Vol.57, n.3: pp. 381-385, May-June 2014
http://dx.doi.org/10.1590/S1516-89132014005000007
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Cell Density and Solvent Are Critical Parameters Affecting Formazan Evaluation in MTT Assay

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ABSTRACT

The aim of this study was to establish the more accurate protocol for fibroblast cell viability using MTT assay. NIH/3T3 fibroblasts were seeded at the following cell densities: $3.125x10^3$; $1.156x10^4$; $3.125x10^4$; $1.156x10^5$ and $3.125x10^5$ cells/cm^2. Following 24h of seeding, MTT was added to the wells. After 4h of the MTT addition, different solvents were added to solubilize the formazan crystals: 1) HCl/SDS group- 20% SDS and 0.01 M HCl; 2) EtOH/ HAc group-50% ethanol and 1% acetic acid; 3) DMSO group- 99.5% dimethyl sulfoxide; and 4) PropOH group- 99.5% isopropanol. The absorbance values were measured using a spectrophotometer at 570 nm. The data were analyzed by 2-way ANOVA ($p<0.05$) and showed that the absorbance average varied according to the number of cells and solvents: HCl/SDS (0 to 0.13), EtOH/HAc (0 to 0.22), DMSO (0.76 to 1.31) and PropOH (0.66 to 1.04). The DMSO and PropOH groups presented the most appropriate protocols for NIH/3T3 fibroblasts cell viability, especially at the density of $1.156x10^4$ cells/cm^2.

Key words: cell viability, MTT assay, fibroblast NIH/3T3, solvents

INTRODUCTION

MTT (3-4,5 dimethylthiazol-2, 5 diphenyl tetrazolium bromide) assay is a wide world known method to evaluate cell viability and toxicity. It is based on the conversion of the soluble tetrazolium, a yellow salt, into purple insoluble formazan, by succinate dehydrogenase enzyme of viable cells (Mosmann 1983). It has been replacing traditional methodologies such as manual cell counting or colony formation in large-scale experiments, when hundreds or even thousands of samples are tested. It presents sensitivity in the detection of cells that stop the division process, but are still considered metabolically active (Gerlier and Thomasset 1986). Besides this, it can be used for monolayers or suspension cell preparations (Henriksson et al. 2006). However, its disadvantages are: 1) the impossibility of keeping the cells alive in the culture after the procedure (Al-Nasiry et al. 2007); 2) this method does not differentiate between non-proliferative cells and death cells; and 3) it is also not able to quantify and detect the type of cell death (Freshney 2005).

Other methods are also employed to evaluate the cell viability, such as Neutral Red (Schweikl and Schmalz 1996; Repetto et al. 2008), Crystal Violet (Chiba et al. 1998; De-Deus et al. 2009), LDH (Issa et al. 2004; Garner et al. 2012), Alamar Blue (Hamid et al. 2004; Hjalmarsson et al. 2011), and trypan blue exclusion (Agarwal et al. 2011), but MTT has still demonstrated to be more trustful among them (Keepers et al. 1991; Schweikl and

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Schmalz 1996; Chiba et al. 1998; Kawada et al. 2002; De-Deus et al. 2009; Alanezi et al. 2010; de Castilho et al. 2012). Since it was described, this indirect colorimetric method underwent several modifications that could be required, depending on the experiment design (Twentyman and Luscombe 1987; Vistica et al. 1991; Mueller et al. 2004; Gonçalves et al. 2008). Some alterations in the original protocol are regarded to the cell densities (Vistica et al. 1991) due to different behavior to reduce the dye presented by distinct cell lines (Carmichael et al. 1987) and some other intra- and inter-assay variations (Plumb et al. 1989).

Cell density is an important factor to be considered when performing viability tests. The cell physiology and response to toxins are affected by the cell density number; cells at a lower density are more affected by the toxin. Adversely, toxin may become bound and unavailable at higher cell densities (Riss and Moravec 2004).

One of the most common modifications of the MTT assay is related to the solubilization of the formazan crystals. Several organic solvents have been employed (Denizot and Lang 1986; Abe and Matsuki 2000) in an attempt to improve the measurements at spectrophotometer. To-date, there is no consensus on the best solvent to be used for each cell line in the MTT assay. In this regard, several organic solvents that were previously addressed in the literature were assessed. NIH/3T3 mice fibroblast cells were chosen because of their broad range of applications in cell culture for several decades (Jainchill et al. 1969), including cell viability and toxicity tests of biomaterials or drugs in dentistry and medicine (Fontes et al. 2013; Vitteková et al. 2013).

