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A comparison of four methods for determining viability in human dermal fibroblasts irradiated with blue light

Daniela S. Masson-Meyers, Violet V. Bumah, Chukuka S. Enwemeka *,[1]

College of Health Sciences, University of Wisconsin-Milwaukee, 2400 East Hartford Ave., Milwaukee, WI 53211, USA

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ABSTRACT

Introduction: Several tests are available for assessing the viability of cells; however, there is a dearth of studies comparing the results obtained with each test. We compared the capability of four viability assays (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), neutral red, trypan blue and live/dead fluorescence), to detect potential toxicity in fibroblasts irradiated with 470 nm blue light.
Methods: Cells were irradiated at 3, 55, 110 and 220 J/cm^2, incubated for 24 h and viability assessed using each test.
Results: MTT assay showed significant decreases in viability when cells were irradiated with 110 and 220 J/cm^2 energy fluence (dose) (89% and 57% viable cells, respectively; $p < 0.0001$, compared to control); likewise the trypan blue assay showed 42% and 46% viable cells ($p < 0.0001$). Neutral red assay revealed significant decrease in viability when cells were irradiated with 220 J/cm^2 (84% viable cells; $p = 0.0008$, compared to control). The live/dead fluorescence assay was less sensitive, evincing 91% and 95% viable cells after irradiation with 110 and 220 J/cm^2 respectively.
Discussion: (1) The four assays differed in their levels of sensitivity to cell viability. (2) The adverse effect of increasing doses seems to manifest as alteration of mitochondrial metabolism, followed by lysosomal dysfunction, membrane disruption and finally loss of cell membrane integrity. (3) Overall, irradiation with 3 J/cm^2 or 55 J/cm^2 did not adversely affect cell viability. Thus, doses below 110 J/cm^2 appear safe.

1. Introduction

A variety of tests are available for assessing the viability of cells. The choice of one test over another depends on a wide range of considerations, including the limitations of each test, ease and speed of use, available resources, and equipment complexity. (Hawkins & Abrahamse, 2005; Stoddart, 2011). In most cell viability tests, biomarker molecules are applied and monitored to estimate the resulting cytotoxicity either in the form of cell membrane damage, mitochondrial injury, dysfunction of lysosomal activity, release of the cytosolic enzyme lactate dehydrogenase, decrease in cell protein content, among others (Riss, Moravec, & Niles, 2011; Zwolak, 2015). Commonly used cell viability tests, such as 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), neutral red, trypan blue and live/dead fluorescence offer great advantage, as they permit rapid measurement of cell growth inhibition resulting from an experimental intervention. Moreover, they engender reliable data that could be used to determine the immediate or short term adverse effects of treatment at low cost (Pescheck, Dürr, Bláha, & Sell, 2014; Riss et al., 2013; Zwolak, 2015).

Viability assays have been carried out on different types of cells such as HeLa, hepatoma cells (HepG2), hamster ovarian cells (CHO-K1 line), Chinese hamster lung fibroblasts (V79), Balb/c 3T3 fibroblasts, L929 mouse fibroblasts, human keratinocytes, skin-derived endothelial cells, and human dermal fibroblasts (Fotakis & Timbrell, 2006; Liebmann, Born, & Kolb-Bachofen, 2010; Mamalis, Garcha, & Jagdeo, 2015; Masson-Meyers, Andrade, Leite, & Frade, 2013; O'Brien, Wilson, Orton, & Pognan, 2000; Pescheck et al., 2014; Seth, Yang, Choi, Sabean, & Roberts, 2004; Stoddart, 2011; Vian et al., 1995; Volpato, Oliveira, Espinosa, Bagnato, & Machado, 2011; Zwolak, 2015). However, there is a dearth of studies comparing the results obtained with each test, even though the physiological and biochemical basis of commonly used cell viability protocols differ. As detailed in Table 1, the specific method used determines the type of data obtained. For this reason, some have suggested using

Abbreviations: MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; EthD-1, ethidium homodimer-1; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; LEDs, light-emitting diodes; DPBS, Dulbecco's phosphate-buffered saline; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

* Corresponding author at: San Diego State University, 5500 Campanile Drive, San Diego, CA 92182, USA.
E-mail addresses: masson@uwm.edu (D.S. Masson-Meyers), bumah@uwm.edu (V.V. Bumah), Enwemeka@mail.sdsu.edu (C.S. Enwemeka).
[1] Present address: San Diego State University, 5500 Campanile Drive, San Diego, CA 92182, USA.

