Resazurinreagent to Determine the Safe Dose of PPAR-γ Agonist for Reducing Activated Gliosis Using 3T3 Cells.

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Abstract:

A method is described spectrophotometric test, for the assessment of the possible toxicity effects of the agent, such as PPAR-γ agonist (rosiglitazone) on 3T3 cells in culture.The last three decades of the nineteenth century the 3T3 became very common use in biology that easy to become confluent. These cells are sensitive and reproducible assay lend themselves to a screening procedure of potential toxicants which can help reduce the use of animals for toxicity testing. The PPAR-γ agonist such as rosiglitazone will be used in further experiments to reduce activated gliosis and inhibits astrocyte proliferation in a mouse model which represents the neurodegenerative disorder.The purpose of the assay is to verify that the reason for reducing the astrocytes is due to the positive effect of the PPAR-γ agonist not for its toxicity.

The results were analysed by a FLUOstar Galaxy software reader, Results showed that Rosiglitazone was non-toxic at the concentrations used in this assay.

Keywords:

In vitro alternative; spectrophotometric analysis; 3T3; Resazurin;Confluent

Abbreviations:

PPAR-γ, Peroxisome Proliferators- Activated Receptor gamma;DMEM, Dulbecco's Minimum Essential Medium; HBSS, Hanks' Balanced Salt Solution.

Introduction:

Resazurin is a reagent that is not toxic to cells and is stable in culture medium. It is used to provide a quantitative measure of cell viability and allows the user to measure cell proliferation *in vitro*, via redox reaction that releases a fluorescent signal. Resazurin is a blue dye and non-fluorescent, also known as alamar Blue Reagent(Czekanska 2011; Anoopkumar-Dukie, et al., 2014). The colour change from blue to red is characteristic of this assay and causes the cells to become fluorescent. This assay was conducted to investigate whether the PPAR-γ has toxic effects on brain tissue (Sarker, et al., 2007).

The Fibroblast 3T3 cell line was used as the standard for toxicity testing, as they are fast growing cells, and are highly sensitive to toxic materials (Seiler, et al., 2004; Eldeniz, et al., 2007).The 3T3 is a cell line culture protocol refers to 3-day transfer. This cell line was originated from the primary mouse embryonic fibroblast cells.

During recent decades, this technique has become frequently used that possibility of cell growth within hours where cells are able to double every 18 hours and become confluent at roughly 50,000 cells/cm^2, to 55,000 cells ultimately (Chugh, et al., 2015). However, in culture, cells should not be allowed to become totally confluent.

Before investigating the actual effects of the agonist, a preliminary assessment was carried out to determine the toxic levels of the drug. This was necessary to determine whether any potential reduction of cell number was not due to toxicity. Before commencing a fundamental experiment that related to a mouse model of neurodegeneration, also in order to investigate the impact of the PPAR-γ, instead of using organotypic slice culture of mouse brain model directly. For this purpose these cells have been used. This type of cells was chosen instead of brain tissue, for three

reasons. They only require 24 hours to grow; they grow in a uniform manner, and are highly sensitive to toxic materials (Chung, et al., 2007; Salic and Mitchison 2008).

Materials and method:

The cells (fibroblast cell line) were grown in several 96-well plates for 24 and 72 hours. The medium was changed every other day. The cells were grown to densities of 1K, 5K, 10K, 20K and 50K. Rosiglitazone was dissolved in in clnde in the abbreviation section at the following concentrations: 25µM, 50µM and 100 µM. The medium was removed and the cells were treated with either Rosiglitazone, or the same concentrations of DMSO vehicle for 24 and 72 hours. Once the time points were reached, Resazurin was added and the plates were subsequently re- incubated from 2 hours, up to 1 week. The stock solution contains resazurin (440µM) and phenol red-free HBSS (Ca^{+2} and Mg^{+2}). The concentration was 12mg of resazurin dissolved in 100ml HBSS. 10% of resasurin mixed with 90% of culture medium. The results have been translated to graphical forms using GraphPad Prism6; two way ANOVA followed by a post hoc student. Fluostar Galaxy software reader was used for resazurin analyzed.

