Use of MTT Assay for Proliferation of U937 Cell Line and its Inter-rater Reliability - Best Taken with a Grain of Salt

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ABSTRACT

Background

Biomedical research frequently employs immortal human cell lines to study various physiological and pathological topics of interest. An important problem that is often ignored is the question of reliability of observations made while using a particular assay on cell lines, especially MTT Assay. Recent studies have questioned its reliability by highlighting the diverse intracellular environmental factors that affect accuracy of MTT assay results.

Objective

To assess the inter-rater reliability of MTT assay while observing the differential effect of addition of fetal bovine serum to the culture medium on proliferation of U937 cell line using Intraclass correlation coefficient.

Method

U937 cell suspension prepared by the tutor to maintain uniformity of immortalized cell line was used. Four trainee investigators who participated in the study underwent rigorous training in pipetting and plating methods for four consecutive days prior to start of the experiment. Each trainee investigator followed the same protocol and all procedures were conducted simultaneously. At the end of the experiment, inter observer reliability was calculated.

Result

Interrater reliability calculated by using intra class correlation coefficient with two way mixed effect model was found to be poor (p = 0.173). For growth with 10% fetal bovine serum, inter-rater reliability was 0.258 for each item and 0.58 for the average of the triplicates, whereas for 0% fetal bovine serum it was 0.374 for each item and 0.70 for average of the values (p=0.104).

Conclusion

Though MTT assay is considered the gold standard test for assessment of cell proliferation and viability, the inter-rater reliability of this assay might be poor and needs further investigation.

KEY WORDS

Cell culture, Cell proliferation, Cell viability, MTT Assay, U937 cell line

INTRODUCTION

Biomedical research frequently employs immortal human cell lines as a substitute for primary cells in order to cost-effectively study various physiological and pathological topics of interest.[1] In comparison to primary cells, cell lines offer diverse advantages like ready availability, easy proliferation, reduced cost and increased durability while also avoiding the possibility of ethical issues.[2] The homogenous, pure population of cells offered by cell lines are thus being increasingly used in wide range of research areas ranging from vaccine development, drug testing and cytotoxicity assay to genetics research.[3]

The U937 cell line is a fine example of routinely used immortal human cell line. It was established from the histiocytic lymphoma of a 37 year old male.[4] It resembles human monocytes and has been an important asset in the research surrounding the mononuclear phagocytic system.[4] Monocytes and macrophages are involved in numerous biological functions in the wound healing process as well as in host response to pathogens and biomaterials.[2] Therefore U937 cells have been extensively used to study apoptosis, cell-mediated cytotoxic activity in mycobacterial infections, efficacy testing of immune-modulatory agents and anti-viral drugs for Influenza and diverse other research questions.[5-8] In most studies involving the U937 cell line, cells are cultured in the Roswell Park Memorial Institute (RPMI) 1640 medium.[9] Serum which contains nutrients, hormones, growth factors, inorganic salts, trace elements, and other compounds is added to enrich the culture medium and improve its efficacy.[9] Fetal bovine serum (FBS) is the most commonly used serum and is appropriate for use in cells that otherwise pose problem in cellular proliferation.[10]

However conducting research using cell lines is also fraught with numerous problems. Since cell lines are not identical to primary cells, the results obtained from research using them may not adequately represent normal physiological or pathological processes.[3] Similarly cell line misidentification, risk of contamination, genotypic and phenotypic instability are other issues frequently encountered with cell lines.[3,11]

Another problem that is often ignored by the scientific community, is the question of reliability of observations while using a particular assay on cell lines. Reliability denotes the replicability of findings.[12] The magnitude of reliability indicates the accuracy of measurements and hence is an important parameter of research quality.[13] Reproducibility of findings and study protocols is indispensable in laboratory science research. High reliability which assures that the difference observed between study subjects can be attributed to a genuine difference in their values rather than merely being a result of error, is essential to avoid misinterpretation and misrepresentation of results of any assay.

