CYTOTOXICITY OF OXIDISED LOW DENSITY LIPOPROTEIN (OxLDL) ON U937 CELLS: A COMPARISON OF VIABILITY MEASUREMENT BY PROPIDIUM IODIDE-FLOW CYTOMETRY AND MTT REDUCTION ASSAYS

[*]Mohd Izani Othman[1], Steven Gieseg[2] , Barry Hock[3]

[1] Faculty of Pharmacy
Universiti Teknologi MARA Cawangan Pulau Pinang, Kampus Bertam
13200 Kepala Batas, Pulau Pinang, Malaysia

[2] School of Biological Sciences
University of Canterbury
8041 Christchurch, New Zealand

[3] Pathology Department
University of Otago
8140 Christchurch, New Zealand

*Corresponding author's email: mohdizani.othman@ppinang.uitm.edu.my

Abstract

Atherosclerosis is a major cause of cardiovascular disease. It is characterised by a development of atherosclerotic plaque in a blood vessel wall that involves a death of macrophages. Oxidized low density lipoprotein (oxLDL) is a toxic and a key player that promotes macrophages recruitment, activation, and foam cell formation in atherosclerotic inflammation. Thus, an understanding of the underlying mechanism of cell death is important to gather information for therapeutic interventions. *In vitro* studies, using cells are common for toxicity investigation where cell viability is measured. While there are different options of assays available for cell viability measurement, choosing the most appropriate assay could be a challenging task. Therefore, picking the right and the best assay tool to suit particular needs requires an understanding of what each assay measures as well as limitations of the assay chemistries. This is significant to generate meaningful data obtained from the experiments. In this study, two cell viability assays i.e. MTT reduction assay and PI-flow cytometry assay were compared.

Keywords: Cell viability; propidium iodide; MTT; flow cytometry; oxLDL

1.0 INTRODUCTION

OxLDL is associated with an initiation and a development of atherosclerosis (Ross, 1999; Smook *et al.*, 2008). One of the characteristics that might mediate this process is its cytotoxic effect to various types of cells including monocytes and macrophages (Gieseg *et al.*, 2010; Katouah *et al.*, 2015). In the *in vitro* studies investigating oxLDL-induced cell death, the measurement of cell viability is common and necessary. There are many cell viability assays available and can be classified into assays that measure metabolic activity, metabolic markers, or loss of membrane integrity. Therefore, a sound understanding of the assays is important in selecting the most appropriate assay to be used.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolinum bromide) reduction assay is a widely recommended method in analysing cell activities such as cell viability and proliferation rates in cell cultures (Denizot & Lang, 1986; Mosmann, 1983). It is one of the metabolic assays that based on the reduction of MTT to water-insoluble purple formazan complex inside the viable cells through mitochondrial dehydrogenases as well as non mitochondrial, cytosolic and microsomal enzymes (Gonzalez & Tarloff, 2001; Liu *et al.*, 1997). Nevertheless, intracellular MTT reduction rate is assumed to be closely related to the number of actively metabolising cells in the cell culture (Gonzalez & Tarloff, 2001; Mosmann, 1983).

On the other hand, propidium iodide (PI) assay is based on the ability of this highly fluorescent dye to penetrate cells that lack membrane integrity. Early apoptotic cells exclude PI but not necrotic and late apoptotic cells. Integrating PI and flow cytometry analysis therefore, it enables the detection of viable cells from dead cells whereby the latter emitted bright red fluorescence compared to the former which do not fluoresce (Ross *et al.*, 1989). The most prominent working principle of flow cytometry assay is that it allows direct counting of cells without being influenced by the metabolic state of the cells (Wang & Zheng, 2002). Integrating PI with flow cytometry method creates a new dimension for the analysis of cell viability. The aim of this study is to compare both types of assay for measurement of cell viability in various aspects.

