Inaccuracies in MTS assays: major distorting effects of medium, serum albumin, and fatty acids

Kuang Tzu Huang, Yen Hao Chen, and Ameae M. Walker
University of California, Riverside, CA, USA

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*Soluble formazan assays are widely used for cell number assessment. However, in our hands, we observed frequent occasions in which the actual cell number was at odds with the assay reading. In this study, we have determined that (*i*) a large proportion of the reading obtained in commonly used culture media can be caused by media component amplification of formazan production in a way that cannot be corrected for by media-only controls; (*ii*) the albumin present in 10% serum can reduce the assay absorbance by 50% so that an actual doubling of cell number can be obscured; and (*iii*) this latter effect is dependent on the concentration of fatty acids. To counter these problems, we have developed a protocol that gives consistent readings that are fully representative of cell number while retaining some of the original advantages of soluble formazan assays.*

INTRODUCTION

Tetrazolium salt reduction has proven to be a convenient, widely used, and cost-effective indirect measure of viable cell number (1–4). When originally described, these assays involved the uptake of tetrazolium salts such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] from culture medium and the analysis of the reduced product that accumulated inside the cells. Intracellular reduction was primarily attributable to mitochondrial dehydrogenases (5–7). After the desired incubation time, the medium was discarded, and the cells were solubilized in order to monitor the absorbance of the formazan product (8,9).

More recently, newer tetrazolium salts have been introduced that can pass back out of the cell after reduction (1,4,6,10–13). These include MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], XTT [sodium 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide], and WST-1 [4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate]. For these salts, the usual protocol is simply to add them to the culture medium and read the absorption of the reduced product at an appropriate time (1,14). This approach obviously saves time and eliminates potential errors such as cell loss that can occur when removing culture medium and subsequently solubilizing cells. However, in our laboratory, prolonged use of these soluble formazan salts for cell assays began to cause concern. Occasions when microscopic examination of cultures gave different impressions from the assay results became increasingly common. We therefore undertook the present study aimed at determining which substances interfere in such assays. We report major contributions to salt reduction by common culture media and effects of usual supplements that can obscure changes in cell number during proliferation assays.

MATERIALS AND METHODS

Materials

MTS was purchased from Promega (Madison, WI, USA). Phenazine methosulfate (PMS), bovine serum albumin (BSA; minimum 98% pure, essentially fatty acid-free), fatty acid supplement, and activated charcoal were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), horse serum (HS), all culture media, and Dulbecco's phosphate-buffered saline (DPBS; without magnesium chloride and calcium chloride) were purchased from Invitrogen (Carlsbad, CA, USA). Charcoal-stripped FBS and HS were from Hyclone (Greeley, CO, USA) and Cocalico (Reamstown, PA, USA), respectively. Sex hormone binding globulin (SHBG) was purchased from Cal-Biochem (San Diego, CA, USA).

Cell Culture

The mouse mammary epithelial cell line, HC11, was a gift from Dr. Hynes (Friedrich Meischer Institute, Basel, Switzerland) through Dr. Neville (University of Colorado, Denver, CO, USA). The cells were grown in RPMI 1640 with 10% FBS, 5 µg/mL insulin (Sigma-Aldrich), and 10 ng/mL epidermal growth factor (EGF; Invitrogen, Grand Island, NY, USA). The cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Cell-Mediated Reduction of MTS

Growing cells were harvested, counted, and seeded at the cell number indicated in Figure 1 (200-µL volume) into 96-well microplates. After 18 h, culture medium was replaced by medium containing experimental agents (200 µL). MTS/PMS solution was freshly prepared at 2/0.92 mg/mL in DPBS. Ten microliters of MTS/PMS reagent (excess MTS/PMS) were added to each well, followed by incubation in a humidified, 5% CO_2 atmosphere. Absorbance at 490 nm was recorded at 1, 2, and 4 h. A blank experiment detecting cell-free background absorbance was also performed in parallel. Absorbance shown in the figures using HC11 cells was obtained by subtracting the absorbance of cell-free equivalents. Trypan blue exclusion showed less than 1% cell death both before and after the assays.