Methylthiazole tetrazolium (MTT) assay which uses tetrazolium salt known as 3-(4,5-dimethyl-2-thia-zolyl)-2, 5-diphenyl-2H-tetrazolium bromide), is a colorimetric method commonly used to assess cellular proliferation and viability of both primary cells and immortalised cell lines.[14,15] It was introduced in 1983 and was hailed as a welcome substitute for traditional methodologies such as manual cell counting or assessment of colony formation in large-scale experiments, has been an important asset in laboratories around the globe for decades.[15,16] Till date it is still considered the gold standard method to assess cell proliferation and viability.[17] However recent studies have questioned its reliability by highlighting the diverse intracellular environmental factors that affect MTT reduction while also demonstrating the questionable accuracy of assay results in immortalized cells compared to primary cells.[18] Furthermore intra and inter-rater reliability of MTT assay results has not been fully explored as yet in scientific literature.

Hence this manuscript attempts to assess the inter-rater reliability of MTT assay while observing the differential effect of addition of FBS to the culture medium on proliferation of U937 cell line. There are many indices which are used to measure reliability such as Pearson correlation coefficient, paired t test, and Bland-Altman plot.[19] However, an ideal estimation of reliability must include assessment of both the extent of correlation as well as the agreement between measurements.[19] Of the aforementioned indices, paired t test and Bland-Altman plot merely assess agreement whereas Pearson correlation coefficient measures only correlation.[19] Intraclass correlation coefficient (ICC) is a statistical test which reflects both the parameters and is thus better suited to assess the reliability of MTT assay. This test has been used in the current study.

METHODS

The present study was carried out at International Centre for Stem Cells, Cancer and Biotechnology (ICSCCB), Pune as part of a training curriculum. The U937 cell suspension used in the study was prepared by the tutor to maintain uniformity of immortalized cell line used and the four trainee investigators who participated in the study underwent rigorous training in pipetting and plating methods for four consecutive days prior to start of the experiment. Each trainee investigator followed the same protocol and all procedures were conducted simultaneously. At the end of the experiment, inter observer reliability was calculated.

The U937 cell line was acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were serially cultured with the Roswell Park Memorial Institute (RPMI) 1640 medium (ATCC, Manassas, VA, USA) and an aliquot from this maintained culture was used for experiments.

From the subculture flask of U937 cell suspension, cell sample was taken in 15 ml tube, centrifuged (125×g, 10 minutes, 4°) and pellet formed was washed with ice-cold

phosphate buffered saline (PBS, pH 7.4). Centrifugation was done to wash the PBS off and the pellet formed was re-suspended in 10 ml of base medium RPMI-1640. From this, cell sample was taken for counting and then for culture, with and without 10% FBS (ATCC, Manassas, VA, USA).

Cell density was adjusted to 2×105 cells/ml. Then two suspensions of 2 ml each were prepared one with only base media (0% FBS) and other with 10% FBS added to the base medium. The trainee investigators pipetted out 100 µl each of the two cell suspensions and plated the sample in triplicates to a 96 well plate in consecutive rows. Three control wells of pure base medium without cells were included as blanks for absorbance reading.

After incubating for 24 hours at 37^0C with 5% CO_2, MTT assay was performed following standard guidelines (15,20) to assess cell proliferation and viability. 10 µl of MTT reagent 5 mg/ml (Hi Media, Mumbai, INDIA) was added to each well, including the control wells and further incubated for 4 hours until purple coloured precipitate of formazan crystals were visible. To solubilize the formed formazan crystals. 100 µl of solubilisation solution (Hi Media, Mumbai, INDIA) was added to each well including the control wells and mixed thoroughly with a pipette. The plate was covered and kept in dark at room temperature for 4 hours. Then the cover was removed, samples were mixed again and absorbance was measured at 570 nm using microplate reader (Thermo Multiscan Ex) with multi-well plate reading ability. Three readings were recorded for each well.