2.0 MATERIALS AND METHODS

2.1 Chemicals and reagents

All chemicals and reagents of analytical grade or better were obtained from Sigma Chemical Company (USA), BDH Chemicals (New Zealand) or Merck (Germany). All solutions were prepared using ion exchanged ultra-filtered water from a NANOpure ultrapure water system supplied by Barnstead/Thermolyne (USA). Cell culture plasticware was supplied by Greiner Bio-one through Raylab Ltd. (New Zealand). Phosphate buffered saline (PBS) solution consists of 150 mM sodium chloride and 10 mM sodium phosphate pH 7.4.

2.2 Blood collection and lipoprotein isolation

Human plasma (anti-coagulated with EDTA) was isolated by venepuncture from healthy male and female donors following an overnight fast (ethics approval CTY/98/07/069 by the Upper South (B) Regional Ethics Committee, New Zealand). LDL was isolated from human plasma by buoyant density gradient ultracentrifugation using an NVTi 60 rotor (Beckman Coulter, USA) (Gieseg & Esterbauer, 1994). The LDL concentration was determined by enzymatic cholesterol assay using a CHOL kit (Roche Diagnostic, USA) assuming an LDL molecular weight of 2.5 MDa and a cholesterol content of 31.6% (Gieseg & Esterbauer, 1994). The remaining potassium bromide (KBr) and EDTA were removed and the LDL concentrated by ultrafiltration using Amicon® Ultra-15 filter tubes (Millipore, USA). LDL preparations were filter sterilised (0.22 µm, Membrane Solutions, USA) and stored in the dark at 4°C under argon gas.

2.3 Lipoprotein (LDL) oxidation

Copper-oxidised LDL was prepared using a method adapted from Gerry *et al.* (2008). LDL at 10 mg/mL total mass (2 mg/mL apoB protein) was placed in a dialysis bag (12–14 kDa, Medicell International, UK) with the addition of $CuCl_2$ to give a final concentration of 0.5 mM. The dialysis bag was placed in a

solution of PBS (100 mL buffer per 5 mg of LDL total mass) containing 0.5 mM $CuCl_2$ and incubated at 37°C for 24 hours. Oxidised lipoprotein was dialysed against three changes of Chelex-containing PBS at 4°C for 24 hours.

2.4 Cell culture

U937 human monocyte-like cells were grown in suspension in culture medium consisting of RPMI-1640 containing penicillin G (100 U/mL) and streptomycin (100 µg/mL) (Gibco Invitrogen, NZ) supplemented with 5% heat inactivated foetal bovine serum (Invitrogen, USA). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 (Sanyo CO_2 Incubator). During experiments, cells were incubated for 24 hours in RPMI 1640 at a concentration of $5x10^5$ cells/mL.

2.5 MTT reduction assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay using the method of Mosmann (1983), but using 10% (w/v) sodium dodecyl sulphate (SDS) to lyse the cells and solubilise the insoluble MTT-formazan salt.

2.6 Propidium iodide (PI) – flow cytometry assay

Propidium iodide (PI) is a highly fluorescent red membrane permeable dye that is generally excluded by viable cells. It binds to double stranded DNA of the cells by intercalating between base pairs. The cell membrane integrity excludes PI in viable and apoptotic cells whereas necrotic cells are permeable to PI (Moller *et al.*, 2005) resulting in non-viable cells having bright red fluorescent while viable cells are non-fluorescent (Ross *et al.*, 1989). PI is excited at 488 nm and emits at a maximum wavelength of 617 nm. Stock solution of 1 mg/mL PI was prepared by dissolving 10 mg PI in 10 mL nano pure water and stored at 4°C, final concentration used was 1.5 µg/mL. U937 cells were incubated with PI (in RPMI-1640 or PBS) for 10 to 15 minutes in the dark before measurement using Accuri C6® flow cytometer. Data were recorded and analysed using the cFlow Plus software (BD Biosciences, USA).