In Vitro Cell-Free Reduction of MTS

The methodology for the in vitro cell-free MTS assay was essentially the same as that described for cells, with the following modifications: 20 µL of 0.5 mM ascorbic acid (prepared in DPBS) were added to each well to create a 0.05-mM final concentration. DPBS/Dulbecco's modified Eagle's medium (DMEM)/RPMI 1640 was then added with different amounts of experimental agents

(various types of serum, SHBG, serum albumin, or fatty acid supplement) to make a final volume of 200 µL and concentrations as indicated in the figures. Ten microliters of MTS/PMS solution were then added, and absorbance at 490 nm was recorded at 1, 2, and 4 h. Blank equivalents with no ascorbic acid were performed in parallel.

RESULTS AND DISCUSSION

The major advantage of MTS assays is the ability to measure cell number without having to disturb the cells by the removal of the experimental culture medium. As can be seen in this study, however, conduct of the assay in culture medium will produce artifacts derived from a variety of interacting factors.

Figure 1 illustrates some of the concerns with the MTS assay. For Figure 1A, HC11 cells were seeded at 2000 or 4000 cells per well of a 96-well plate in RPMI 1640 containing 10% FBS and incubated overnight. MTS/PMS was then added to the cultures, and absorbance at 490 nm was recorded at 1–4 h of incubation. There are several concerns: first, the absorbance reading, while greater with 4000 cells, is never twice the reading of 2000 cells, and second, the difference between 4000 and 2000 cells is very small at 1 h, increases to 3 h, and then falls again at 4 h. This is illustrated more clearly in the starred line of Figure 1B, which plots the 4000 to 2000 cell absorbance ratio. In Figure 1C, the culture medium (with 10% FBS) was replaced with either RPMI or RPMI with 2% FBS for the MTS assay. One can see that increased incubation times now continue to result in increased differences between 2000 and 4000 cells, although the reading for 4000 cells still did not reach double that for 2000 cells. The absorbance ratio of 4000 to 2000 cells was greatest in the RPMI without serum (Figure 1B). If, instead, the assay was conducted in DPBS or DPBS with 2% FBS, much lower absorbance readings were obtained, suggesting that some components of RPMI contributed either to the activity/viability of the cells during the assay or to the amount of formazan produced. This is not due to absorbance of the medium itself since this has been subtracted. This is not a small concern because 75% of the reading at 1 h is due to these phenomena (compare 1 h readings in RPMI containing 2% serum with DPBS containing 2% serum). Incubation in DPBS without serum gave the worst 4000 to 2000 cell ratio. This was improved by adding 2% FBS. Since 2% FBS reduced the RPMI response, this suggests that mitochondrial activity in DPBS was significantly enhanced by 2% serum, despite the counterbalancing negative effects of serum on the assay reading.

From the study illustrated in Figure 1, it appeared that serum could influence the assay. A dose-response curve using RPMI as the base medium for the HC11 cells was therefore performed.