There is no previous study addressing the effect of both cell density and type of solvent at the same experiment on the results of the viability assay. Thus, this study analyzed the effect of 1) the cell plating number and 2) the four most employed solvents for MTT assay to better establish an adequate protocol for NIH/3T3 cells in viability tests.

MATERIALS AND METHODS

Material

The materials were obtained from the following companies: culture media, antibiotics and fetal bovine serum from Nutricell (Campinas, Brazil); plastic ware and pipettes from TPP (Trasadingen, Switzerland) and SPL Life Sciences (Pochen City, Korea); MTT and trypsin from Sigma Chemical (St. Louis, USA); DMSO from Nuclear (Ribeirão Preto, Brazil); ethanol and isopropanol from LabSynth (Diadema, Brazil); and sodium dodecyl sulfate (SDS) from Merck (Darmstadt, Germany). NIH/3T3 fibroblasts were obtained from American Type Culture Collection (ATCC). All reagents were of analytical grade.

Cell culture

The cells (NIH/3T3, ATCC® CRL1658™) were seeded in Dulbecco's Modified Eagle's Medium (DMEM) with antibiotics (1% of a solution containing 10,000 UI/mL penicillin and 10 mg/mL streptomycin) and 10% fetal bovine serum. They were maintained at 37°C in a humidified atmosphere of 5% CO_2. The enzymatic digestion with 0.25% trypsin was used to harvest the cells. The cells were counted using Neubauer modified camera and optical inverted microscope (x32, Leica DM IL). The mean values obtained in the quarters were multiplied by 10^4 to obtain the total number of cells in 1.0 mL of culture medium (Freshney 2010). To evaluate the best plating cell number, different cell densities ($3.125x10^3$; $1.156x10^4$; $3.125x10^4$; $1.156x10^5$ and $3.125x10^5$ cells/cm^2) were plated in 96-well plates in six replicates. After 24h, the medium was removed, the cells were washed two times in pH 7.4 phosphate buffered saline (PBS), and the different protocols of MTT were performed.

MTT Assay

MTT dissolved in DMEM (0.5 mg/mL, 110 µL) was added to each well, with the exception of the HCl/SDS group, in which 10 µL MTT diluted in 1x PBS (5 mg/mL) and 160 µL of MEM without bromophenol red were added. After the addition of MTT, plates were wrapped in aluminum foil and incubated at 37°C for 4h in a humidified atmosphere of 5% CO_2. Then, the MTT solution was removed from the wells (with the exception of the HCl/SDS group). Each MTT technique applied a different crystal solvent for each cell density as described below:

- Group HCl/SDS: 50 µL solution containing 20% SDS and 0.01M HCl (solubilization with 170 µL MTT+MEM)
- Group EtOH/HAc – 200 µL 50% ethanol and 1% acetic acid

- Group DMSO: 200 µL dimethyl sulfoxide
- Group PropOH: 200 µL pure isopropanol

The absorbance was read in a spectrophotometer (Fluorstar Optima- BMG Labtech, Ortenberg, Germany) at 570 nm, 30 min after the solubilization of the crystals.

Statistic Analysis

All of the values are given as the mean±S.D (n=6). The Kolmogorov-Smirnov test was performed to verify whether the samples followed the Gaussian distribution. The results were subjected to two-way repeated-measures ANOVA, followed by the Bonferroni post-hoc test. The statistical tests were performed using statistical software GraphPad Prism 4.0 (San Diego, CA, USA). A level of significance of 5% was applied.

RESULTS AND DISCUSSION

Table 1 shows the mean values of absorbance for each MTT protocol, considering the different cell densities. Two-way ANOVA test revealed a significant difference among cell densities ($p < 0.0001$), MTT assays ($p < 0.0001$), and an interaction between these factors ($p < 0.0001$), There was an increase in the absorbance means only for the cell densities higher than 3.125 x 10^4/cm^2 for HCl/SDS and EtOH/HAc solvents. For DMSO and PropOH, a better relationship between the cell density and optical absorbance was seen (Table 1). The HCl/SDS and EtOH/HAc groups presented differences only when the cells were seeded at 3.125 x10^5/cm^2. Both the HCl/SDS and EtOH/HAc produced significant lower optical densities in comparison to DMSO and PropOH for all the cell densities. On the other hand, DMSO produced the highest optical densities for all the cell densities that were studied (Table 1).

