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Standardization of a resazurin-based assay for the evaluation of metabolic activity in oral squamous carcinoma and glioblastoma cells

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ABSTRACT

The widely accepted resazurin-based assay can be used, prior to in vivo studies, as an inexpensive method to determine cytotoxicity. The aim of this study was to evaluate and standardize the assay conditions for oral squamous cancer cell (OSCC) and glioblastoma (U87-MG) lines by UV–vis spectroscopy. The cells were treated with 25 $\mu g mL^{-1}$ of resazurin sodium salt and then incubated for 4 h, 6 h, and 6.5 h. All absorbance measurements were carried out at 21 ± 1 °C on a spectrophotometer with a microplate reader. After 4-hs of incubation, resazurin was completely reduced by OSCC cells, as demonstrated by the suppression of the absorbance at 380 nm. However, the U87-MG cells needed 6.5 h of incubation to demonstrate the same behavior. The Statistical analysis did not indicate significant differences between the OSCC and U87-MG cell lines' viability after 4 and 6.5 h respectively. We concluded that spectroscopic analysis is an efficient method for the standardization of the resazurin assay. In addition, without the implementation of suitable protocols, there could be an increase in the chance of errors or false positives or negatives that would reduce the usefulness of the data.

1. Background

In vitro tests allow fast and inexpensive screening of drug cytotoxicity prior to *in vivo* studies [1]. The resazurin-resorufin assay (commercially available as CellTiter blue® and Alamar Blue®), based on an oxidation-reduction indicator that fluoresces and changes color when reduced by the living cells, has gained prominence [2–4]. The dye reduction is promoted by NADH reductase and carnitine dehydrogenase [1,5,6]. However, studies show that this reduction can be carried out by other subcellular fractions [7]. Therefore, the assay conditions can be influenced by inherent features of different cell lines [8]. This study optimizes the UV–vis spectroscopy resazurin assay.

2. Aims

The main objectives of our study were to analyze and validate both the analytical and biological parameters of the resazurin-based assay, for cell viability and cytotoxicity of different cancer cell lines, to attain an improved transition from *in vitro* to *in vivo* studies.

3. Methods

3.1. Cell culture

Oral squamous cell carcinoma (OSCC; American Type Culture Collection [ATCC]-SSC-CRL-1623) and glioblastoma (U87-MG; ATCC® HTB14™) cell lines were cultured in Dulbecco´s Modified Eagle Medium (DMEM) (Gibco, New York, USA) supplemented with 10% fetal bovine serum (FBS).

3.2. Resazurin and spectroscopy analyses

Cells were seeded into a 24-well plate (7.5×10^4 cells/well) and after 24 h, 92% of cells death was induced by the addition of 70% (v/v)

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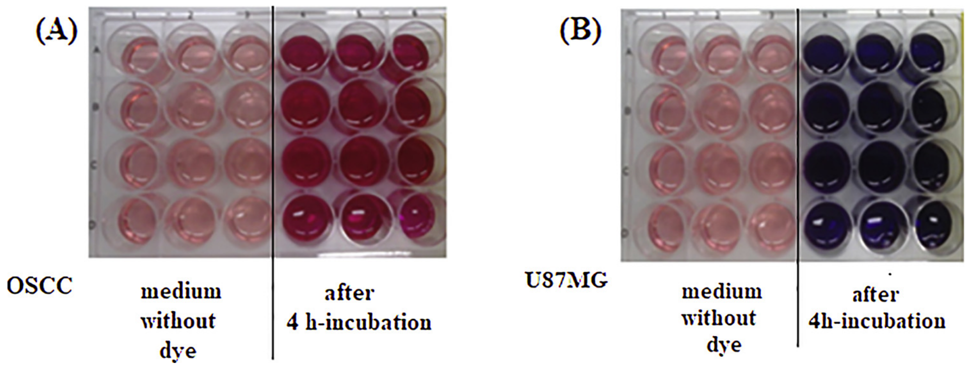

Fig. 1. Resazurin assays with a resazurin concentration of 0.25 μmmL^{-1}. Observed coloration after 4 h of incubation, for (A) oral squamous cell carcinoma cells and for (B) glioblastoma cells.