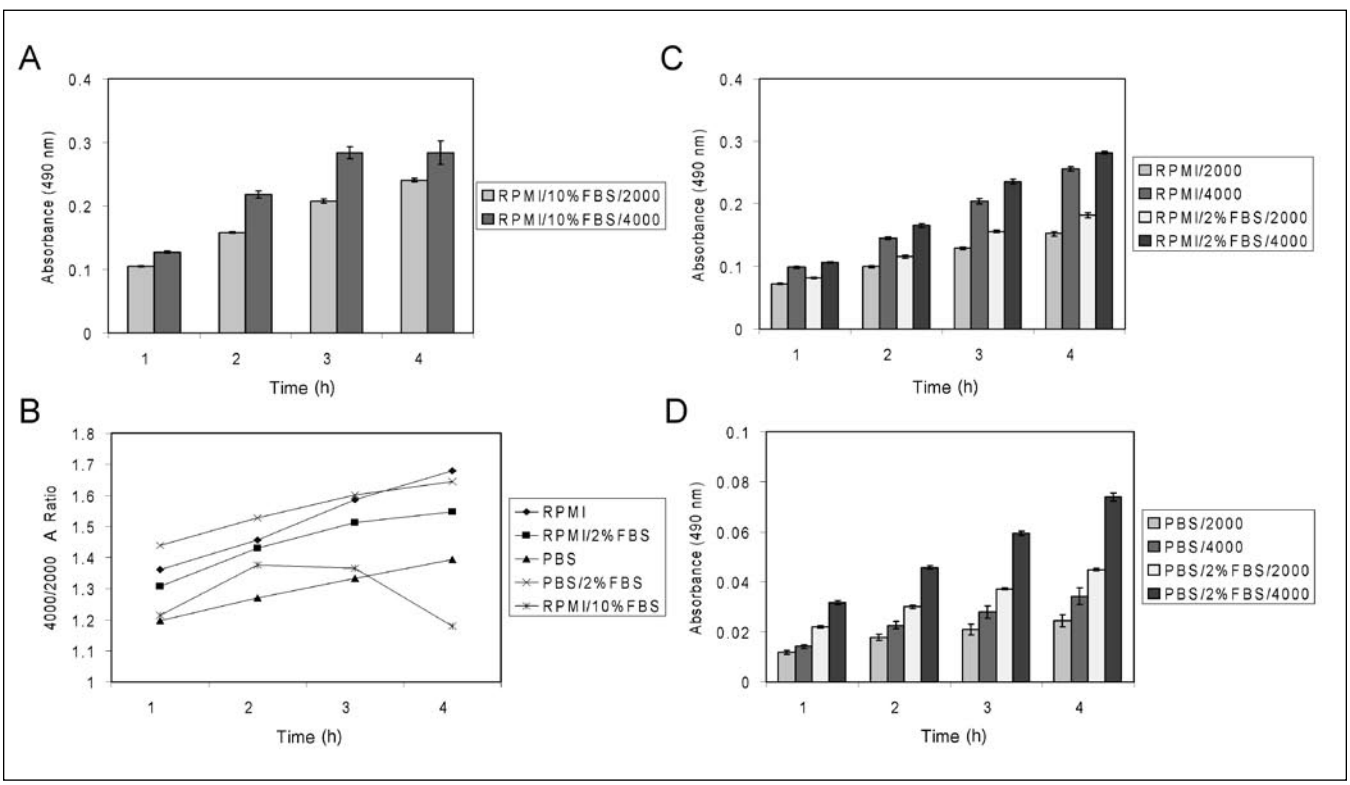

Figure 1. Absorbance generated in cell-mediated MTS assays. (A) HC11 cells were seeded at 2000 or 4000 cells per well into 96-well microplates. After 18 h, the MTS assay was performed. (B) Diagram of the 4000/2000 cell absorbance ratio using different media in the MTS assay. In an additional study, the cells were seeded as described in panel A. After 18 h, medium was removed and replaced with (C) RPMI or RPMI containing 2% fetal bovine serum (FBS), (D) Dulbecco's phosphate-buffered saline (DPBS) or DPBS containing 2% FBS, followed by the MTS assay. Results shown were means (±SD) of quadruplicate wells obtained by subtraction from cell-free equivalents, to eliminate A_{490} produced by the media alone. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; A Ratio, absorbance ratio.

Figure 2A, using cell-free controls for each condition, showed that increasing concentrations of FBS had a deleterious effect on the reading such that 10% serum gave a reading about 40% lower than 2.5% serum. The same effect was seen with HS (data not shown). Charcoal stripping of the serum exaggerated the deleterious effect regardless of whether the charcoal stripping was performed by the distributor or in our laboratory (data not shown).