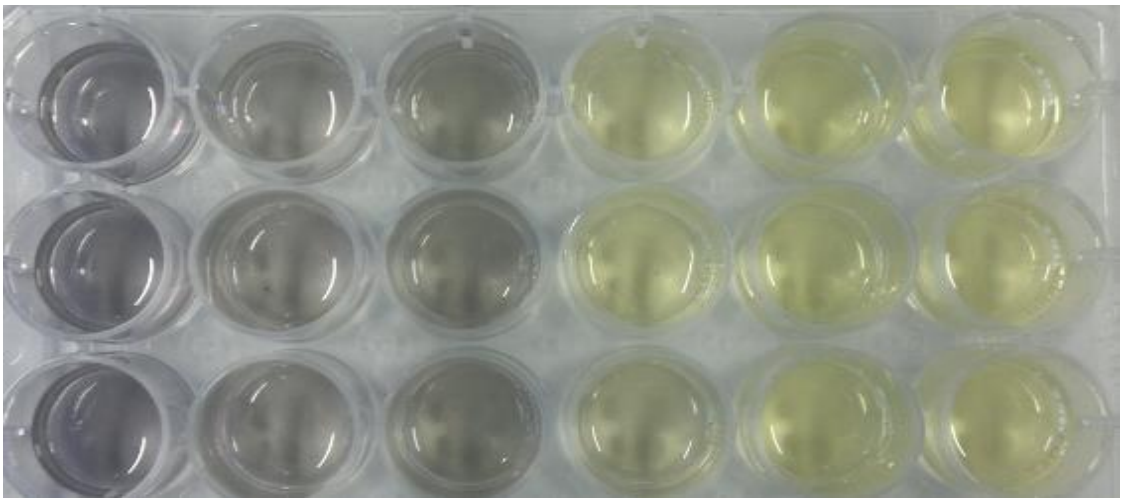
2.7 Statistical analysis

Data shows results of a single experiment and a representative of five separate experiments. Mean and standard error of the mean (SEM) shown within each experiment were calculated from triplicate samples in every case. The data was analysed using the Prism software package (version 6.0, GraphPad Software Inc., USA).

3.0 RESULTS

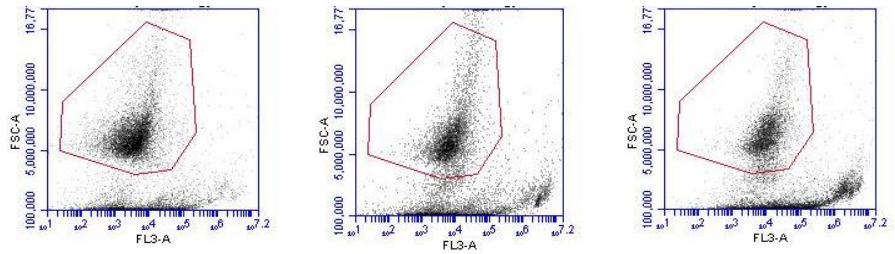
An example of the experiment and the results obtained from MTT reduction assay and PI-flow cytometry assay are shown in Figure 1 and 2 respectively. Figure 1 shows the end result of MTT reaction with U937 cells after solubilisation of MTT formazan with 10% SDS in a cell culture plate. Depending on the reaction between MTT reagent and the cells, this then resulted in different colours (intensities) being formed which is a basis of measuring cell viability using MTT reduction assay. MTT will remain yellow if the cells are unable to metabolise the reagent that reflects dead cells.

Figure 2 shows the flow cytometry dot plot histograms [FSC (forward scatter) vs FL3-PI] for U937 cells treated with different concentrations of oxLDL after 24 hours has been stained by PI. With the concentrations of oxLDL increased, more cells have shifted downwards and to the right of the histogram. This shifting shows that more cells are being stained by PI which depicts decreasing cell viability when increasing oxLDL concentrations. Based on the histograms, there are almost no cells viable when they were treated with higher oxLDL concentrations of 0.8 and 1.0 mg/mL. Although both assays (MTT and PI) are not in the same of category for cell viability measurements, the results obtained by each method were comparable (R^2= 0.9766) (Figure 3).

