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TECHNICAL NOTE

Toxic effects of resazurin on cell cultures

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Abstract Resazurin, introduced as a cell viability indicator under the trade name alamarBlue®, is generally regarded as nontoxic when used according to manufacturer's suggested shorter-term incubation time specifications. However, problems arise when exposure times are extended to longer-term cultures on the order of days. To assess the effect of resazurin over longer incubation times, MCF7 (HTB-22), MCF10A (CRL-10317), 3T3-L1 (CL-173), and D1 (CRL-12424) cultures were tested with varying amounts of resazurin over 4- and 8-day periods. MCF7, 3T3-L1, and D1 cells cultured for 8 days with 20 % alamarBlue® had significantly less cell survivability. Specifically, levels of metabolic activity, deoxyribonucleic acid (DNA) concentration, and glucose consumption of the cell lines cultured for 8 days in medium with 20 % alamarBlue® were significantly lower ($p < 0.05$) than metabolic activity, DNA concentration, and glucose consumption of MCF7 cells cultured for 8 days in medium with no alamarBlue®. MCF7, 3T3-L1, and D1 cells used less glucose at concentrations as low as 5 %. Data also suggests the toxic effects are more pronounced in the cancerous cell line as compared to the noncancerous cells.

Keywords alamarBlue · Hydroresorufin · Resazurin · Resorufin · Toxic

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Introduction

Metabolic activity assays, including alamarBlue®, are key determinants in assessing the state of culture systems. Resazurin, or alamarBlue®, is generally nontoxic during typical, manufacturer-recommended assay incubation times of a few hours (Fields and Lancaster 1993; Zhang et al. 2004). However, some studies deviate from these shorter time periods (Gloeckner et al. 2001). Resazurin is the fully oxidized active component in alamarBlue®, which is blue and nonfluorescent (Fields and Lancaster 1993; Zhang et al. 2004). Resazurin undergoes a redox reduction to resorufin, a pink, fluorescent chemical (O'Brien et al. 2000). The redox reaction in resazurin-based assays provides information about the intercellular processes and metabolic activity. Under typical assay conditions, the active component, resazurin, is reduced to resorufin (Candeias et al. 1998). This chemical change, changes the physical properties of the medium allowing detection via fluorescence or spectrophotometry. However, if the assay is incubated too long, resorufin will further reduce to hydroresorufin, a product of the second reduced state (Ramsdell et al.

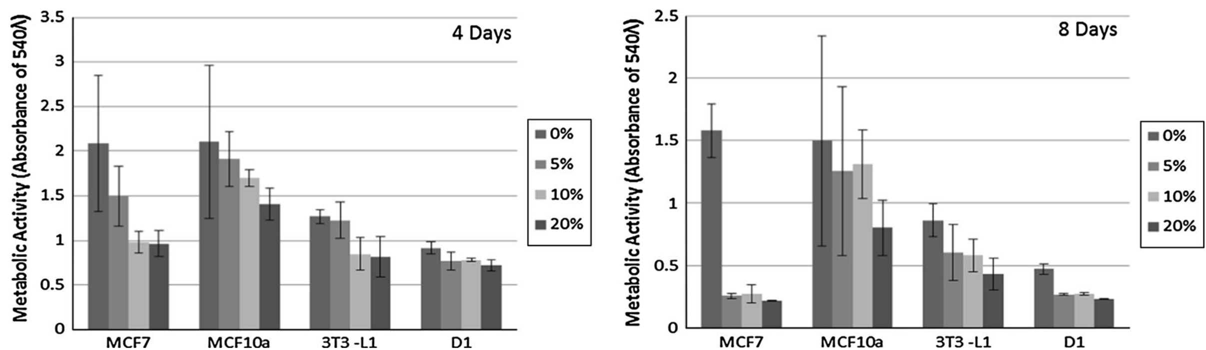

Fig. 1 Metabolic activity after 4 and 8 days of culture with alamarBlue concentrations listed. The assay was conducted after a 2-day recovery period in fresh alamarBlue free medium, then an MTT assay was performed. Three repeats were conducted per condition. *Bars* illustrate two standard deviations or 99.5 % confidence

1935). Caution must be taken to avoid incubation times leading to the production of hydroresorufin as this product is nonfluorescent and colorless and will interfere with colorimetric or fluorescent assays.

Although only commercially available since 1993 (Lancaster and Fields 1996), resazurin detection systems have been investigated since at least 1934 (Eichler 1934). During this time, few people have noted toxic effects. Motivation for this study originated from the desire to use resazurin as an in situ assay for testing tissue engineered cellular systems over time, rather than as an endpoint assay.