The MTT assay, a widespread colorimetric biological technique, is used to test the viability of various cell types, such as fibroblasts (Scelza et al. 2012), odontoblasts (de Castilho et al. 2012), and transformed cells (Metsios et al. 2012), and to research applications such as dental material cytotoxicity (Garner et al. 2012), and drug testing (Odabas et al. 2011; Dateoka et al. 2012). It is also useful for small-scale experiments testing expensive and toxic drugs, as well as plating cells whose growth is slow and the livelihood in the medium is critical (Hamid et al. 2004). The MTT assay is able to distinguish between the viable cells and senescent or dead cells, but it is not sensitive to distinguish between the senescent or dead cells. It is also not able to quantify or detect the type of cell death. One methodological limitation is that the tested agent must not affect the ability of the cell to reduce the dye or to interfere on the color range of the reaction. Also, it is important to perform the assay when the cells are in their exponential phase. If the cells reach the stationary phase or the absorbance is not linear, the assay should be shortened or the cells concentration changed. If the drug or agent to be evaluated present longer terms toxicity, it is advisable to choose other viability assays (Freshney 2005).

Table 1 - Mean and S.D. of absorbance values for the 24h-MTT protocols and cell densities (n=6).

	3.125 x 10^3	**1.156 x 10^4**	**3.125 x 10^4**	**1.156 x 10^5**	**3.125 x 10^6**
HCl/SDS	0.00±0.00^{A,a}	0.00±0.01^{A,a}	0.01±0.01^{A,a}	0.13±0.05^{B,a}	0.08±0.05^{B,a}
EtOH/HAc	0.00±0.00^{A,a}	0.00±0.00^{A,a}	0.00±0.00^{A,a}	0.15±0.02^{B,a}	0.22±0.02^{C,b}
DMSO	0.76±0.11^{A,c}	0.80±0.04^{AB,c}	0.86±0.03^{B,c}	1.31±0.04^{D,c}	1.18±0.04^{C,d}
PropOH	0.66±0.05^{A,b}	0.70±0.03^{AB,b}	0.72± 0.03^{B,b}	1.04±0.06^{D,b}	0.88±0.06^{C,c}

Between columns, different upper-case superscript letters indicate significant difference among the cell densities for the same MTT assay. Within columns, different lower-case superscript letters indicate significant different among different MTT assay for each cell density (cells/cm^2) (two-way ANOVA, $p<0.0001$).

Accordingly, some modifications have been proposed in order to adjust the method to the field of research. Cell density has been demonstrated to be an important factor to alter any cell culture technique, including cell viability, toxicity and apoptosis assays (Metsios et al. 2012). The present study showed that the highest cell concentration (such as 3.125x10^5 cells/cm^2) presented a reduction in the absorbance values, probably because cells had stopped dividing at higher densities as a result of cell crowding, shape change and growth factor depletion (Freshney 2010). These results indicated that NIH/3T3 should be seeded at a density lower than 3.125x10^5/cm^2 and

that the best results were around 3.125x 10^3 $3.125x10^4/cm^2$. The experiments employing longer periods of time should be carefully planned in order to avoid an over confluence that could result in exhaustion of the medium, and as a consequence, lower cell viability.

In order to improve the achievements of the method, this study also aimed to verify which solvent could be more effective when NIH/3T3 fibroblasts were used. Results indicated that DMSO and PropOH were better solvents for MTT cell viability assays to be applied for NIH/3T3 cells under a density of $3.125x10^4/cm^2$ in a 96-well plate. Using these solvents, the experiment can be performed using fewer cells, which is an important point to be considered when planning a large experiment, containing many batches. The lower density at seeding is crucial for the cells with rapid proliferative rate, such as the fibroblasts. These cells should not reach confluence before the assay endpoint; otherwise, they withdraw from the cell cycle or even die. Also, rare cells lineages or cells derived from the patient biopsies need an assay in which they could be plated at the lowest cell density as possible.

