Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2014; 6(4): 136-139

ISSN: 0975-5160

Research Article

Prediction of Cytoxic Effects of SMA-DMSO in Mouse Fibroblast Cell Line Using MTT Assay

*Singh P[1], Bansode F.W[2], Ganguly C[3]

[1]*Dept of Biotechnology, IILM Academy of Higher Learning,Gr.Noida,U.P,India*
[2]*Div of Endocrinology,CDRI, Lucknow, U.P, India*
[3]*Dept of Biotechnology IILM Academy of Higher Learning,Gr.Noida,U.P,India*

Available Online: 29th November, 2014

ABSTRACT

Biologically active polymer of SMA-DMSO(RISUG) formulated in ratio 1:2 and known to work as non hormonal contraceptive for males was analyzed for cytotoxicity invitro in mouse fibroblast adherent cell line, L929 using MTT Assay. Cells were exposed to different concentrations (10^{-3}–10^{-9}M) of SMA-DMSO (RISUG) for different periods of time i.e. 24, 48, 72 and 96 hr. preceded by addition of Tetrazolium salt 4 hours prior to completion of each incubation period. Percentage cell viability was calculated as per the MTT assay.A dose dependent statistically significant decrease in the percent cell viability was observed following the 24 hr exposure of test compound between 10^{-3}M to 10^{-5}M concentrations.Further, A recovery trend was observed in cells following an incubation periods of 48, 72 and 96 hr. This investigative study has signified that doses of 10^{-6}M to 10^{-9}M were non-cytotoxic in L929 cells on overall analysis.

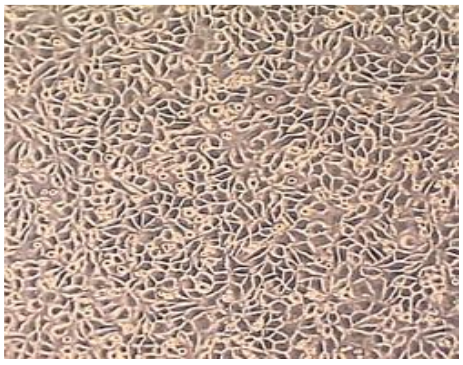
Keywords: SMA-DMSO, MTT, Cell viability, Cytoxicity,Mouse fibroblast cell line

INTRODUCTION

The non-hormonal contraceptive, named RISUG (an acronym for Reversible Inhibition of Sperm under Guidance) has been expected to provide a valuable addition to the current options of male contraception (1). A single injection (therapeutic dose of 60 mg SMA in 120 ml of DMSO) bilaterally into the vas deferens of the male in a minimally invasive manner has been shown to cause the disintegration of sperms and leads to necrozoospermia (2,3). SMA is one such agent which when placed in the vas deferens lumen lowers the pH of the luminal micro-environment and inhibits sperm acrosomal reaction within 3h post-injection before the ejaculation in human (4,5).Earlier studies carried on with Langur Monkeys have shown that vas deferens with SMA results in severe oligospermia in first two ejaculations and continuous azoospermia in subsequent ejaculations.(6). Nature of action of RISUG is reversible in primates (7).In addition to contraception, RISUG also possesses antimicrobial activity (8,9).