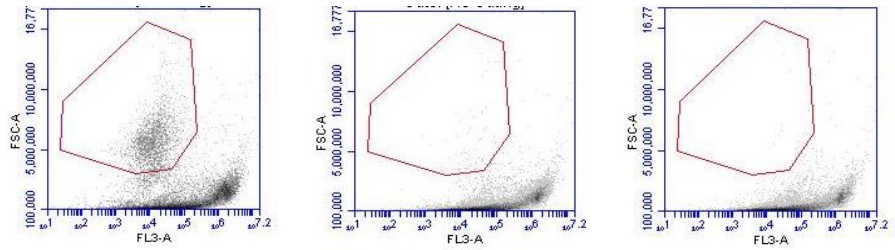


Control	0.2 mg/mL oxLDL	0.4 mg/mL oxLDL	0.6 mg/mL oxLDL	0.8 mg/mL oxLDL	1.0 mg/mL oxLDL

Figure 1: Cell viability measured using MTT reduction assay



Control 0.2 mg/mL oxLDL 0.4 mg/mL oxLDL



0.6 mg/mL oxLDL 0.8 mg/mL oxLDL 1.0 mg/mL oxLDL

Figure 2: Flow of cytometry dot plot profiles of U937 cells treated with different concentrations of oxLDL (FSC vs FL3) (Gated area shows viable cells)

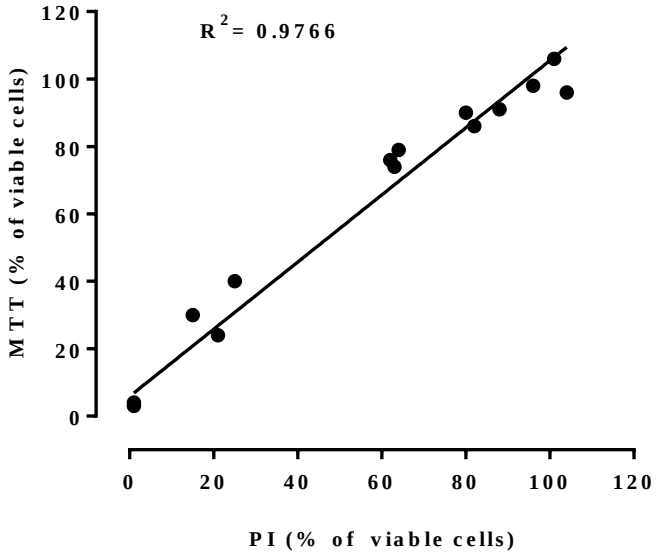


Figure 3: Comparison of cell viability measurements (MTT vs PI-flow cytometry)

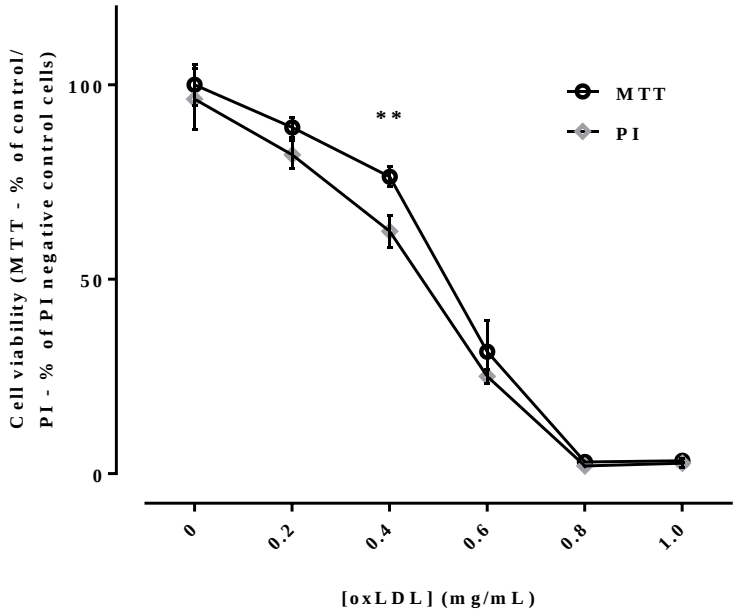


Figure 4: Effects of different oxLDL concentrations, significance is indicated between both methods at LDL concentration of 0.4 mg/mL, **, p<0.01 (two-way ANOVA, Sidak's multiple test)

