International Endodontic Journal

doi:10.1111/j.1365-2591.2010.01796.x

Comparative analysis of two colorimetric assays in dental pulp cell density

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Abstract

Nedel F, Soki FN, Conde MCM, Zeitlin BD, Tarquinio SBC, Nör JE, Seixas FK, Demarco FF. Comparative analysis of two colorimetric assays in dental pulp cell density. *International Endodontic Journal*, **44**, 59–64, 2011.

Aim To compare and contrast two colorimetric assays used for the measurement of proliferation using two dental pulp cell types: dental pulp stem cells (DPSC) and human dental pulp fibroblasts (HDPF).

Methodology Dental pulp stem cells or HDPF were seeded at 0.25×10^4 cells per well in 96-well plates. Cell proliferation was evaluated after 24–72 h. At the end of the experimental period, the sulforhodamine B (SRB) assay or a water-soluble tetrazolium salt (WST-1) assay was performed. Optical densities were determined in a microplate reader (Genius; TECAN). Data were analysed by Student's *t*-test (comparison between cell types) and one-way ANOVA followed by Tukey test (time-point intervals). Pearson' correlation tests were performed to compare the two assays for each cell line.

Results Both assays showed that DPSC had higher proliferation rates than HDPF. A positive significant correlation between the two colorimetric assays tested for both cell types DPSC (Pearson's correlation coefficient = 0.847; $P < 0.05$) and HDPF (Pearson's correlation coefficient = 0.775; $P < 0.05$).

Conclusion Both tests demonstrated similar trends of cell proliferation, and thus are both appropriate for the evaluation of DPSC and HDPF. The choice of assay is therefore one of the practical applications. SRB stained plates can be dried and stored so may have utility in laboratories where data may require review or when access to analytical equipment is limited. WST-1 assays have the benefit of both ease and speed and may have utility in laboratories requiring either high throughput or rapid analyses.

Keywords: endodontics, methods, stem cells, tissue engineering.

Received 6 October 2009; accepted 20 August 2010

Introduction

Measurement of cell proliferation has become an essential technology in the life sciences. Dental materials research has been driven by an understanding of toxicity limitations and biocompatibility of new materials with dental and other oral tissue (Schweikl *et al.* 2005). In particular, biocompatibility of novel endodontic materials is important for the clinical success of endodontic treatment. Clearly, toxic or irritant materials may induce or even exacerbate inflammatory reactions in the periapical tissues, which may in turn weaken the reparative materials themselves (Geurtsen 2001, Gorduysus *et al.* 2007). Furthermore, advances in tissue engineering are providing potential new biological therapies, especially with dental stem cells, which will allow the creation or reconstruction of dental tissue

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using biodegradable scaffolds and growth factor panels (Cordeiro *et al.* 2008, Nedel *et al.* 2009). Thus, it is evident that effective and appropriate methods must be available to dental materials and tissue research to assess toxicity and biocompatibility but also that with the broad spectrum of materials, reagents and cells involved, one methodology alone may perhaps be insufficient.

Modern colorimetric cell-based proliferation or toxicity assays have been optimized for the use of microtitre plates, allowing many samples to be analysed rapidly and simultaneously using compounds that stain the cells directly or that are metabolized into coloured products (Weyermann *et al.* 2005). A spectrophotometer is used to quantify the intensity of the colour, producing numerical data (absorbance) that correlate with the number of cells present (Givens *et al.* 1990). Notably, significant differences may exist in the sensitivity of colorimetric methods, which use different labelling systems to assay the same final growth parameters, so comparative assessment of the assay methods themselves is vital (Weyermann *et al.* 2005).

The sulforhodamine B (SRB) assay is used in *in vitro* anticancer drug screening (Papazisis *et al.* 1997, Lin *et al.* 1999, Vichai & Kirtikara 2006). Indeed, the SRB assay is an American National Cancer Institute and National Institute of Health standard assay for testing novel anticancer drugs (http://dtp.nci.nih.gov/branches/btb/ivclsp.html). It has been widely employed for both the evaluation of cells other than cancer cells in a 96-well microplate-based assay format (Lin *et al.* 1999). SRB is a bright pink aminoxanthene dye that binds to basic amino acids of cellular proteins under mild acidic conditions and dissociates under basic conditions (Vichai & Kirtikara 2006). The binding of SRB is stoichiometric, and the amount of dye extracted from stained cells is directly proportional to the total protein mass and therefore correlated with cell number (Papazisis *et al.* 1997, Vichai & Kirtikara 2006).