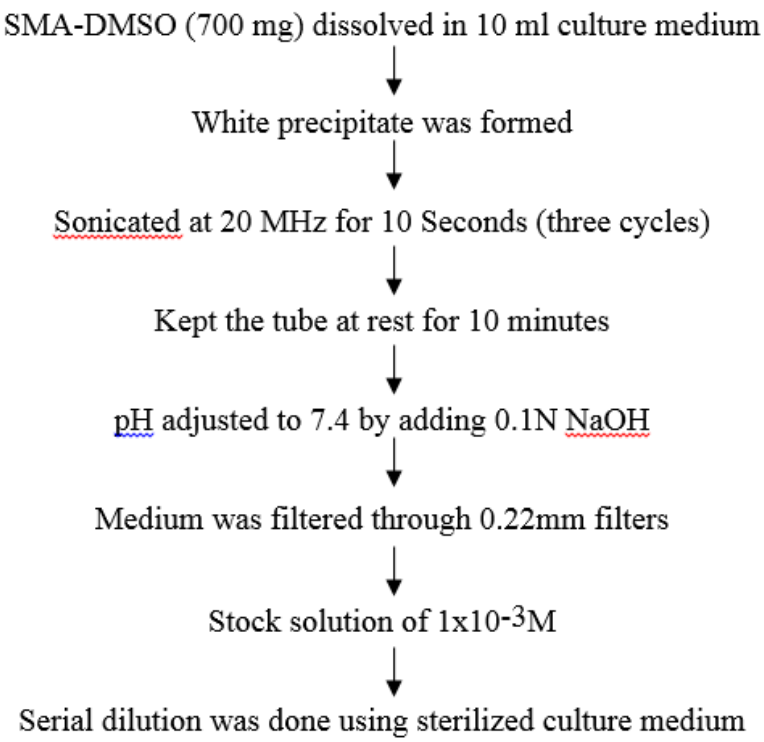
Cytotoxicity testing is a rapid, standardized, sensitive, and inexpensive means to determine whether a material contains significant quantities of biologically harmful extractables. The high sensitivity of the tests is due to the isolation of the test cells in cultures and the absence of the protective mechanisms that assist cells within the body (10). A mammalian cell culture medium is the preferred extractant because it is a physiological solution capable of extracting a wide range of chemical structures, not just those soluble in water. Antibiotics can be added to the medium to eliminate potential interference from microbial contamination that may be present on the test material and control samples. Results of cytotoxicity tests correlate reasonably well with short-term and long term implant studies(11). However, they do not necessarily correlate well with other standard tests of biocompatibility that are designed to examine specific end points (such as sensitization) or that use extracts prepared under more rigorous conditions (for example, at 121°C in saline or cottonseed oil). Cytotoxicity test methods are useful for screening materials that may be used in medical devices because they serve to separate reactive from non-reactive materials, providing predictive evidence of material biocompatibility(12). The ISO 10993-5 standard, "Guidance on the Selection of Tests," considers these tests so important that they are prescribed for every type of medical device, along with sensitization and irritation testing.

The MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl



Cell line

*Author for correspondence



Preparation of Stock culture of SMA-DMSO

tetrazolium bromide) cleavage assay, originally described by Mosmann (13) for measuring cell survival/proliferation, has been widely applied for estimation of cytotoxicity(14). This assay is most commonly employed for the detection of cytotoxicity of cell viability following exposure to toxic substance and to determine IC50 value.(15)

Cytotoxicity test methods are also useful for lot-to-lot comparison of materials, for determining whether a potential replacement material is equivalent to that currently being used, and for troubleshooting and exploring the significance of changes in manufacturing processes.(16)