Materials and methods

Resazurin was tested with MCF7 (ATCC, Manassas, VA, USA, HTB-22), MCF10A (ATCC, CRL-10317), 3T3-L1 (ATCC, CL-173), and D1 (ATCC, CRL 12424) cell lines. MCF7 cells are human epithelial mammary adenocarcinomas cells, MCF10A cells are human epithelial mammary fibrocystic cells, 3T3-L1 cells are mouse embryonic fibroblast stem cells, and D1 cells are mouse bone marrow mesenchymal stem cells. AlamarBlue® (Invitrogen, Grand Island, NY, USA) was used as the resazurin source. Medium for the MCF7s, was Dulbecco's Modified Eagle's Medium (ATCC) supplemented with 10 % fetal bovine serum (Cellgro, Manassas, VA, USA), 1 % antibiotic–antimycotic (Cellgro), 1 % fungizone (Cellgro), and 0.01 mg/ml insulin (Gibco, Grand Island, NY). Medium for the MCF10A cell line was Dulbecco's Modified Eagle's Medium (ATCC) supplemented with 10 % fetal bovine serum (Cellgro) and Singlequots (Lonza, Basel, Switzerland). Medium for the 3T3-L1 and D1 cell lines was Dulbecco's Modified Eagle's Medium (ATCC) supplemented with 10 % fetal bovine serum (Cellgro), 1 % antibiotic–antimycotic (Cellgro), and 1 % fungizone (Cellgro).

All cells were seeded at a density of 10,000 cells/ml in each well of four 96-well plates (Greiner Bio-one, Monroe, NC, USA). Each well was filled with 200 µL of medium, specific to each cell type. The plates were incubated for 3 days in a 37 °C and 5 % CO_2 incubator. After the initial seeding time, the wells were changed with new media which were supplemented with concentrations of resazurin ranging from 0 to 20 % (v/v) of stock alamarBlue® solution to medium. Two of the seeded plates were then incubated at 37 °C and 5 % CO_2 for 3 h, while one plate was incubated for 4 days, and the last plate was incubated for 8 days. Medium was changed every 2 days.

After the time points were reached for each plate, the treated medium was removed and replaced with fresh medium, free of any resazurin. Cells were then incubated for 2 days further to perform assays. This additional 2 days of incubation allowed insight into each condition and cell type while the groups were unstressed.

After the two additional recovery days, the medium was removed and stored while fresh medium was added. An MTT assay (Invitrogen) was performed by adding 100 µL medium + 10 µL of 12 mM MTT stock solution to each well. The plates were incubated for 3 h, as per protocol instructions. The 3-h time point plates were treated using the sodium-dodecyl-sulfate (SDS) method, where 100 µL of 350 mM SDS was

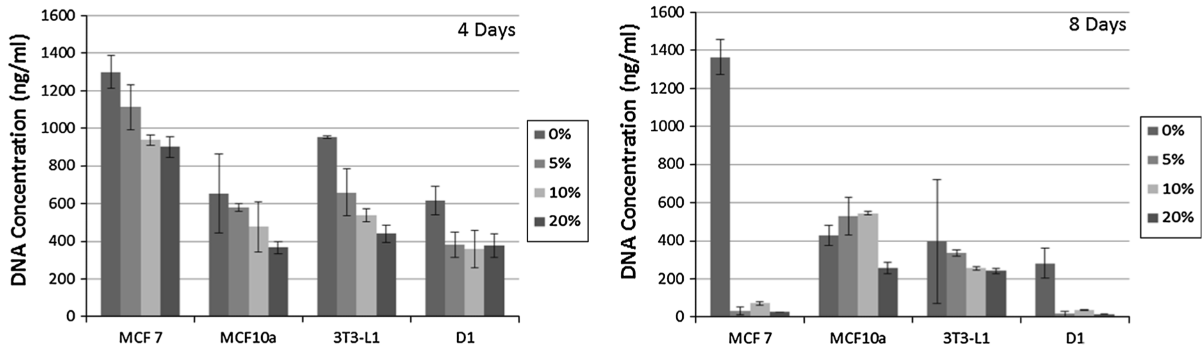

Fig. 2 DNA concentrations after 4 and 8 days of culture with alamarBlue concentrations listed. The assay was conducted after a 2-day recovery period in fresh medium. Two repeats per condition. *Bars* illustrate two standard deviations or 99.5 % confidence