All data in this study has been expressed as means ± standard deviation (SD) and statistical significance has been set at $p < 0.05$. The average value of triplicate readings was adjusted with control reading and difference in absorbance was compared and plotted. Wilcoxon signed rank test was used to compare the test and control groups. Inter observer reliability was calculated by using intraclass correlation coefficient (ICC). ICC estimates and their 95% confidence intervals were calculated using SPSS V 16 statistical software based on mean rating (k=4), consistency, 2-way mixed-effects model.

RESULTS

In the first part of the experiment assessing the influence of adding FBS in the growth medium, U937 cell suspension grown with 10% FBS had a significantly increased cell proliferation rate as compared to 0% FBS (OD 0.765 ± 0.038, Vs 0.468 ± 0.0388, p = 0.002) as shown in figure 1.

In the second part of the experiment, using the optical density of MTT assay of four trainee investigators we assessed the inter-rater variability and reliability and shown in figure 2. Interrater reliability calculated by using intra class correlation coefficient with two way mixed effect

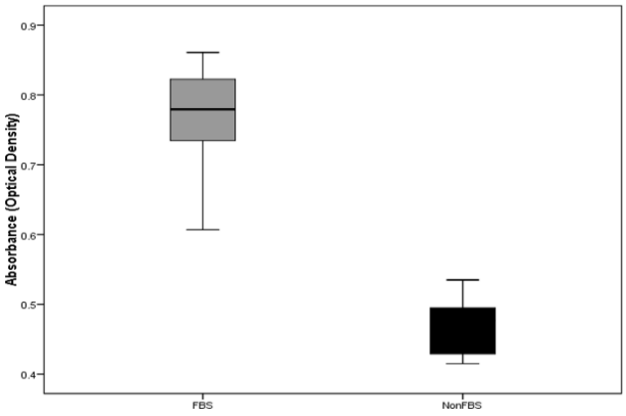

Figure 1. Comparison of optical density between cell suspensions containing 10% FBS and 0%.

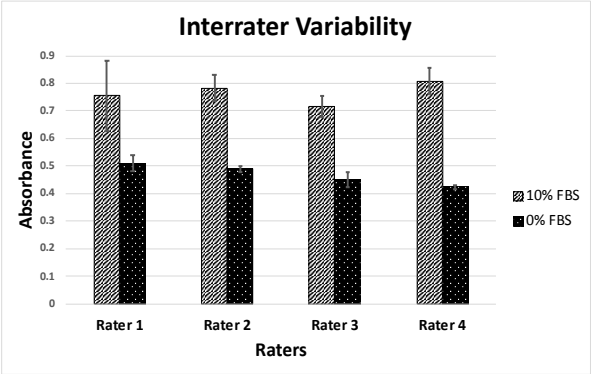

Figure 2. Comparison of interrater variability

model was found to be poor (p = 0.173). For growth with 10% FBS, inter-rater reliability was 0.258 (95% CI -2.02 to 0.959) for each item and 0.581 (95% CI -2.039 to 0.989) for the average of the triplicates, whereas for 0% FBS it was 0.374 (95% CI - 0.154 to 0.971) for each item and 0.70(95% CI -1.144 to 0.992) for average of the values (p=0.104).

DISCUSSION

Even though the exact composition of FBS is unknown as it contains varying concentrations of unidentified, naturally occurring substances which act as growth stimulatory factors.[21,22] It has long been used by laboratory scientists worldwide as a cell culture supplement for countless types of primary and immortalized cells.[10,23] Many previous studies have explored the effect of different concentrations of FBS on cell line proliferation.[24,25] Study conducted by Chen et al. has shown significant fall in cellular viability with absence of FBS, whereas with addition of 5% FBS it reached 90% of that attained with 10% FBS in the absence of Phorbol 12-myristate 13-acetate (PMA).[2] Our study reiterated the fact that supplementation of 10% FBS aided the proliferation of U937 cell as confirmed by increased absorbance seen on MTT assay.