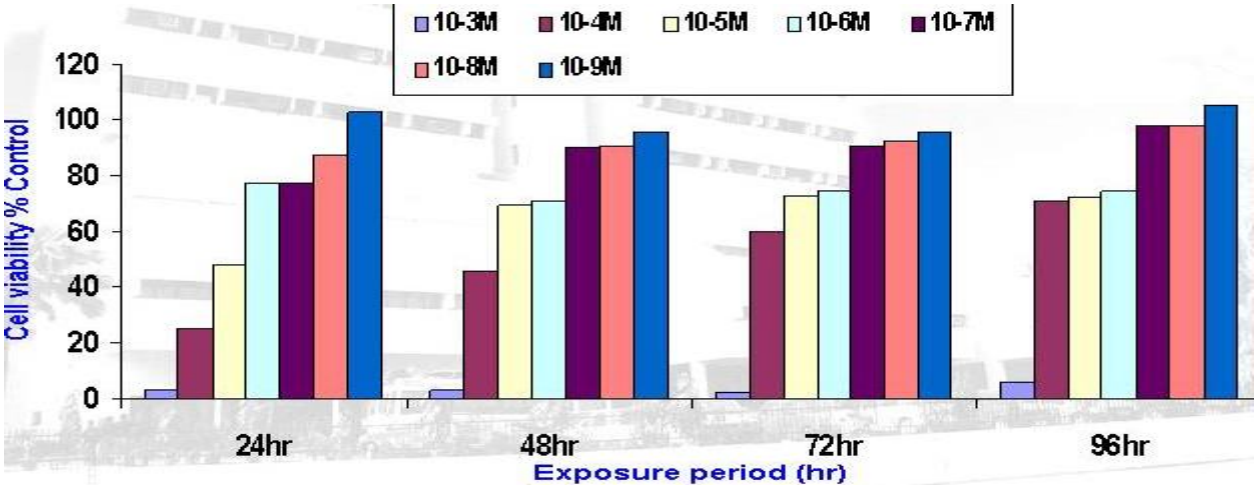
MATERIAL AND METHODS

Cell line: L929, an adherent type mouse fibroblast cell line, recognized by ISO 10993 for the cytotoxicity assessment of biomedical devices and materials which are supposed to come in contact with human subject directly or through indirect means. L929 cells used in all the experiments of the present investigations were originally procured form NCCS, Pune and since then has been maintained at *In Vitro* Toxicology Laboratory, CDRI, Lucknow.

Initiation of culture from frozen cells: Frozen vial of L929 cells were taken out from the liquid nitrogen and immediately transferred to a pre warmed (37°C) water bath for thawing. The cells were then transferred to sterile centrifuge tube containing complete medium (MEM + 10% FBS + Antibiotic-Antimycotic solution (1.5 ml/100 ml of medium)) and centrifuged at 600 rpm for 10 minutes at room temperature. The supernatant was discarded & the loosely bound pellet was suspended in 5 ml of complete medium & appropriate numbers of cells were seeded in T-75 tissue culture flask, incubated in a CO_2 incubator (5% CO_2 - 95% air at high humidity) at 37°C. Initially the medium was changed post 24 hr. and subsequently at an intervals of 2-3 days till sufficient growth was observed.

Cell viability test:

Dye exclusion test: In principle, the cells with damaged membrane allow the trypan blue dye to pass into the cytoplasm whereas undamaged cells are capable of dye exclusion. This dye exclusion method was used to see the cell viability by assessing the loss of membrane integrity following the method of Thompson *et aI.*, 1991 with little modifications. This test was done for every batch of cells before the start of experiments to ascertain that how much cells are viable in the batch specific and batches showing the cell viability more than 95% were used for the experiments. In brief, well-mixed cell suspension (0.8 ml) was added to a test tube already containing 0.2 ml of trypan blue (0.4%) and mixed the contents by gentle shaking. In continuation, without wasting the time, 20 ml of dye-cell mixture was placed on the edge of both the chambers of the haemocytometer prefixed with the cover slip and allowed the cell suspension to fill the chambers by capillary action. The counting of unstained (viable cells) and stained cells (nonviable cells) in the four large corner squares in both counting chambers was made using a 10X microscope objective. The percent cell viability was calculated by deducting the number of all stained cells from total number of cells counted (stained cells and unstained cells) over the haemocytometer. The



only batches showing more than 95% cell viability were used in further experimentation.

Plating of L929 cells: Cells were harvested by trypsinization from the flasks having the high confluency and peletted by centrifugation at 600 rpm for 5 minutes. The pellet was re-suspended in fresh culture medium and clumps were broken if any by a tip of 20 ml by up and down several times. Cells were then counted with the help of coulter electronic cell and particle counter and plated in the frequency of 10,000 cells per well in 100ml of culture medium.

Percent Cell Viability by MTT Assay: The MTT assay showed the ability of the cells to convert the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide tetrazolium salt into a purple MTT formazon, by mitochondrial dehydrogenase activity of live cells, which is measured spectrophotometrically at 530 nm. MTT assay provides an indication of mitochondrial integrity & activity, which is interpreted as a measure of percent cell viability. The assay was done following the method of Mosmann *et al* (1994) with desired modifications. In brief, cells (10,000 in number) were seeded in 96-well tissue culture plates and incubated in the CO_2 incubator for 24 h. at 37°C prior to experiment for the proper attachment of the cells. Then the medium was aspirated & cells were exposed to different concentrations (10^{-3}–10^{-9}M) of SMA-DMSO (RISUG) prepared as discussed earlier for different periods of time i.e. 24, 48, 72 and 96 hr. Tetrazolium (10ml/well containing 100ml of cell suspension; 5mg/ml of stock in PBS) salt was added 4 hr. prior to completion the each incubation period. Then, the reaction mixture was carefully taken out and 200 ml of DMSO was added to each well by pipetting up & down several times unless the content get homogenized. After 10 minutes, the color was read at 530 nm, using Multiwell microplate reader. The untreated sets were also ran parallel under identical conditions & served as basal control, whereas cells treated with manganese (10^{-4} M) were served as positive control.