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Table 1
Characteristics of some commonly used methods to determine cell viability.

	Cell metabolism	Membrane integrity	Cell morphology	Cell distribution
MTT	+	+	−	−
Neutral red	+	+	−	−
Trypan blue	−	+	−	−
Live/dead fluorescence	+	+	+	+

+appropriate, −not appropriate.

more than one assay to determine cell viability in order to improve reliability and avoid overestimation or underestimation of the toxicity of an experimental intervention (Fotakis & Timbrell, 2006).

One of the most frequently used methods for measuring cell viability is the MTT assay which detects cells that are still metabolically active (Riss et al., 2013; Vega-Avila & Pugsley, 2011). The MTT assay is used to assess how effectively the mitochondrial dehydrogenases of viable cells metabolically reduce pale yellow MTT salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to insoluble purple formazan product [1-(4,5-dimethylthiazol-2-yl)-3,5 diphenylformazan]. Formazan crystals are impermeable to cell membranes, hence the resulting accumulation within healthy cells can be spectrophotometrically analyzed to estimate cell viability (Fotakis & Timbrell, 2006; Godley et al., 2005; Hawkins & Abrahamse, 2005; Mosmann, 1983; Pires-Oliveira, Oliveira, Machado, Zângaro, & Pacheco-Soares, 2010; Riss et al., 2013; Stoddart, 2011).

Similar to MTT, the neutral red assay is also a colorimetric assay. However, it is based on the uptake of the dye neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) by active transport, and incorporation into the lysosomes of viable cells. Damage to lysosomes decreases cellular accumulation of neutral red dye (Seth et al., 2004; Weyermann, Lochmann, & Zimmer, 2005). The incorporated dye is then released from the cells and spectrophotometrically quantitated to estimate cell viability (Fotakis & Timbrell, 2006).

One of the earliest cell viability methods which remains widely used is the trypan blue exclusion assay. With about 960 Da molecule, trypan blue is a polar dye that is cell membrane impermeable. It is absorbed only by cells with compromised membranes. Upon entry into the cell, the dye binds to intracellular proteins turning the cell dark blue in color. Since viable cells do not absorb the dye, they appear clear under a microscope with a refractive blue ring around them (Hawkins & Abrahamse, 2005; Louis & Siegel, 2011; Tran, Puhar, Ngo-Camus, & Ramarao, 2011). Thus, it is possible to estimate cell viability by quantitating and comparing the ratio of cells with compromised membranes with those with intact membranes.

A combination of fluorescent dyes, in which one dye stains live cells (usually in green) and the other stains dead cells (usually in red) is another approach to assay cell viability. Known as live/dead fluorescence test, this assay gives indications of membrane integrity and cell enzyme activity. An example of this combination is calcein AM with ethidium homodimer-1 (EthD-1). The polyanionic dye calcein AM is membrane permeable and is cleaved by esterases in live cells to yield cytoplasmic green fluorescence (Stoddart, 2011). EthD-1 enters cells with damaged membranes and binds to nucleic acids, producing red fluorescence in dead cells. The determination of cell viability is based on biochemical (intracellular esterase activity) and physical (membrane integrity) properties of cells (Cao et al., 2015; Jones & Senft, 1985; Stoddart, 2011).

Light microscopy remains a valuable age-old tool for visibly observing structural changes in cells, thereby yielding complementary information on potential toxicity resulting from an experimental intervention. Gross modifications such as blebbing or vacuolization can be observed using light microscopy, whereas fine ultrastructural modifications require analysis with transmission or scanning electron microscopy (Ekwall, Silano, Paganuzzi-Stammati, & Zucco, 1990). In either case, microscopy provides complimentary pictorial evidence of cell viability.