Metabolism of tetrazolium salts, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) and 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1), form the basis of other colorimetric assays. In contrast to the SRB, tetrazolium salt assays are based on the metabolic reduction of the salt to highly coloured formazan end-products (Berridge *et al.* 1996). Despite several studies examining methods for measuring reductase enzymes, the precise nature of the bioreduction of tetrazolium salts is still being investigated (Berridge *et al.* 1996, Tan & Berridge 2000). These colorimetric assays are used extensively in cell proliferation and cytotoxicity analysis, enzyme analysis and bacteriological screening (Berridge & Tan 1993, Berridge *et al.* 1996, Ngamwongsatit *et al.* 2008). More specifically WST-1 has been used in precursor cells such as human embryonic stem cell (Dvorak *et al.* 2005) and mesenchymal stem cells (Dang *et al.* 2006).

A review of the literature revealed that no report has directly compared these two colorimetric assays for evaluation of the proliferation rates of human dental pulp stem cells (DPSC). Such data are critically important for the determination of dental pulp cell function in biocompatibility studies and studies of novel dental tissue engineering methodologies, particularly where one assay relies on metabolic function, which may differ from cell type to cell type. The aim of this study was to perform a comparative analysis of SRB and WST-1 for measurement of the proliferation of DPSC and human dental pulp fibroblasts (HDPF).

Material and methods

Cells

Human DPSC were obtained from Dr Songtao Shi (University of Southern California, USA), and human HDPF were retrieved from permanent teeth extracted in the University of Michigan Oral Surgery department. The research protocol was approved by the Research Ethics Committee of the Federal University of Pelotas, Brazil. Both cell types were cultivated in Dulbecco's Modified Eagle Medium (DMEM) low glucose (DPSC) or high glucose (HDPF) (Invitrogen, Grand Island, NY, USA) containing 10% foetal bovine serum (Invitrogen) and 1% penicillin/streptomycin solution (Invitrogen) at 37 °C in 5% CO_2. Cells from passage 4–6 were used for these experiments. Subconfluent (80%) DPSC and HDPF were detached with 0.25% trypsin–EDTA (Invitrogen). Each cell lineage was seeded at 0.25×10^4 cells per well in 96-well plates. Immediately after seeding, samples were placed in an incubator (37 °C in 5% CO_2). Absorbance was then determined after 24, 48 and 72 h using the WST-1 or the SRB assay.

Sulforhodamine B assay (SRB)

At the end of the experimental period, cells were washed with PBS and fixed with 10% trichloroacetic acid (TCA) for 1 h at 4 °C. Then, plates were washed five times under running water and dried at room temperature. To

stain cells, 50 μL of the SRB premixed solution (0.4% SRB dissolved in 0.1% acetic acid) were added to each well and incubated for 30 min at room temperature. Cells were washed four times with 1% acetic acid to remove unbound excess dye and then dried at room temperature. One hundred and fifty microlitres of 10 mmol L^{-1} unbuffered trizma-base were added to solubilize the bound dye, followed by incubation at room temperature for 1 h. Finally, the plates were placed in a shaker for 1 min. The absorbance was determined in a microplate reader (Genius; TECAN, Männedorf, Switzerland) at 565 nm (Lin *et al.* 1999, Houghton *et al.* 2007).

WST-1 assay

At indicated time-points, 20 μL of the WST-1 premixed reagent (cell proliferation reagent WST-1 (Roche Molecular Biochemicals, Basel, Switzerland) were added to each well, and the plates were incubated at 37 °C for 1 h, as recommended by the manufacturer. Then, the plates were placed on a shaker for 1 min, and the absorbance was determined in a microplate reader at 450 nm.

Statistical analysis

Data were analysed by Student's *t*-test (comparison between cell types) and one-way ANOVA followed by Tukey test (time-point intervals). Pearson's correlation tests were performed to compare the two assays for each cell line. Statistical analyses were carried out using the SIGMASTAT 2.0 software package (SPSS, Chicago, IL, USA). The significance level was set at $P < 0.05$. Triplicate wells were analysed for each experimental data point, and the experiments were repeated at least three times to verify reproducibility of results.