4.0 DISCUSSION

MTT reduction and PI-flow cytometry are among the cell-based viability assays used to distinguish the viable cells from the dead cells. The percentage of viable cells measured using MTT assay is proportional to the amount of purple formazan (Figure 1) being generated by cellular NADPH-converting enzymes, thus depending on the intracellular metabolism that reflects the mitochondrial activity of the cells (Mosmann, 1983) . Since the measurement of formazan is based on the optical density value (absorbance) measured by spectrophotometer, therefore, the MTT assay can represent the cell number and can be compared among the samples only when the cells in the particular sample are in the same metabolic rate. In contrast, the flow cytometry assay was directly counted the number of cells that flow through a laser beam of the flow cytometer. Hence, the number of cells counted are proportional to the cell concentration and not the metabolic state of the cells (Wang & Zheng, 2002).

In this study, an excellent agreement between MTT and PI-flow cytometry assays for cell viability determination is illustrated in Figure 3. The reasons are that the cells used for the two assays were of the same type, being treated with the same concentrations of oxLDL, and were in the same metabolic state (Wang & Zheng, 2002) when the measurements were done. The percentage of cytotoxicity obtained using both MTT and PI-flow cytometry assays also showed an increasing trend with increasing oxLDL concentrations (Figure 4) (n = 18). Nevertheless, cell viability measurement using MTT assay yielded higher percentage of viable cells compared to PI-flow cytometry. The contributing factor to the specificity of the assays i.e. MTT assay is measuring the metabolic activity of the cells. This means that some dying cells might still be able to metabolise MTT and contribute to the higher percentage of live cells as compared to PI, which only binds to the DNA of the dead cells. Since the plasma membrane is the first to be exposed to oxLDL, it may be more readily and easily affected compared to the mitochondria, thus explains why the PI-flow cytometry assay is more sensitive (Liao *et al.*, 2011). The cell viability data

obtained by MTT assay is therefore impacted by the changes in intracellular metabolic activity that indirectly reflects overall cell viability (Wang & Zheng, 2002).

However, there are distinct characteristics of the PI-flow cytometry assay that may provide additional advantages over MTT reduction assay. First, it requires only a few treatment steps (Wang & Zheng, 2002) before the measurement of cell viability is carried out. A required volume of cells need to be transferred into a vial before PI is added and incubated in the dark prior to the analysis. It requires only a short incubation time period of 15 minutes compared to the MTT assay which needs approximately 1 to 2 hours of incubation time with the MTT reagent. The MTT assay requires an extra step of dissolving the purple formazan complex with SDS before an absorbance can be measured by using a spectrophotometer. The PI-flow cytometry assay also has an extra feature whereby it allows counting of the cells (quantitative analysis of the cells can be done).

Moreover, the PI-flow cytometry assay has fast turnaround time with only a few seconds lesser than a minute to run a sample. Notably, a relatively small volume of sample is required for the analysis (approximately 150-200 µL) as compared to the MTT assay. Another feature exclusive to PI-flow cytometry assay is that the cell profile as well as the dynamic changes of the cells can be obtained and distinguished through the dot plots and histograms. This is very useful as we are able to see the size changes of the cells (forward scatter) and fluorescence intensities when the cells moved from a 'live phase' to a 'dead phase' as shown in Figure 2. The number of the viable and dead cells in each sample is able to be quantified as opposed to the MTT assay. Another benefit of using the PI-flow cytometry is that it allows repeated measurements of the same sample. For the MTT assay, this is a limitation because the cells become non-viable with the chemical treatment used in the assay, thus repeating or complementary assays cannot be carried out on the same plate of the cells.