RESULTS

The highlights of the data of cytotoxicity are summarized in Fig.-1. A dose dependent statistically significant decrease in the percent cell viability was observed following the 24 hr exposure of test compound between 10^{-3}M to 10^{-5}M concentrations, when compared with control. Interestingly, a recovery trend was observed in further incubation periods i.e., 48, 72 and 96 hr. The highest concentration used in the study i.e., 10^{-3}M was found to be statistically highly cytotoxic (<0.001) at all the time intervals of observation. Whereas, doses of 10^{-6}M to 10^{-9}M were found overall non-cytotoxic in L929 cells.

INFERENCE

The results indicate that the test compound SMA-DMSO is safe for all the time intervals at the dose levels of 10^{-6} to 10^{-9}M, however the higher concentrations posed a range of statistically significant cytotoxic response in L929 cells under experimental conditions. The dose of 10^{-3}M causes a loss in cell viability more than 90% at all the time intervals selected in the study. Incubation period of 24 hr was found to be most critical followed by a recovery trend till the end of experiment i.e., 96 hr.

REFERENCES

1. Singh R.K, Bansode F.W, Meena A.K.,Studies on Testis, Sperm characteristics and Teratogenic aspects after vas occlusion by a copolymer of styrene maleic anhydride- RISUG and its reversal in rats.*IJIRSET*,2014, vol. 3,12474-12485.
2. Chaudhury K, Sharma U, Jagannathan NR, Guha SK.Effect of a new injectable male contraceptive on the seminal plasma amino acids studied by proton NMR spectroscopy. *Contraception* , 2002,66:199-204.
3. Guha SK .Biophysical mechanism-mediated time-dependent effect on sperm of human and monkey vas implanted polyelectrolyte contraceptive. *Asian J Androl* ,2007,9:221–227.
4. Sharma RS, Rajanna A, Singh BK, Mathur AK, Mukherjee AK.Current status of development of RISUG: an intravasal injectable male contraceptive. In: Recent Advances and Challenges in Reproductive Health Research. Indian Council of Medical Research, New Delhi,2008, pp. 135–149.

5. Guha SK, Non-invasive reversal of intraluminal vas deferens polymer injection-induced azoospermia--technology. *Asian J Androl* ,1999, 1:131-134.
6. Lohiya NK, Manivannan B, Mishra PK. Ultrastructure changes in the spermatozoa of langur monkeys Presbytis entellus entellus after vas occlusion with styrene maleic anhydride. Contraception 1998;57, 125-132.
7. Lohiya NK, Manivannan B, Mishra PK. Repeated vas occlusion and non-invasive reversal with styrene maleic anhydride for male contraception in langur monkeys. Int J Androl 2000; 23(1): 36-42.
8. Guha SK. RISUG_ (reversible inhibition of sperm under guidance) -An antimicrobial as male vas deferens implant for HIV free semen. Medical hypothesis 2005; 65: 61-64.
9. Sharma U, Chaudhury K, Jagannathan NR, Guha SK. A proton NMR study of the effect of a new intravasal injectable male contraceptive RISUG on seminal plasma metabolites.Reproduction 2001; 122: 431-436
10. Borenfreund, E., Puerner, J.A.A simple quantitative procedure using monolayer culture for toxicity assays. J. Tissue Cult. 1984, Vol.9, 7–9.
11. Decker, T., Lohmann-Matthes, M.,A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J. Immunol.1988, Meth. 15, 61–69.
12. Denizot, F., Lang, R., Rapid colorimetric assay for cell growth and survival. J. Immunol. Meth. 1986,vol 89, 271–277.
13. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, 1983, 65, 55.
14. Soldateschi, D., Censini, S., De Gori, V., Antoni, G., Tagliabue, A. and Boraschi, D. ,Macrophage antitumor activity in vitro. Comparative analysis of cytolytic, cytostatic and cytotoxic activities of mouse macrophages and human monocytes. Immunobiology ,1984,vol 166 ,251-255.
15. Fotakis, G., Timbrell, J.A., In vitro cytotoxicity assays: Coparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride.*Toxicology Letters*, 2006, 160: 171-177.
16. Dewi Fatma, S., et al. In vitro cytotoxicity of *Jatropha curcas* in epithelial and fibroblast cells. Journal of Natural Products, 2012, vol.5, 214- 221