Results

The SRB assay showed that DPSC displayed significantly greater dye incorporation than HDPF ($P < 0.05$) for each time-point evaluated ($P < 0.05$) (Fig. 1). Similar results were observed when cells were analysed with the WST-1 assay ($P < 0.05$) in each time-point ($P < 0.05$), which revealed that DPSC produced significantly more dye product than HDPF (Fig. 1). The overall trends of cell proliferation were similar using both methods. To test whether both colorimetric methods displayed the same proliferation trends using both cell lines, a Pearson's correlation test was used; this demonstrated a strong correlation between the two assays for DPSC (correlation coefficient = 0.847, $P < 0.05$) (Fig. 2). For HDPF cells, a positive correlation between the two assays was also observed (correlation coefficient = 0.775, $P < 0.05$) (Fig. 2).

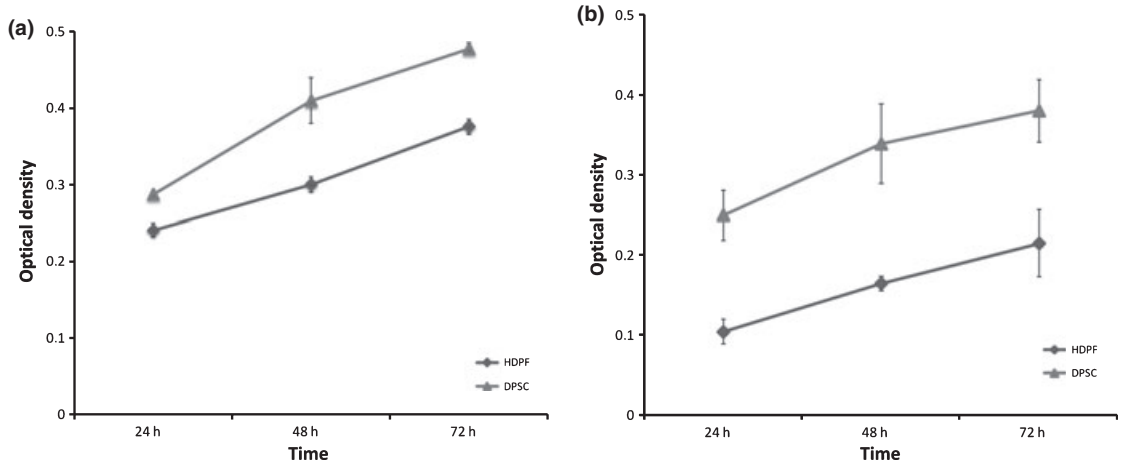

Figure 1 Proliferation of human dental pulp fibroblasts (HDPF) and dental pulp stem cells (DPSC) over time. Cells (DPSC or HDPF) were seeded in 96-well plates at 0.25×10^4 cells per well and grown for indicated time periods. (a) For sulforhodamine B (SRB) assay, cells were fixed with trichloroacetic acid (TCA) stained and evaluated at 565 nm in a microplate reader. (b) For water-soluble tetrazolium salt (WST-1) assay, cells were incubated 1 h in the premixed solution of WST-1. Dye intensity was measured at 450 nm using a microplate reader. Data was analysed from triplicate wells per experimental point and cell line, and reflect the results of three independent experiments.

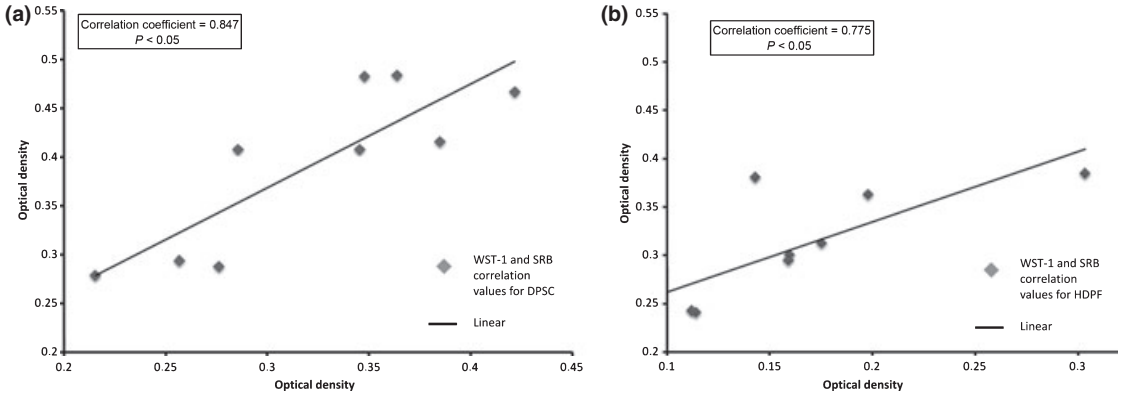

Figure 2 Correlation between the results obtained for water-soluble tetrazolium salt (WST-1) and sulforhodamine B (SRB) assays for dental pulp stem cells (DPSC) and human dental pulp fibroblasts (HDPF). (a) Graph depicting the correlation between WST-1 and SRB for DPSC cells (correlation coefficient = 0.847, $P < 0.05$). (b) Graph depicting the correlation between WST-1 and SRB for cells (correlation coefficient = 0.847, $P < 0.05$).