The increase in optical density of the cell suspensions upon addition of 10% FBS was detected using the MTT assay which measures cell viability in terms of reductive activity of enzymatic conversion of Tetrazolium compound to coloured formazan crystals by dehydrogenases occurring in mitochondria of living cells.[15] Published literature attests to the effectiveness of using the MTT assay for the detection of proliferation and viability of the U937 cell line.[26–28]

However, results of the said assay might be subject to variability secondary to compromised accuracy of measurements. The concept of inter-rater reliability has been widely used in clinical research especially in fields like mental health, orthopaedics and so on. Such popular use of the concept has not been seen in laboratory science which is surprising given the need for repeatability and reproducibility of measurements.[29-38] Few studies have analysed the inter and intra-rater reliability of commonly used assays like Trypan Blue Assay, while similar reliability assessment has been done for histochemical tests used in Epstein–Barr Virus detection.[39-41] But inter-rater reliability of MTT assay has not been evaluated as yet.

In our study the inter-reliability of MTT assay was calculated by using intra class correlation coefficient. Since the experimental setting was part of a pre-planned workshop, it was not possible to randomly choose a sample of raters from a larger population of raters with similar characteristics. We thus chose the two way mixed effect model of ICC for our study.[19] In In this study, interrater reliability calculated by using intra class correlation coefficient with two way mixed effect model was found to be poor ($p = 0.173$). Though it has been said that the results of the two may mixed effect model cannot be generalized to other raters even if those raters have similar characteristics as the selected raters in the reliability experiment, a recent analysis by Liljequist et al. found that irrespective of the context-specific appropriateness of the ICC model chosen, both the two-way random and the two-way mixed models lead to precisely the same sample ICC formulas and thus same values for a given data matrix.[19,42]

We followed standard guidelines for interpretation of ICC according to which values less than 0.5 indicate poor reliability, values between 0.5 and 0.75 indicate moderate reliability, values between 0.75 and 0.9 indicate good reliability, and finally values greater than 0.90 indicate excellent reliability.[19] Accordingly overall inter-rater reliability was found to be poor. Inter rater reliability when tests were done with 10% FBS was found to be poor for each absorbance reading whereas it can be interpreted as moderate when the average of the triplicate absorbance readings is considered. Similar findings were seen when the assay was performed without addition of FBS.

The interpretation of the aforementioned findings should be undertaken keeping in mind the limitations of the study. Firstly a low ICC value while reflecting the lack of rater agreement could also be attributed to other reasons. Loss of liquid due to evaporation in combination with variations in growth rate among different wells may lead to variations. Furthermore, the investigators performing the assay were only trained for short period of time and it can be reasonably expected that repeating this reliability assessment with more rigorously trained investigators might yield higher ICC values. Although there are no specific guidelines which state the minimum number of raters to be included when assessing reliability, Koo and Li 2016 provide a rule of thumb stating that at least 30 heterogeneous samples and least 3 raters should be tested.[19] We have conducted our assessment on four trainee investigators. Secondly, it has been argued that using 95% confidence interval ranges for interpretation of the degree of reliability is better than absolute ICC values.[19] The small sample size of our study yielded a wide confidence interval, thus limiting the precision of our findings. Finally due to resource limitation, we could not focus on intra-rater reliability. Future work should attempt to assess both inter and intra-rater agreement in a larger randomly selected sample of trained laboratory scientists to ensure precise estimation of generalizable results.

The main aim of this work was to provide researchers with the novel information regarding the questionability of the inter-rater reliability of MTT assay results and to make them aware of unexpected measurement errors when the assay is being conducted. But for the sake of clarity and completeness, we highlight the fact that through this study we do not intend to question the overall accuracy of the MTT assay which is being widely used in different contexts and with different cell lines. We also do not lay claim to evaluation of the quality of the U937 cell line.

CONCLUSION

MTT assay is considered the gold standard colorimetric test for assessment of cell proliferation and viability. However, no study has been published so far with regard to validation of the assay in terms of inter-rater reliability while assessing cell line proliferation and viability. The results obtained in this highlight the fact that inter-rater reliability of the assay might be poor and thus needs further investigation.

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