Characterisation of the Cellular Reduction of 3-(4,5-Dimethylthiozol)-2,5-Diphenyltetrazolium Bromide (MTT): optimization of Dose of Azadiracta Indica (Neem) For The Viability of Macrophages in Vitro

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Abstract- Rapid and accurate assessment of viable cell number and cell proliferation is an important requirement in many experimental situations within in vitro cell culture studies. In vitro techniques for drug screening, determination of the cytostatic potential of different compounds in toxicology testing are very useful. Also it helps to evaluate the cytotoxic, mutagenic and carcinogenic effects of the respective compound. The activation of macrophages in vitro was studied by using Azadiracta indica commonly known as Neem and standard macrophage activator like LPS. Screening of the drug and optimization of drug dose was carried out initially on log scale and then to the linear scale by MTT assay.

I. INTRODUCTION

Initial morphology of J774A.1 cells was observed during cell culturing. Macrophages are of various shape and size. The cells are irregularly shaped, with surface folds, ruffles, and projections. The cells were surface adherent (11). Macrophages are oval to spindle shaped and also spreads with the pseudopodia. Macrophages in their sub-confluent phase were treated by the drug at different concentrations to check their effect on viability (by trypan blue dye exclusion test and MTT assay) at different time intervals.

MTT assay is a colorimetric assay used to check the metabolic activity of viable cells. It is a microtiter assay which uses the tetrazolium salt MTT (succinate dehydrogenase inhibition or SDI test) to quantitate cell proliferation and cytotoxicity (6,10). MTT assay exclusively detect viable cells because tetrazolium salts are cleaved only by metabolically active cells. In the SDI assay MTT is reduced by viable cells to a colored, water-insoluble formazan salt. After it is solubilized, the formazan formed can easily and rapidly be quantitated in a conventional ELISA plate reader.

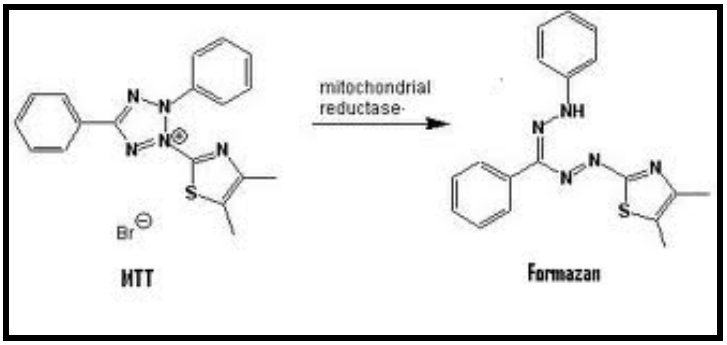

Fig.1: MTT reduction
Fig1: Reduction of a tetrazolium to a formazan (fig source: biotek.com).

[note:MTT is cleaved to formazan by the 'succinate tetrazolium reductase' system which belongs to the mitochondrial respiratory chain and is active only in viable cells (Slater, 1963). Interestingly however, recent evidence suggests that mitochondrial electron transport may play a minor role in the cellular reduction of MTT. Since most cellular reduction occurs in the cytoplasm and probably involves the pyridine nucleotide co factors NADH and NADPH, (2) the MTT assay can no longer be considered strictly a mitochondrial assay.]

II. MATERIALS AND METHODS

Reagents: Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and 25 mM HEPES buffer were purchased from (HiMedia Pvt. Ltd. India.) Fetal bovine serum was purchased from Hyclone (Logan, USA) and heat inactivated at 56^0C for 30 min.

The plant leaves was subjected to extraction with 200 ml methanol at 50^0 C for 8 cycles by Soxhlet extraction process. The extract was then concentrated with rotator vacuum evaporator and used for further analysis. The drugs

prepared in incomplete DMEM were tested for endotoxin contamination by limulus amebocyte lysate assay which showed insignificant levels [0.0007ng/mg]. Necessary precautions were taken to avoid endotoxin contamination through out the investigation, by using endotoxin free buffers, reagents and sterile water. All other chemicals and solvents used in this study were obtained from Sigma Chemical Company (St. Louis, USA) and were of analytical grade or the highest grade available.

III. LAB PREPARATIONS

Phosphate buffer saline (PBS): PBS is one of the very important solutions required in tissue culture. To maintain the cell culture environment saline with the constant pH (7.2-7.4) was applied. The saline solution was prepared in sterile conditions. Sterilization was done by autoclaving or by filtering with nitrocellulose filters of 0.22µm diameter. This sterile solution was used further for preparation or dilutions of several chemicals.