Results:

Results showed that Rosiglitazone was non-toxic at the concentrations used in this assay. The results were analysed by a FLUOstar Galaxy software reader. Subsequent bar charts represent the cell viability after treatment with Rosiglitazone.

Resazurin Test for the investigation of cell viability (3T3) Treated with Rosiglitazone (PPARγ agonist) for 2h, 24h and 72 h:

Results showed that Rosiglitazone was non-toxic at all the concentrations when incubated for 24 hour using resazurin. The lower limit of detection was 3500 cells/well. For the 24-hour incubation period, there is a gain in assay sensitivity with a lower limit of detection of 3300 cells/well (Figure 1).

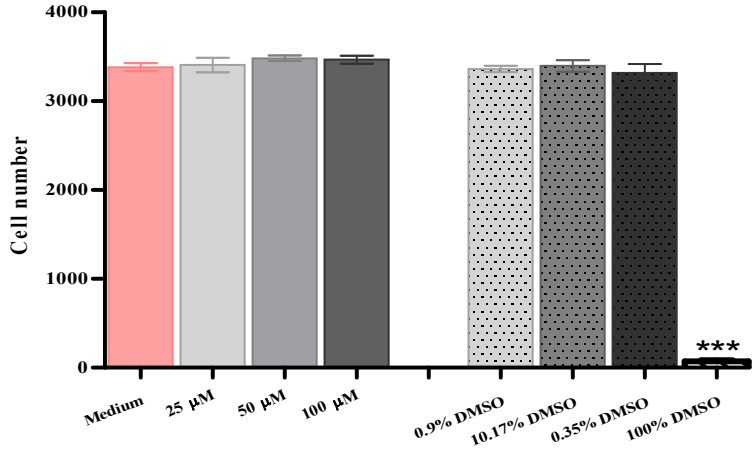

Figure 1;The Resazurin assay was performed and fluorescent cells were checked 2 hours later. Results of a one way ANOVA (post-hoc Dunnett's multiple comparison tests) show that all three concentrations are not toxic. Data are expressed as mean ± SD (n=6)100% DMSO positive control showed a significant difference from the control medium, indicated with an asterisk ($P < 0.05$).

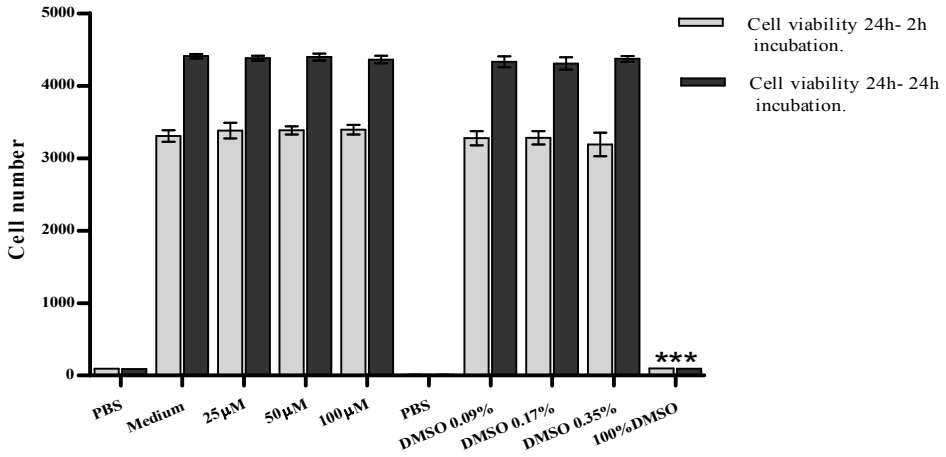

Figure 2; Bar chart shows the results obtained when reading the plates after 2 and 24 hours (24 hours post-seeding).Cell viability of 3T3 cells was measured after 2 and 24 hours, using the Resazurin assay, after being treated with Rosiglitazone (PPAR-γ agonist) for 24hours. Two ways ANOVA shows all three concentrations are not toxic. Data are expressed as mean ± SD (n=6. The 100% DMSO positive control showed significant difference from the control medium indicated with an asterisk ($P < 0.001$).