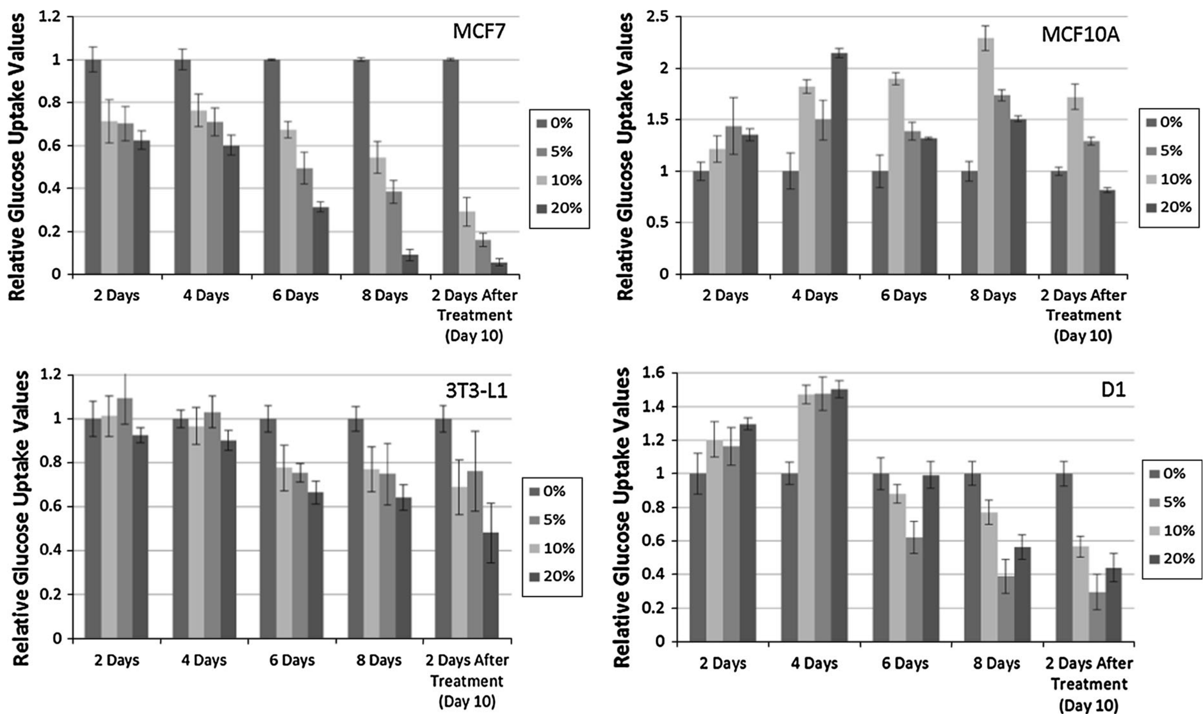

Fig. 3 Glucose uptake results at each day of medium change. Cultures with alamarBlue were used at concentrations listed. Day 10 measurements were made after a 2-day recovery period in fresh medium. Five repeats per condition. *Bars* illustrate two standard deviations or 99.5 % confidence

added to the plates, while the 4- and 8-day plates were treated using the dimethyl sulfoxide (DMSO) method, where 100 μL of DMSO was added to the plates. To reduce bubbles which would obstruct the spectrophotometer, 100 μL of the 210 μL solution in each well was removed and added to black-walled 96-well plates. These black-walled 96-well plates were assessed in a SynergyMX multi-well plate reader (Biotek, Winooski, VT, USA). The SDS-treated plates were observed at 570 λ while the DMSO-treated method plates were observed at 540 λ.

Two more assays were performed in addition to the metabolic activity assessment. Wells were rinsed in phosphate buffered saline and loaded with 200 μL 1× TE buffer (Invitrogen). The plates were then subjected to three freeze/thaw cycles, with an hour between each