Trypsin (TPVG): Adherent cells can not be detached simply by hand tapping. To detach the cells from the culture flasks during subculture or assaying they were treated with sterilized trypsin (pH7.2-7.4) for around 1-2 minutes.

Cells: The macrophage J774A.1 cell line, obtained from National Center for Cell Sciences (NCCS, Pune), was used as source of macrophages (Origin: BALB/c mouse; Nature: Mature), grown and maintained in the DMEM (pH 7.5) enriched with 10% fetal bovine serum, at 37^0C and 5% CO_2 environment.

Viability assay: Cell viability was determined by the Trypan blue dye exclusion technique. Equal volumes of cell suspensions were mixed with 0.4% Trypan blue in PBS, and the unstained viable cells were determined. These cells were further used for cytotoxicity assay in 2 x 10^6 densities per ml in the 96 well tissue culture plates.

Stimulation of macrophages: The macrophage cells (cell line J774A.1) from late log phase of growth (subconfluent) were seeded in 96 well flat bottom microtiter plates (Tarsons, India) in a volume of 100µl under adequate culture conditions. Drug was added in different concentrations in a volume of 100µl in triplicate. The cultures were incubated at 37^0 C and 5% CO_2 environment. After 24 hr and 48 hr incubation percent viability was checked.

Cytotoxicity assay: In order to detect the toxicity of herbal preparation the cytotoxicity assay was standardized by using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at different time intervals for 24 hrs and 48 hrs (6) using the drug at various concentrations. After 24 hrs and 48 hrs of incubation, supernatants were collected and ten microlitres of MTT (3 mg/ml) was added to each well and plates were further incubated for 2 hrs. The enzyme reaction was then stopped by addition of 150µl of dimethyl sulphoxide (DMSO). Plates were incubated for 10 min under agitation at room temperature before the optical density at 570nm was read under an ELISA plate reader. Three independent experiments in triplicate were performed for the determination of sensitivity to each drug. Cells treated with medium alone were considered as Control.

Percent viability was calculated by the given formula.

$$\text{Percent viability} = \frac{E}{C} \times 100$$

Where, E is the absorbance of treated cells and C is the absorbance of untreated cells.

Statistical analysis:

Statistical significance of difference between the control and experimental samples were calculated by Student's *t*-test. All the experiments were done in triplicate samples. Conclusions were drawn from 3 independent experiments.

IV. RESULTS

Viability and proliferation of J774A.1 cells:

J774A.1 cells showed 100% viability before the drug treatment by trypan blue dye exclusion test. The cells were treated with drugs initially on log scale and then on linear scale. J774A.1 cells were incubated in medium alone or drug for 24 h and 48 h and checked for percent viability as described in Materials and Methods.

Fig2: macrophages in culture.

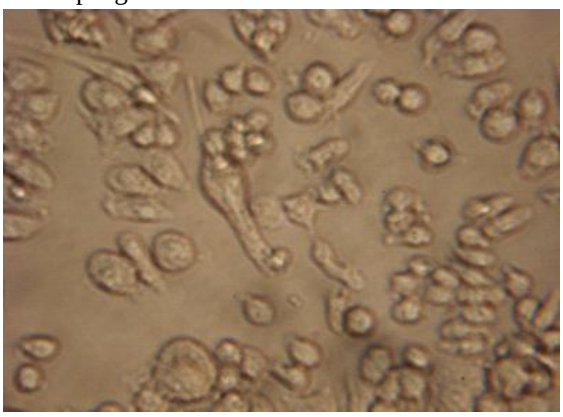

Fig2: J774A.1 (Oriin: BALB/c Nature:mature (fig: image taken from inverted microscope)

Fig3: log scale results of macrophages

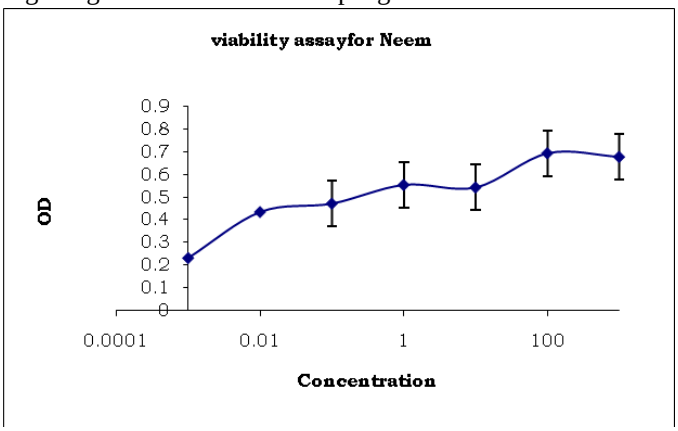

Fig3: J774A.1 viability assay; on log scale

Neem in concentration 100µg/ml, and LPS at 10µg/ml each, showed maximum viability of macrophages as compared to medium alone, thereby proving that these drug concentrations were not cytotoxic to the cells. The assay results are mentioned with the respective IC_{10} values of the drugs (Table 1 and 2).