In order to separate the effects of serum on the cells versus on the assay reading, we developed a cell-free assay system. A reduced formazan product was generated in the absence of cells using ascorbic acid (15). MTS assays were conducted in three different commonly used culture media: DMEM, DMEM/F-12, and RPMI 1640. The results showed that, in a 10-min incubation, more formazan was generated in DMEM/F-12 and RPMI than in DMEM (data not shown). In other words, some constituent of F12 and RPMI amplified the rate of reduction by ascorbic acid but had no effect on its own. Both glutathione and inositol were considered likely candidates but were determined to be without effect. These results necessitated the use of DPBS for further analysis so that we could test one component at a time. A 0.05-mM concentration of ascorbic acid was chosen for subsequent assays so as to produce formazan readings in the range obtained in the cell assays.

Using ascorbic acid to reduce the MTS in DPBS, we then demonstrated that there was a dose-related reduction in A_{490} with increasing FBS. At the 4 h time point, the absorbance reading was reduced by half when comparing 10% with 0% FBS (Figure 2B). The same was true for HS (data not shown). Commercially produced charcoal-stripped HS, however, seemed to have an even greater effect. The commercial charcoal-stripped serum obviously contained very fine charcoal that had not been removed by filtration. Given that charcoal binds a large number of small molecules, we considered the possibility that the formazan product was binding the remnant charcoal and thus the A_{490} was reduced. To test this possibility, DPBS was charcoal-treated and then added to fresh PBS in the wells as if it were serum. The charcoal-treated DPBS was slightly colored, showing that some charcoal was present after filtration. That charcoal was without effect on the A_{490} in the absence or presence of 0.05 mM ascorbic acid (data not shown).

Structural similarities between formazan and steroid hormone metabolites suggested the possibility that the key element in serum might be SHBG. SHBG is present in serum at about 60 µg/mL. Concentrations up to an equivalent of 10% serum were therefore tested but had no effect (data not shown).

Another possible binder of formazan was albumin. Albumin binds fatty acids and has a lower affinity for steroids than SHBG but is present in very large quantities in serum. The concentration in human serum is about 4.5 g/dL and in FBS is 2.5 g/dL. A solution of 2.5 g/dL BSA was prepared and considered equivalent to 100% FBS. Addition to the assay at 1.25%–10.0% of this showed a dose-