Furthermore, the flow cytometry assay also enables more than one dye (dyes that are not using the same filter for measurements) to stain the cells at the same time and a measurement can be carried out simultaneously (e.g. PI and Annexin V for the measurements of necrotic and apoptotic cells). Another benefit of PI-flow cytometry is the use of a very small volume of reagent compared to the MTT assay. In this study, approximately 4 µL of 100 µg/mL PI was added to a 250 µL sample and this volume is significantly lesser than the volume needed for the MTT assay.

5.0 CONCLUSION

The flow cytometry assay of cell viability using PI dye has more advantages and benefits over the conventional MTT reduction assay. The PI-flow cytometry assay is rapid, reliable, accurate, easy, and cheap for cell viability studies.

References

Denizot, F., & Lang, R. (1986). Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of Immunological Methods, 83,* 271-277.

Gerry, A. B., Satchell, L., & Leake, D. S. (2008). A novel method for production of lipid hydroperoxide- or oxysterol-rich low density lipoprotein. *Atherosclerosis, 197,* 579-587.

Gieseg, S. P., & Esterbauer, H. (1994). Low density lipoprotein is saturable by pro-oxidant copper. *FEBS Letters, 343*, 188-194.

Gieseg, S. P., Amit, Z., Yang, Y.-T., Shchepetkina, A., & Katouah, H. (2010). Oxidant production, oxLDL uptake, and CD36 levels in human monocyte-derived macrophages are downregulated by the macrophage-generated antioxidant 7,8-dihydroneopterin. *Antioxidants & Redox Signaling, 13*(10), 1525-1534.

Gonzalez, R. J., & Tarloff, J. B. (2001). Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity. *Toxicology in Vitro, 15*, 257-259.

Katouah, H., Chen, A., Othman,I. and Gieseg, S. P. (2015). Oxidised low density lipoprotein causes humn macrophage cell death through oxidant generation and inhibition of key catabolic enzymes. *The International Journal of Biochemistry and Cell Biology, 67, 34-42.*

Liao, T. T., Jia, R. W., Shi, Y. L., Jia, J. W., Wang, L., & Chua, H. (2011). Propidium iodide staining method for testing the cytotoxicity of 2,4,6-trichlorophenol and perfluorooctane sulfonate at low concentrations with Vero cells. *Journal of Environmental Science and Health. Part A: Toxic/Hazardous Substances and Environmental Engineering, 46*(14), 1769-1775. doi: 10.1080/10934529.2011.624016

Liu, Y., Peterson, D. A., Kimura, H., & Schubert, D. (1997). Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *Journal of Neurochemistry, 69*, 581-593.

Moller, W., Brown, D. M., Kreyling, W. G., & Stone, V. (2005). Ultrafine particles cause cytoskeletal dysfunctions in macrophages: role of intracellular calcium. *Particle and Fibre Toxicology, 2*, 7. doi: 10.1186/1743-8977-2-7.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*, 55-63.

Ross, D. D., Joneckis, C. C., & Ordonez, J. V. (1989). Estimation of cell survival by flow cytometric quantification of fluorescein diacetate/propidium iodide viable cell number. *Cancer Research, 49*, 3776-3782.

Ross, R. (1999). Atherosclerosis - an inflammatory disease. *The New England Journal of Medicine, 340*(2), 115-126.

Smook, M. L., van Leeuwen, M., Heeringa, P., Damoiseaux, J. G., Theunissen, R., Daemen, M. J., Lutgens, E., & Tervaert, J. W. (2008). Anti-oxLDL antibody isotype levels, as potential markers for progressive atherosclerosis in APOE and APOECD40L mice. *Clinical & Experimental Immunology, 154*(2), 264-269. doi: 10.1111/j.1365-2249.2008.03746.x

Wang, Y.-Y., & Zheng, X.-X. (2002). A flow cytometry-based assay for quantitative analysis of cellular proliferation and cytotoxicity in vitro. *Journal of Immunological Methods, 268*, 179-188.