Results showed that the best relationship between the absorbance and cell numbers for DMSO were in accordance to Carmichael et al. (1987), which compared several solvents in the tumor cell lines. However, these authors found that the use of acid isopropyl alcohol (PropOH) as a solvent was unable to achieve the solubilization of the formazan crystals and that the minimal absorbance was detected at 570 nm. The type of cell line might explain this different result for PropOH compared to the present study. DMSO was very appropriate when an undetectable residual medium could be left in the wells (Sieuwerts et al. 1995). Another advantage is that optical density is stable for several hours when this solvent is applied (Sieuwerts et al. 1995).

Although many advanced molecular techniques have been discovered in the past, MTT is still a useful, trustful and economic tool for cell viability and cytotoxicity assays. For this reason, this work is relevant since it studied which solvent could reach the best results using different densities of NIH/3T3 fibroblast cells, the most useful cell lineage for experiments involving dental materials cytotoxicity.

CONCLUSIONS

Based on the results, DMSO and PropOH could be recommended as the most suitable solvents for viability tests in NIH/3T3 fibroblast at a density of $1.156x10^4$ cell/cm^2.

ACKNOWLEDGMENTS

The authors are grateful to Centro Integrado de Pesquisa (CIP) – Faculdade de Odontologia de Bauru - USP for providing some work facilities. This work was supported by FAPESP (10/14984-4 a grant for the last author and 09/17360-4 a scholarship for the second author).

REFERENCES

Abe K, Matsuki N. Measurement of cellular 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction activity and lactate dehydrogenase release using MTT. *Neurosci Res*. 2000; 38: 325-332.

Agarwal SK, Praveen G, Gupta S, Tandon R, Gupta S. In vitro evaluation of cytotoxicity of denture adhesives. *Indian J Dent Res.* 2011; 22: 526-529.

Al-Nasiry S, Geusens N, Hanssens M, Luyten C, Pijnenborg R. The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells. *Hum Reprod*. 2007; 22(5): 1304-1309.

Alanezi AZ, Jiang J, Safavi KE, Spangberg LSW, Zhu Q. Cytotoxicity evaluation of endosequence root repair material. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2010; 109: 122-125.

Carmichael J, DeGraff WG, Gazdar AF. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.* 1987; 47: 936-942.

Chiba K, Kawakami K, Tohyama K. Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells. *Toxicol In Vitro*. 1998; 12: 251-258.

Dateoka S, Ohnishi Y, Kakudo K. Effects of CRM197, a specific inhibitor of HB-EGF, in oral cancer. *Med Mol Morphol.* 2012; 45(2): 91-97.

de Castilho AR, Duque C, Negrini T de C, Sacono NT, de Paula AB, Sacramento PA, et al. Mechanical and biological characterization of resin-modified glass-ionomer cement containing doxycycline hyclate. *Arch Oral Biol*. 2012; 57: 131-138.

De-Deus G, Canabarro A, Alves G, Linhares A, Senne MI, Granjeiro JM. Optimal cytocompatibility of a bioceramic nanoparticulate cement in primary human mesenchymal cells. *J Endod*. 2009; 35: 1387-1390.

Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods.* 1986; 89: 271-277.

Fontes ST, Fernández MR, Ogliari FA, de Carvalho RV, de Moraes RR, Pinto MB, et al. Tetrahydrofuran as solvent in dental adhesives: cytotoxicity and dentin bond stability. *Clin Oral Investig.* 2013; 17(1): 237-242.

Freshney RI. Database of misidentified cell lines. *Int J Cancer*. 2010; 126(1): 302.

Freshney. RI. Culture of animal cells: a manual of basic techniques. 5th Ed. New York: Wiley-Liss; 2005.

Garner AD, Tucci MA, Benghuzzi HA. Functional and structural responses associated with potential restorative dental adhesives using human gingival fibroblasts as a model. *Biomed Sci Instrum*. 2012; 48: 141-148.

Gerlier D, Thomasset N. Use of MTT colorimetric assay to measure cell activation. *J Immunol Methods*. 1986; 94(1-2): 57-63.

Gonçalves TS, Schmitt VM, Thomas M, Souza MAL, Menezes LM. Cytotoxicity of Two Autopolymerized Acrylic Resins Used in Orthodontics. *Angle Orthod.* 2008; 78: 926-930.