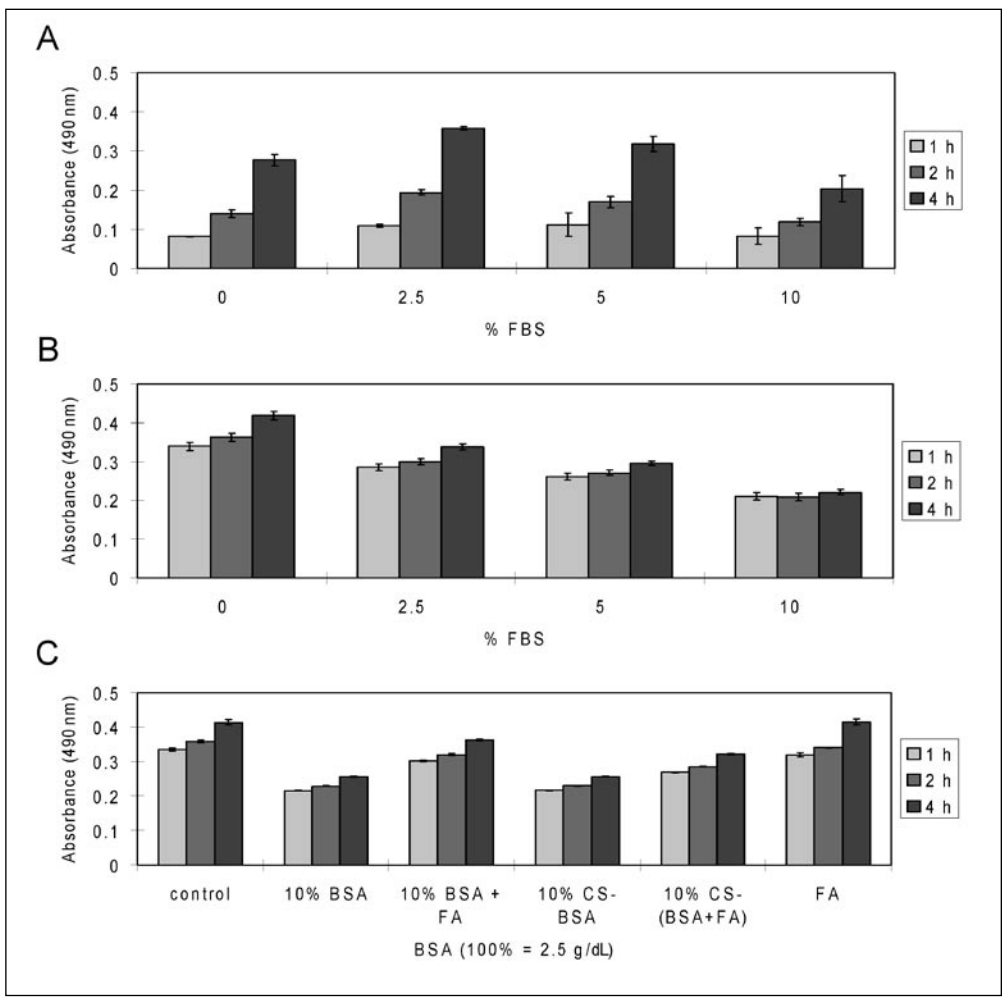

Figure 2. Absorbance generated in cell-mediated and cell-free MTS assays. (A) HC11 cells were seeded at a concentration of 10^4 per well into 96-well microplates. After 18 h, medium was changed to that with 2.5%, 5%, or 10% fetal bovine serum (FBS), followed by the MTS assay. (B) Effect of serum and (C) effect of bovine serum albumin (BSA) with and without fatty acids in the cell-free assay. Dulbecco's phosphate-buffered saline (DPBS) was added to each well of a 96-well microplate with (B) 2.5%, 5%, or 10% FBS or (C) BSA in the presence of 0.05 mM ascorbic acid, followed by the MTS assay. Results shown were means (±SD) of quadruplicate wells obtained by subtraction from (A) cell-free equivalents or (B and C) media equivalents without ascorbic acid. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

related decrease in A_{490}. This decrease was about 33% at the dose equivalent to 10% FBS at 4 h (Figure 2C). This is remarkably similar to the effect of serum shown earlier in Figure 2B.

If BSA binds formazan, thereby reducing A_{490}, we argued that fatty acids and formazan would compete for binding. We showed that the effect of BSA was almost entirely reversed by 2 mg/mL of mixed fatty acids. Charcoal stripping of the BSA-fatty acid mixture produced BSA, which once again inhibited A_{490} development, thereby demonstrating that charcoal stripping produces a serum that binds even more formazan product (Figure 2C).

In Figure 2, we have shown that the detrimental effects of serum on A_{490} were due to adsorption of the reduced formazan product onto serum albumin. This adsorption was dependent on the level of fatty acids present because the fatty acids competed for the binding sites. One theoretical approach for eliminating the effect of serum during an MTS assay would therefore be to supplement the culture medium with fatty acids. However, fatty acids are toxic to a variety of cells (16–18), including cancer cell lines. Fatty acids also have the potential to alter mitochondrial function.