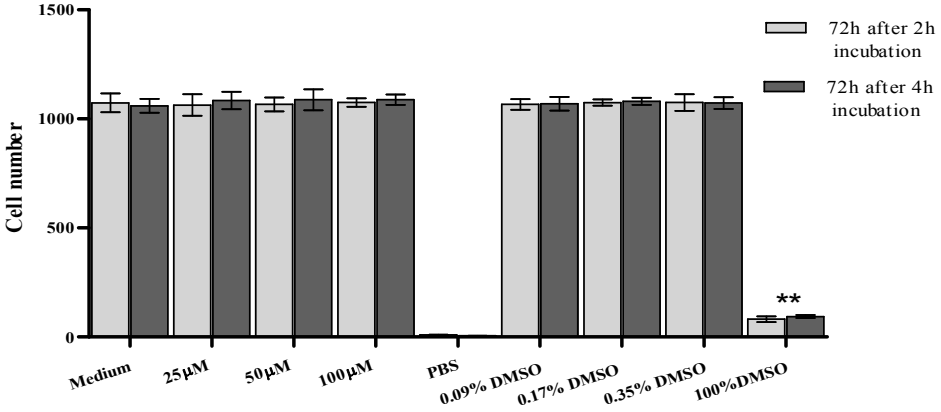

Figure 3; Bar chart shows the results obtained after reading the plates after 2 and 4 hr, (72 hours post-seeding).Cell viability of 3T3 cells was measured after 2 and 24 hours, using the Resazurin assay, after being treated with Rosiglitazone (PPAR-γ agonist) for 72h. Two way ANOVA shows all three concentrations are not toxic. Data are expressed as mean ± SD (n=6.100% DMSO positive control showed significant difference from the control medium, indicated with an asterisk ($P < 0.001$).

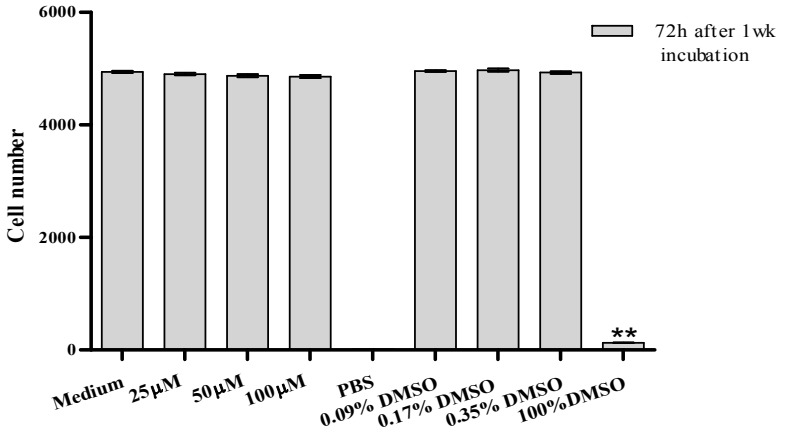

Figure 4; Bar chart shows the results of the survival of the cells after seeding for 72 hours.Cell viability of 3T3 cells after 1 week incubation was measured using the Resazurin assay, after being treated with Rosiglitazone (PPAR-γ agonist) for 72h. Two ways ANOVA shows all three concentrations are not toxic. All data are expressed as mean ± SD (n=6) 100% DMSO positive control showed significant difference from the control medium, indicated with an asterisk ($P < 0.001$).