Discussion

Tissue engineering is an emerging and multidisciplinary field with potential for designing and constructing tissues or organs to restore their function or even completely replace them. One of the main components of tissue engineering is stem cell that responds to morphogenic signals, proliferating and differentiating to give rise to tissue and organs (Nakashima & Akamine 2005, Nedel *et al.* 2009). Therefore, the certainty of effective and simple methods for the evaluation of stem cell proliferation is critical to expedite the progress of research in the field of dental pulp tissue engineering.

Sulforhodamine B is a technique that requires several steps with one very critical step, subject to error, being the initial addition of trichloroacetic acid (TCA). The acid must be added gently so that the cells are not dislodged before they become fixed. Once fixation occurs, the cells become fairly resistant to damage (Papazisis *et al.* 1997). In addition to mechanical error, exogenous proteins present in foetal bovine serum can potentially be fixed along with the cells, and this may affect the results causing high background values and high coefficients of variation (Papazisis *et al.* 1997). In contrast, the WST-1 assay is less technique sensitive. Specifically, WST-1 is available as a ready-to-use solution and, once applied, the plates can be read generally without further manipulation.

Notably, timing is a major factor separating the methods. The SRB assay takes approximately five or more hours to perform, irrespective of cell type, whilst the WST-1 assay requires between 0.5 and 4 h, depending on cell type. For DPSC, the WST-1 assay was typically performed in approximately 1 h. Therefore, the WST-1 assay is a more rapid method for the evaluation of DPSC proliferation. However, an important benefit of the SRB assay is that at any point after TCA-fixation, plates can be dried and stored under dark and cool conditions (Vichai & Kirtikara 2006).This may be of benefit, for example, in laboratories that require review, or reanalysis, of original data after the end of experimental studies. Alternatively, in laboratories with limited equipment, the plates may be stored until such time as equipment becomes available or, for example, where absorbance analysis must be performed at another physical site.

It has been observed that the SRB assay displays lower variation between different cell lines when compared to colorimetric assays relying on cell metabolism such as MTT, a dye similar to WST-1 (Keepers *et al.* 1991). This is because of the different reduction capacity of each cell type, resulting in varying formazan product levels and thus in varying measured optical density (Keepers *et al.* 1991). The reduction of WST-1 has been associated with superoxide, and it seems to occur in the extracellular environment or associated with the plasma membrane (Berridge *et al.* 1996, Tan & Berridge 2000). Therefore, depletion of NADH or some other mechanism that involves superoxide may potentially interfere with the reduction process of WST-1 (Berridge *et al.* 1996). Also, studies have indicated that different human cell types have varied extracellular superoxide content (Marklund

1984, Fattman *et al.* 2003). This variation could interfere in the reduction capacity of each cell type to different degrees. Since the discovery of stem cells in the dental pulp, many studies have been carried out using these cells (Gronthos *et al.* 2000, Cordeiro *et al.* 2008, Yang *et al.* 2009). Therefore, it is important to test the response of DPSC towards colorimetric assays that are being used, to ensure the quality of results. In this study, a positive correlation was demonstrated between both colorimetric assays (WST-1 and SRB) in the two cell lines tested. This suggests that possible variations in DPSC metabolism did not interfere significantly in the linearity of WST-1 results compared to the more robust SRB assay.

Conclusion

Both tests demonstrated similar trends, with increasing absorbance apparently relating to increasing cell densities over time. Both assays are therefore suitable for analysis of DPSC. Therefore, the choice between one assay or another (WST-1 or SRB) should be based on the practical advantages or disadvantages of each method. The SRB may be more useful in laboratories where results may require re-examination at a later date, whereas WST-1 could be used in daily laboratory routine where a more rapid and easier method is required.

Acknowledgements

This study was supported by a scholarship to the first author and a grant (484329/2007-3 – FFD) provided by two Brazilian Government Agencies (CAPES and CNPq).

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