The current experiments have demonstrated assay effects of albumin, a common constituent of defined media as well as media with serum. Also problematic are fatty acids in the presence of albumin or serum. Greater concentrations of fatty acids prevent binding of the formazan to albumin, thereby increasing the A_{490}. This would result in a reduction of the apparent effect of the fatty acid on cell number. Reducing and oxidizing agents also have the potential to dramatically affect assay results. Ascorbic acid, for example, converted 80% of the MTS to the reduced formazan product in 10 min at 0.05 mM. Ascorbic acid is often part of the diluent for small amines, such as dopamine (19). Reduction of MTT by ascorbic acid has been previously reported by Chakrabarti et al. (15), as has the observation that some constituent of serum influenced the tetrazolium assays. The identity of the responsible serum constituent, however, was not explored in their study. As yet unidentified in our studies is the constituent of RPMI and F-12 that amplifies the effect of ascorbic acid but is without effect on its own.

Having established two components of serum that had major effects on the assay, we therefore turned to the development of a simple, defined medium in which to conduct the assay and achieve results representative of cell number. Because of the lower readings obtained in DPBS versus culture medium, we increased the number of the cells per well to 5000 and 10,000 to decrease the influence of absorbance reading errors. HC11 cells were then plated at 5000 and 10,000 cells per well and incubated overnight. The following day, the medium was removed and replaced with DPBS supplemented with 2 or 4.5 g/L glucose. We demonstrated that the 10,000 to 5000 cell ratio was equal to 2 and consistent as a function of time in DPBS containing 4.5 g/L glucose (Figure 3). These results also demonstrate that the ratios of less than

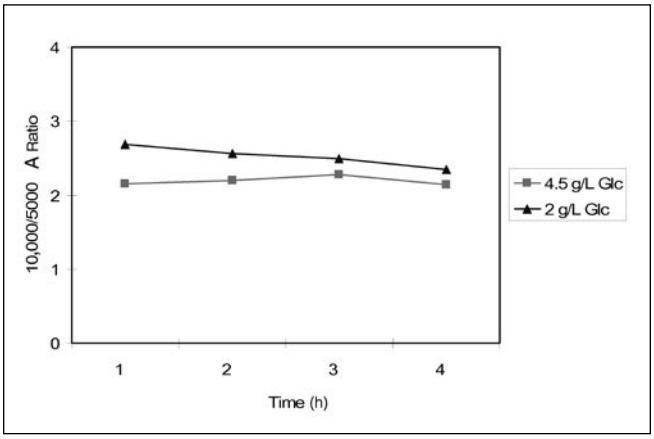

Figure 3. Effects of glucose. Ratio of absorbance generated in cell-based MTS assays performed in Dulbecco's phosphate-buffered saline (DPBS) in the presence of 2 or 4.5 g/L glucose. HC11 cells were seeded in culture medium at 5000 or 10,000 cells per well into 96-well microplates. After 18 h, the culture medium was replaced with DPBS supplemented with 2 or 4.5 g/L glucose, followed by the MTS assay. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Glc, glucose; A Ratio, absorbance ratio.

2 obtained in earlier experiments were not related to differential overnight viability of cells plated at different densities. By comparing results in Figure 1B with those in Figure 3, one can appreciate how very important the addition of glucose is to the achievement of an appropriate ratio (i.e., only in the presence of substantial quantities of glucose was the reading truly representative of cell number).

The effects documented in the study are not trivial. Ten percent serum, for example, can eliminate the difference between 2000 and 4000 cells after a 4-h incubation. Thus, a doubling or halving in cell number in response to a treatment can be totally missed. In addition, less dramatic effects on cell number can be severely dampened if the assay is conducted in a medium such as RPMI in which some constituent of the medium amplifies the reduction to formazan and A_{490} is not correlated with cell number at some time points. We suggest that these collective factors have contributed to a number of controversies in the literature regarding the antiproliferative efficacy of potential cancer therapeutics. We recommend either the original MTT assay or the MTS assay conducted in DPBS-glucose as suitable indirect measures of viable cell number. Analysis of test agents in ascorbic acid cell-free controls is also crucial. A medium-only control is clearly insufficient because all data shown here had been corrected using such controls before presentation.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

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Address correspondence to Ameae M. Walker, Division of Biomedical Sciences, University of California, Riverside, CA 92521-0121, USA. e-mail: Ameae.Walker@ucr.edu