Discussion:

Cell culture can be used to monitor for toxicity by estimation of the basalfunctions of the cell such as those processes common to all types of cells or by tests on specialized cell functions (Tran, et al., 2013). General toxicity tests were aimed mainly at detection of the biological activity of test substances which can be carried out on many the cell types' such as fibroblasts(Ahuja and Sharma 2014). This principle is consistent with this current work, where a 3T3 cells were used to investigate toxicity of drugs; PPAR specifically which will be used in further work, Results showed that the amount of drug was safe to use (Aguirre Rueda, et al., 2015).The main objective of this study was to make sure that the drug is not toxic for this type of cells which known have high sensibility toward toxicity. The results showed that the drug is safe, and this finding is consistent with Yim, 2011 (Yim, et al., 2011).

Conclusion:

The present study showed that Rosiglitazone (PPARγ agonist) is non-toxic at the concentrations (25µM, 50 µM and 100 µM) that used with 3T3 cell. Therefore rosiglitazone can be used safely on the astrocytes in subsequent experiments, and positive changes presumably resulting in efficiency of the rosiglitazone.

References:

1. *Aguirre Rueda, D., S. Guerra Ojeda, et al., (2015). Astrocytes protect neurons from Aβ1-42 peptide-induced neurotoxicity increasing TFAM and PGC-1 and decreasing PPAR-γ and SIRT-1. International Journal Of Medical Sciences, 2015, vol. jan 1, num. 12 (1), p. 48-56.*
2. *Ahuja, V. and S. Sharma (2014). Drug safety testing paradigm, current progress and future challenges: an overview. Journal of Applied Toxicology 34(6): 576-594.*
3. *Anoopkumar-Dukie, S., J. Carey, et al., (2014). "Resazurin assay of radiation response in cultured cells." The British journal of radiology.*
4. *Chugh, R. M., M. Chaturvedi, et al., (2015). Occurrence and sontrol of sporadic proliferation in Growth Arrested Swiss 3T3 Feeder Cells. PLoS One 10(3): e0122056.*

5. *Chung, T.-H., S.-H. Wu, et al., (2007). The effect of surface charge on the uptake and biological function of mesoporous silica nanoparticles in 3T3-L1 cells and human mesenchymal stem cells." Biomaterials 28(19): 2959-2966.*
6. *Czekanska, E. M. (2011). "Assessment of cell proliferation with resazurin-based fluorescent dye. Mammalian Cell Viability: Methods and Protocols: 27-32.*
7. *Eldeniz, A., K. Mustafa, et al., (2007). "Cytotoxicity of new resin-, calcium hydroxide- and silicone-based root canal sealers on fibroblasts derived from human gingiva and L929 cell lines. International endodontic journal 40(5): 329-337.*
8. *Salic, A. and T. J. Mitchison (2008). "A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proceedings of the National Academy of Sciences 105(7): 2415-2420.*
9. *Sarker, S. D., L. Nahar, et al., (2007). "Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the< i> in vitro</i> antibacterial screening of phytochemicals." Methods 42(4): 321-324.*
10. *Seiler, A., A. Visan, et al., (2004). Improvement of an in vitro stem cell assay for developmental toxicity: the use of molecular endpoints in the embryonic stem cell test. Reproductive toxicology 18(2): 231-240.*
11. *Tran, T. B., S. Cho, et al., (2013). Hydrogel-based diffusion chip with electric cell-substrate impedance sensing (ECIS) integration for cell viability assay and drug toxicity screening. Biosensors and Bioelectronics 50: 453-459.*
12. *Yim, M.-J., M. Hosokawa, et al., (2011). Suppressive effects of amarouciaxanthin A on 3T3-L1 adipocyte differentiation through down-regulation of PPARγ and C/EBPα mRNA expression. Journal of agricultural and food chemistry 59(5): 1646-1652.*