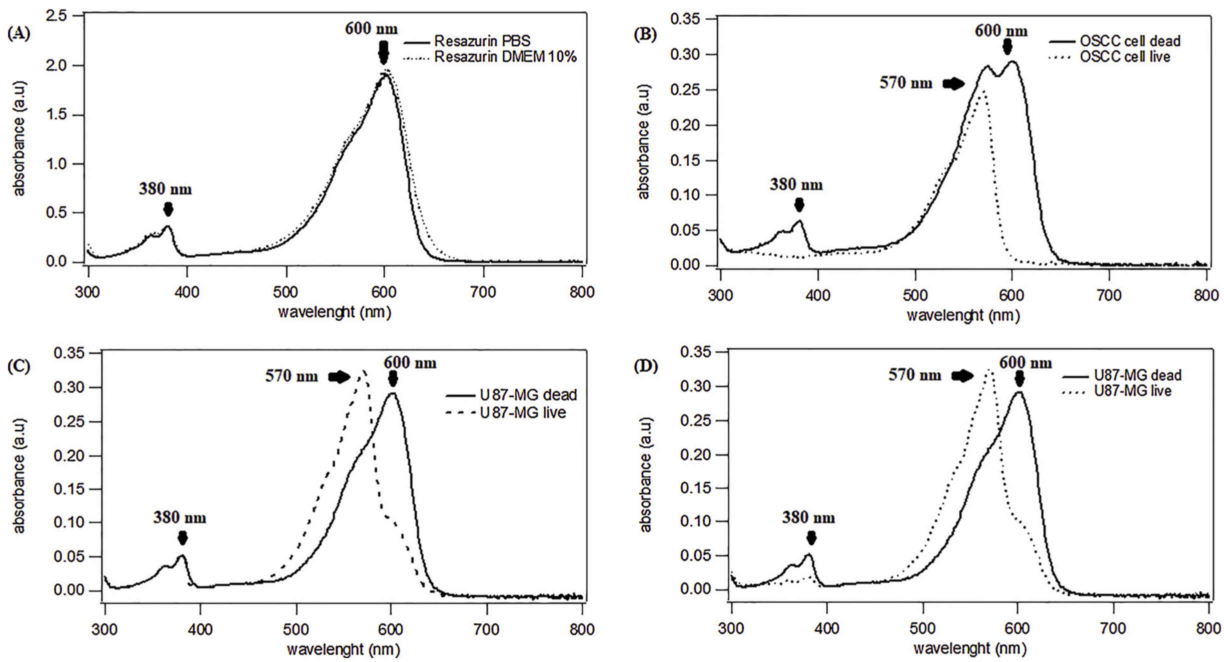

Fig. 2. UV–vis spectra of resazurin from 300 to 800 nm wavelengths: (A) Absorption profiles of resazurin (0.25 μmmL^{-1}) in PBS (—) and DMEM with 10% FBS (······) with a 1–6 dilution of samples in PBS; (B) Oral squamous cell carcinoma (OSCC) control (······) and OSCC dead cells (—) after 4 h of incubation; (C) glioblastoma (U87-MG) control (——) and U87-MG dead cells (—) after 4 h of incubation; (D) U87-MG control (······) and U87-MG dead cells (—) after 6.5 h of incubation. All measurements were carried out at 21 ± 1 °C.