Hamid R, Rotshteyn Y, Rabadi L, Parikh R, Bullock P. Comparison of alamar blue and MTT assays for high through-put screening. *Toxicol In Vitro.* 2004; 18: 703-710.

Henriksson E, Kjellén E, Wahlberg P, Wennerberg J, Kjellström JH. Differences in estimates of cisplatin-induced cell kill in vitro between colorimetric and cell count/colony assays. *In Vitro Cell Dev Biol Anim*. 2006; 42(10): 320-323.

Hjalmarsson L, Smedberg JI, Aronsson G, Wennerberg A. Cellular responses to cobalt-chrome and CP titanium--an in vitro comparison of frameworks for implant-retained oral prostheses. *Swed Dent J*. 2011; 35: 177-186.

Issa Y, Watts DC, Brunton PA, Waters CM, Duxbury AJ. Resin composite monomers alter MTT and LDH activity of human gingival fibroblasts in vitro. *Dent Mater*. 2004; 20: 12-20.

Jainchill JL, Aaronson SA, Todaro GJ. Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. *J Virol.* 1969; 4: 549-553.

Kawada K, Yonei T, Ueoka H, Kiura K, Tabata M, Takigawa N, et al. Comparison of chemosensitivity tests: clonogenic assay versus MTT assay. *Acta Med Okayama*. 2002; 56: 129-134.

Keepers YP, Pizao PE, Peters GJ, Van Ark-Otte J, Winograd B, Pinedo HM. Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for in vitro chemosensitivity testing. *Eur J Cancer*. 1991; 27: 897-900.

Metsios A, Verginadis I, Simos Y, Batistatou A, Peschos D, Ragos V, et al. Cytotoxic and anticancer effects of the triorganotin compound [(C(6)H(5))(3)Sn(cmbzt)]: an in vitro, ex vivo and in vivo study. *Eur J Pharm Sci.* 2012; 47(2): 490-496.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983; 65: 55-63.

Mueller H, Kassack MU, Wiese M. Comparison of the usefulness of the MTT, ATP, and calcein assays to predict the potency of cytotoxic agents in various human cancer cell Lines. *J Biomol Screen*. 2004; 9: 506-515.

Odabaş ME, Ertürk M, Çınar Ç, Tüzüner T, Tulunoğlu Ö. Cytotoxicity of a new hemostatic agent on human pulp fibroblasts in vitro. *Med Oral Patol Oral Cir Bucal*. 2011; 16: e584-587.

Plumb JA, Milroy R, Kaye SB. Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. *Cancer Res.* 1989; 49: 4435-4440.

Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat Protoc.* 2008; 3(7): 1125-1131.

Riss TL, Moravec RA. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay Drug Dev Technol.* 2004; 2: 51-62.

Scelza MZ, Coil J, Alves GG. Effect of time of extraction on the biocompatibility of endodontic sealers with primary human fibroblasts. *Braz Oral Res.* 2012; 26(5): 424-430.

Schweikl H, Schmalz G. Toxicity parameters for cytotoxicity testing of dental materials in two different mammalian cell lines. *Eur J Oral Sci.* 1996; 104: 292-299.

Sieuwerts AM, Klijn JG, Peters HAM, Foekens JA. The MTT tetrazolium salt assay scrutinized: How to use this assay reliably to measure metabolic activity of cell cultures *in vitro* for the assessment of growth characteristics, IC50-values and cell survival. *Eur J Clin Chem Clin Biochem.* 1995; 33: 813-823.

Twentyman PR, Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *Br J Cancer.* 1987; 56: 279-285.

Vistica DT, Skehan P, Scudiero D, Monks A, Pittman A, Boyd MR. Tetrazolium-based Assays for Cellular Viability: A critical examination of selected parameters affecting formazan production. *Cancer Res*. 1991; 51: 2515-2520.

Vitteková M, Dragúňová J, Kabát P, Božiková M, Bakoš D, Koller J. Cytotoxicity testing of scaffolds potentially suitable for the preparation of three-dimensional skin substitutes. *Cell Tissue Bank.* 2013; DOI 10.1007/s10561-013-9390-0.

Received: April 12, 2013;
Accepted: November 25, 2013.