In this study, we compared the outcome of four cell viability assays—MTT, neutral red, trypan blue and live/dead fluorescence—in terms of their ability to detect potential toxicity in human dermal fibroblasts irradiated with 470 nm blue light. Our research group and others have shown that blue light in the range of 400 to 470 nm has antimicrobial effects, suppressing the growth of a wide range of bacteria (Bumah, Masson-Meyers, Cashin, & Enwemeka, 2013, 2015; Bumah, Masson-Meyers, & Enwemeka, 2015; Bumah, Masson-Meyers, Quirk, et al., 2015; Enwemeka, 2013; Enwemeka, Williams, Hollosi, Yens, & Enwemeka, 2008; Enwemeka, Williams, Enwemeka, Hollosi, & Yens, 2009; Maclean, MacGregor, Anderson, & Woolsey, 2009; Masson-Meyers, Bumah, Biener, Raicu, & Enwemeka, 2015). These findings indicate that blue light could be a viable alternative to antibiotic therapy, particularly in cases of infected cutaneous wounds. However, its potential cytotoxic effects on cutaneous cells, such as fibroblasts, particularly at high doses, have been questioned. Thus, a secondary purpose of our study was to document the effect of various doses of 470 nm light on the viability of human dermal fibroblasts using each of the four methods. Fibroblast, a major cell type in the dermis, plays important roles in tissue healing, connective tissue integrity and skin pathology (Frigo et al., 2010; Oplander et al., 2011; Sorrell & Caplan, 2004).

2. Methods

2.1. Cell culture

Human dermal fibroblasts isolated from adult skin (Cat. No. C-013-5C) were obtained from Life Technologies Corporation (Carlsbad, CA). Cells were grown in 75 cm^2 cell culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, in a controlled humidified cell culture incubator (37 °C, 5% CO_2/95% air). The medium was changed every two days. When cells became confluent, the medium was removed, the cell layer was washed with phosphate-buffered saline (PBS) and trypsinized with 0.25% trypsin in buffered ethylenediaminetetraacetic acid (EDTA). Cells were counted in automated cell counter [Cellometer® Auto T4 (Nexcelom Bioscience, Lawrence, MA)] and experimental cultures prepared before the viability tests described below were carried out.

2.2. Experimental design

Fibroblasts were suspended in DMEM to yield 3×10^4 cells/well and added to flat bottom 96-well microplates in a final volume of 200 μL/well (MTT and neutral red assays) (Frigo et al., 2010) and 1×10^5 cells/mL to sterile 35 mm Petri dishes in a final volume of 2 mL/dish (trypan blue, fluorescence and light microscopy assays). A higher cell concentration of 1×10^5 cells/mL in Petri dishes was used to allow more detectable cell counting either with the trypan blue assay or fluorescence and a better visualization of cells under the microscope.

The plates were incubated in a controlled humidified cell culture incubator (37 °C, 5% CO_2/95% air) to obtain confluent cell growth. After 24 h, the presence of cell growth monolayer was confirmed by inverted microscopy [Olympus® IX51 (Olympus America Inc., Melville, NY)] and five groups were prepared for each of the four assays tested: four different irradiation groups (3, 55, 110 or 220 J/cm^2 of 470 nm blue light which corresponds to 30 s, 9, 18 and 36 min respectively as programed in the device used) and a control non-irradiated group, as shown in Fig. 1.

For the assays carried out in 96-well plates (MTT and neutral red), eight wells were used (4 for treated and 4 for control groups) for each treatment/replicate. For each microplate, cells were seeded with the maximum possible distance between wells, in order to prevent cross-irradiation of cells. Samples (irradiated and non-irradiated controls) were subjected to the same standard environmental conditions such as ambient light, humidity, temperature, and time outside the incubator.