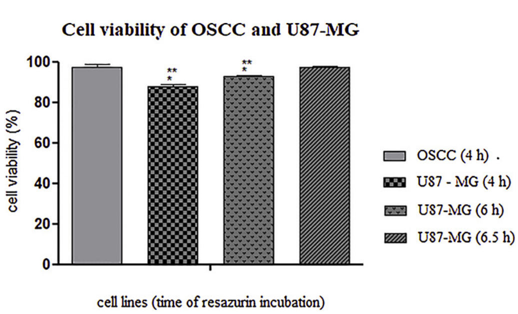

Fig. 3. Cell viability studies of oral squamous cell carcinoma (OSCC) and glioblastoma (U87-MG) cells after the resazurin assay for different time periods of dye incubation. Significant difference to OSCC, after a 4 -h dye incubation period (*) (One-Way ANOVA, Bonferroni post-test; $p < 0.05$). Significant difference to U87-MG, after a 6.5 h of dye incubation (**) (One-Way ANOVA, Bonferroni post-test; $p < 0.05$) (n = 3).

dimethyl sulfoxide (DMSO; Sigma Aldrich, MO, USA), which was quantified by Trypan blue assay. After 4 h, the cells were treated with 25 μgmL^{-1} of resazurin sodium salt (7-hydroxy-3H-phenoxazin-3-one 10-oxide, Sigma-Aldrich Co., MO, USA) in PBS and were further incubated for 4, 6, and 6.5 h. Viable cells with an active metabolism can reduce resazurin, a blue compound, to form resorufin, a pink molecule with absorbance at 570 nm. The absorption analysis was using a microplate reader at 570–600 nm (Safire II, TECAN, Grödig, Austria) and a UV–vis (UV–vis) spectrophotometer at 300–800 nm (Ultrospec 7000, GE, MO, USA), based on previously published protocols [11,12].

3.3. Statistical analysis

Cell viability was calculated according to Jayme et al. [9] and expressed as the mean ± standard deviation (SD). The experiments were triplicated for each parameter. Statistical analysis was performed using one-way ANOVA. Significant comparisons were tested by the Bonferroni correction test. A p-value < 0.05 was considered significant.