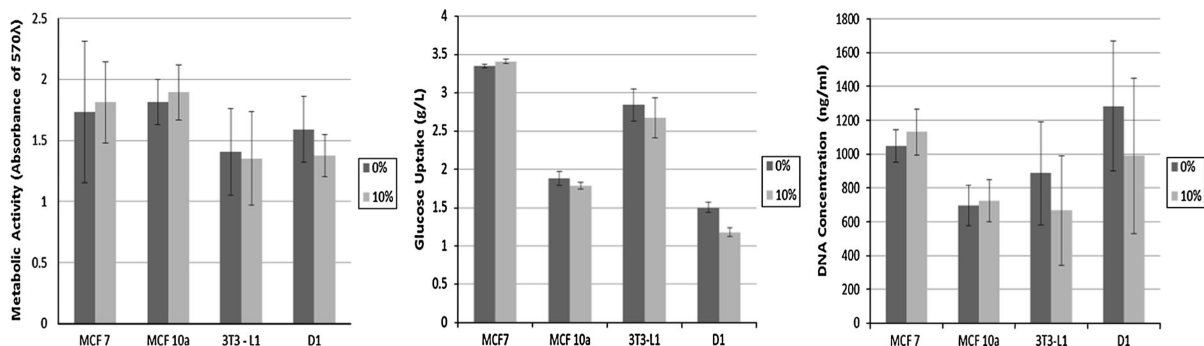

Fig. 4 This figure shows three assays performed on cell cultures which had undergone an alamarBlue assay following the manufacturer's recommended concentrations and times. The assays were conducted after a 2-day recovery period in fresh medium. The spent glucose is that of the recovery medium. Ten repeats were conducted per condition. *Bars* illustrate two standard deviations or 99.5 % confidence

step. Quanti-IT PicoGreen® (Invitrogen) assay was then performed on each well, as per manufacturer instructions. A third assay measured the glucose levels in the medium at each medium change, using a YSI 2900 Biochemistry Analyzer (YSI Life Sciences, Yellow Spring, OH, USA) Four acellular blanks per cell line were used as the control for glucose uptake calculations.

Results and discussion

Resazurin, purchased as alamarBlue®, was tested in culture with a mammary epithelial adenocarcinoma cell line, MCF7, a non-tumorigenic mammary epithelial fibrocystic cell line, MCF10A, a mouse embryonic fibroblast stem cell line, 3T3-L1, and a mouse bone marrow mesenchymal stem cell line, D1. Metabolic activity of the cell cultures after 4 and 8 days of culture with various amounts of resazurin are shown in Fig. 1. These data suggest lower cell survivability with increasing concentrations of resazurin for MCF7, 3T3-L1, and D1 cell lines. Results from a two-tailed Student's *t* test of equal variance suggest MCF7, 3T3-L1, and D1 cultures with 20 % resazurin were significantly less metabolically active than cultures with 0 % resazurin after 8 days in the medium (p value <0.05). No statistical claim can be made regarding the MCF10A cell line.

To investigate the apparent reduction in cellular metabolism, DNA concentration was measured for each sample. These data are shown in Fig. 2, and appear to follow the general patterns seen in Fig. 1. A two-tailed equal variance Student's *t* test reveals that the DNA concentrations of MCF7, and D1 cultures supplemented with 20 % resazurin were significantly different from that of un-supplemented cultures after 8 days (p value <0.05). This suggests the cells were less numerous in cultures with resazurin. No statistical comparisons can be made for MCF10A cultures based on DNA concentration. A two-tailed equal variance Student's *t* test on 3T3-L1 at day 4 shows cultures supplemented with 20 % resazurin were significantly different from un-supplemented cultures (p values <0.05).

Values shown in Fig. 3 are the glucose levels of blanks minus cellular sample glucose levels. The glucose uptake calculation was performed on the medium at each medium change. These data are perhaps the most informative. A two-tailed Student's *t* test, shows MCF7, 3T3, and D1, with either 5, 10 and 20 % resazurin in medium, used less glucose than untreated controls. Statistical claims cannot be made for MCF10A cells.

These data suggest resazurin-based assays are toxic to several cell lines. It cannot yet be said which state of resazurin is causing this effect but it is worth investigating. It is known that both resazurin and its reduced state, resorufin, were in contact with the cells. It cannot be said if the twice reduced state of resazurin, hydroresorufin, was in contact with the cells. However, it is imperative for users of resazurin-based, non-destructive in vitro assays to be aware of potential longer-term toxic effects caused by resazurin or its reduced states, and to be aware that different cell types may have differing levels of tolerance for resazurin.

Since resazurin-based assays have been shown to have toxic effects over longer than manufacturer-recommended incubation times, the recommended

short incubation times were investigated. Cells were treated using the manufacturer's recommendation of 10 % alamarBlue stock solution for 3 hours. After the assay incubation time, the medium was refreshed and the cells were cultured for 2 more days, then the metabolic activity, glucose, and DNA levels were measured (Fig. 4). There is no statistical difference between control and sample results for any cell line. These results support the manufacturer's claim that the assay is non-destructive during the recommended assay times.

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