase, NAD(P)H dependent oxidoreductases like NQO1 and cytochrome P450. Mitochondria (Ψ_{PM} -150 to –170 mV) is established as the main cellular site of tetrazolium reduction(Berridge et al., 2005). It is also reduced by other

non-mitochondrial sites like endoplasmic reticulum(Lu et al., 2012; Stockert et al., 2012).This crystal need to be solublize using organic solvent with DMSO or HCl/isopropanol before taking absorbance. This procedure allows only one measurement as it kills the cells. MTT formazan crystals could activate apoptosis-related factors such as caspase-8, caspase-3 or accelerate the leakage of cell content (Lu et al., 2012).

Other tetrazolium salts are developed such as XTT, MTS more recently WST-1 are used in conjunction with intermediate electron acceptors (IEAs)

because WST-1 is a negatively charged disulphonated inner salt. So it cannot enter cell membrane, it get electron with the help of IEAs and reduced to water soluble yellow-orange coloured formazan outside the plasma membrane. It can be directly measured in ELISA reader (Berridge., 2005., Berridge et al., 2014., Berridge et al., 2008).

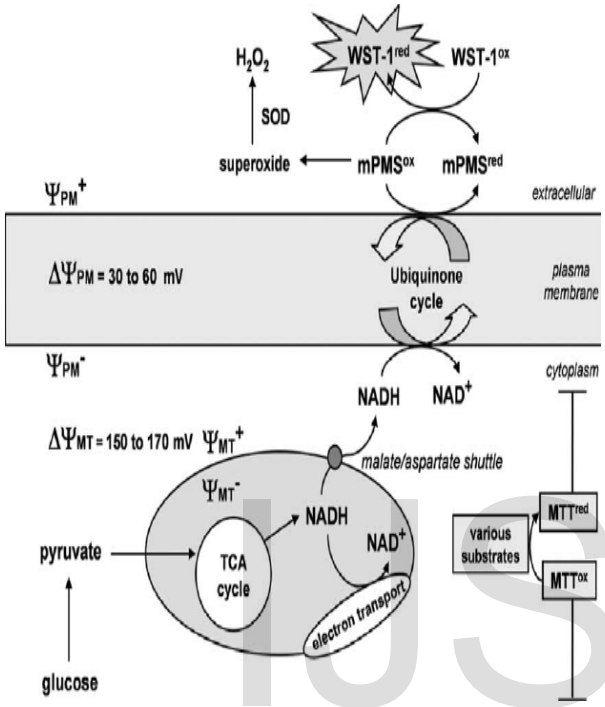

Fig:1 MTT and WST action inside the cell. (Berridge et. al., 2005)

Recently, a new agent is developed called PrestoBlue (PB) foe detecting cell-mediated cytotoxicity. It is a resazurin based compound similar to AlamarBlue which serve as indicator of mitochondrial metabolic activity. It is water soluble, compatible with phenol red and most importantly non-toxic to the cells, allowing continuous monitoring of culture. This reagent requires only 10 min incubation period (Boncler et al., 2013). Cell viability can be detected colorimetrically or fluorometrically, with high sensitivity (below 100 cells per well). PB is regarded as fastest live assay and has been used in the tests to measure viability of tumor cells (Istivan et al., 2011; Lall et al., 2013) and primary cortical neurons (Huang et al., 2012)

Sulforhodamine B total protein assay

It is a fluorescent dye, uses spanning from laser-induced fluorescence to quantify cellular proteins of cultured cells. It is based on the binding of dye to the basic amino acids of cells fixed on trichloroacetic acid. Colorimetric evaluation provides an estimated value of total protein mass, which is related to cell number. It is used for adherent and suspension cultures (Kuete et al., 2017). Cell enumeration is dependent on protein content thus no test compound interference and it is highly reproducible (Van Tonder et al., 2015). It has better linearity, high sensitivity, a stable end point that does not require time-sensitivity measurement. Only disadvantage that it require trichloroacetic acid to fix the cells and low sensitivity with non-adherent cells (Van Tonder et al., 2015).

Conclusion:

Concluding the result, we found the difference in the viable cells,dead cells and unhealthy cells depending on the treatment based on the agents of cytotoxicity test.before using drugs it should be tested as it can cause severe impact on body.Different assay depends on different factor such as enzymatic activity, permeability of plasma membrane etc. It might be influenced by various inhibitors like MTT by chloroquine.One has to be carefull with the suitablility of the assay as it can give false result.

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