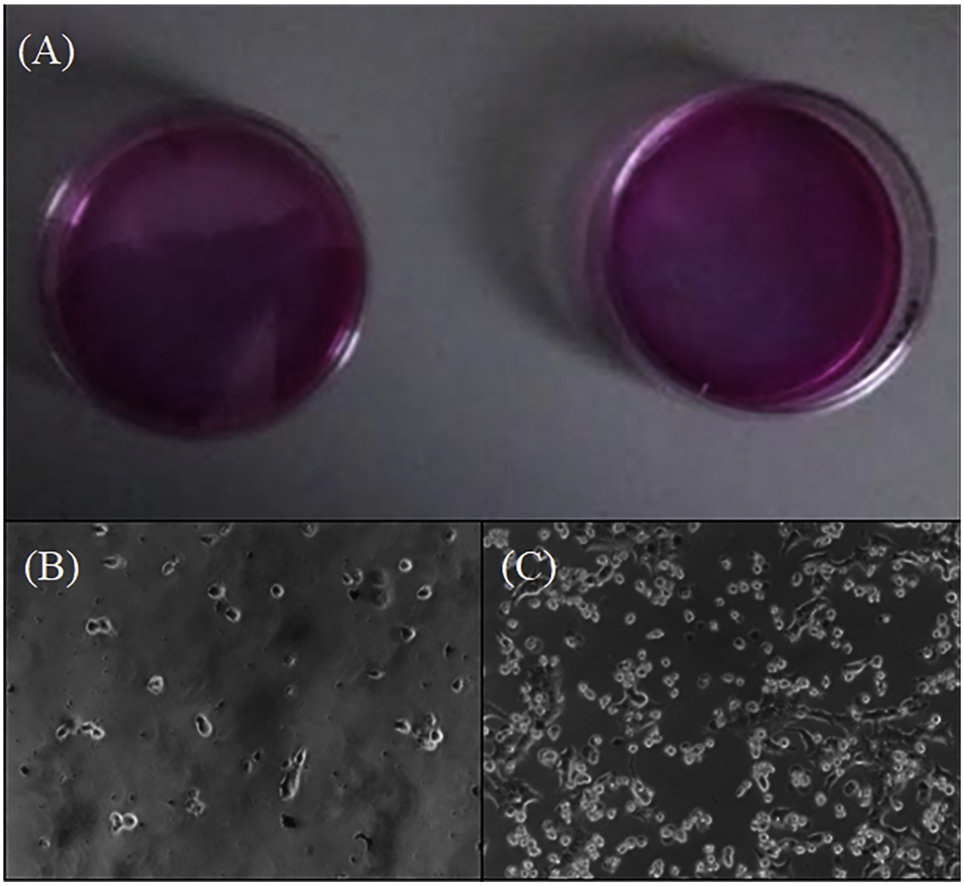

Fig. 4. Total resazurin reduction after a 24 -h dye incubation period: (A) Both cell lines changed the media color to pink. Cell line's micrographs recorded by a Carl Zeiss microscope operating to magnification (10×) coupled to an Axiocam-40 CFL high resolution digital camera. Prolonged exposure to the reduced form of the dye induces cell death in (B) glioblastoma and (C) oral squamous cell carcinoma cells.

4. Results

OSCC cells caused the media to turn pink after 4 h of incubation (Fig. 1A). However, U87-MG cells changed the color of the media to purple, also after 4 h of incubation (Fig. 1B). To understand this difference in behavior, we performed spectroscopy analysis. The UV–vis spectrum for resazurin has significant and relatively low absorbance values at 600 nm and 300–400 nm, respectively (Fig. 2A). For comparison, the spectrum for resorufin showed a shift in λ_{max} from ˜600 nm in the oxidized form, to ˜570 nm after dye reduction by viable cells (Fig. 2B, C, and D). The UV–vis spectrum of OSCCs (Fig. 2B) demonstrated total suppression of the absorption band at 380 nm, after 4 h of resazurin incubation; in addition, we observed a shift in λ_{max} to 570 nm. These data indicate the presence of metabolically active cells, in which the total reduction of the dye takes place. Although the U87-MG UV–vis spectrum shows a shift in λ_{max} of 570 nm after a 4 -h period (Fig. 2C), we found that the total suppression of the band at 380 nm only occurred after a 6.5 -h incubation period (Fig. 2D). Significant differences were not found between the viability of OSCC and U87-MG cells, for the 4- and 6.5 -h periods of resazurin incubation, respectively (Fig. 3). Although resazurin is non-toxic, cell death was observed for both cell lines after a 24 -h incubation period (Fig. 4). These results were intended for the standardization of the resazurin assay.

5. Conclusion

Resazurin-based assays are important for the evaluation of the viability and cytotoxicity in different cell lines. However, to broaden the use of this method, it is essential to evaluate how reducing dye incubation times affects the cell, through effects mediated by lineage metabolism. Authors have reported a reduction in resazurin, caused by diaphorase enzymes including dihydrolipoamide dehydrogenase, quinine oxidoreductase, and flavin reductase [3,8,10,11]. Milano et al. determined the tumoral to non-tumoral dihydropyrimidine dehydrogenase activity ratio from patients with head and neck cancers [12,13]. Furthermore, overexpression of lactate dehydrogenase has been reported in the saliva and tissue of OSCC patients [14]. This was corroborated by fast dye reduction of lineages in our experiments; it is possible that some diaphorases are differentially regulated in expression and activity by the U87-MG cells. Researchers have demonstrated that chemical functional groups (thiols, amines, amides, and carboxylic acids) may interfere with colorimetric assays that involve resazurin and formazan dye in the absence of metabolic activity [2]. These assays have been noted to behave differently depending on the culture media employed in the study and, more specifically, the protein concentration within media [15]. The findings from our study confirm that spectroscopic analysis is an efficient method for the standardization of the resazurin assay, because prolonged exposure to the reduced form of the dye leads to cytotoxicity. Without the implementation of properly controlled studies, there could be a significant increase in errors or false positives or negatives and, therefore, minimize the usefulness of the acquired information.

Disclosure of interest

All authors declare any conflict interests.

Acknowledgments

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