The log scale result explains the effect of drugs at their 10 fold concentrations (fig3). It was found that the drug Neem being herbal preparation did not show cytotoxic effect on macrophages (J774A.1). In fact even the high concentrations of this drug could only reduce the viability upto 10 percent (Table2) and showed negligible cytotoxicity of macrophages (Fig3). Since the work is *in vitro*, Optimum drug concentration for Neem 100µg/ml (Table1). LPS is the standard positive stimulator of macrophages. Higher concentrations of this compound are cytotoxic. Their IC_{10} values were 10-15µg/ml and the optimum drug concentration for macrophage activation was 10µg/ml (Table 1 and 2).

Table1: Optimum drug concentrations

Treatment	Optimum Concentration	% viability (±S.E.M.) (24 hrs)	% Viability (±S.E.M.) (48 hrs)
Medium alone	-	99 ± 0.75	99 ± 0.25
LPS	10µg/ml	93±1*	92±2*
Azadiracta indica (Neem)	100 µg/ml	99 ± 0.72	98 ± 1

Table1: The macrophages treated with medium alone or (with LPS, Neem and the % viability was checked using MTT assay. Values represent SDs of means for triplicate cultures. Data are representative of three separate experiments (*p<0.05, * *p<0.001).

Table2: Drug concentrations with their IC_{10} value :

Treatment	Concentration (IC_{10})	% viability (±S.E.M.) (24 hrs)	% Viability (±S.E.M.) (48 hrs)
Medium alone	-	99 ± 0.75	99 ± 0.25
LPS	(15µg/ml)	91±1*	92±2*
Azadiracta indica (Neem)	250 µg/ml	93±1*	93±1*

Table2: The culture supernatants were collected from macrophages treated with medium alone or with LPS and Neem and the % viability was checked using MTT assay. Values represent SDs of means for triplicate cultures. Data are representative of three separate experiments (*p<0.05,* *p<0.001).

V. DISCUSSION

The most convenient assay for determination of cell viability and cell proliferation has been developed in a micro-titer plate or 96 well plates. This miniaturization allows many samples to be analyzed rapidly and simultaneously. It also reduces the amount of culture medium and cells required. It is cost effective and colorimetric assays allow samples to be measured directly in the micro-titer plate with an ELISA plate reader.

One of the most promising recent alternatives to classical antibiotic treatment is the use of immunomodulators for enhancing host defense responses (9). Plant derived immunomodulatory compounds have also been used in traditional remedies for various medical problems and the investigation of these sources has grown exponentially in recent years. India has a rich tradition in the treatment of many diseases by therapy with ‘Rasayans’. In Ayurveda, ‘Rasayans’ are concerned with nourishing body and boosting immunity. They are also modulators of the immune system and one such cell modulated by them is the macrophage. J774A.1 cells are adherent and spread out when cultured in DMEM, however, when cultured in RPMI 1640, the cells are rounded and relatively non-adherent. Different types of tissue culture plates, sera, and media supplements were not responsible for these changes. In DMEM the cells become more adherent, acquired an amoeboid shape with pseudopods, a more vacuolated cytoplasm and a higher cytoplasmic to nuclear ratio. Interestingly, when the cell is placed back into RPMI, it regains its more ovoid monocytic characteristics. Hence the growth mediums were standardized acoordingly and the cells were grown in DMEM culture during this study (4). J774A.1 cells showed 100% viability before the drug treatment by trypan blue dye exclusion test. The cells were treated with drugs initially on log scale and then on linear scale. J774A.1 cells were incubated in medium alone or drugs for 24h and 48h and checked for percent viability as described in Materials and Methods. The drug Neem (100µg/ml) showed maximum

viability of macrophages as compared to medium alone, thereby proving that the drugs were not cytotoxic to the cells. The macrophage cells (J774A.1) were treated with the drug and the inhibitory concentration (IC_{10}) of the drug for the cells was estimated by MTT assay and trypan blue dye exclusion test.

VI. CONCLUSION

The drug was observed not to have cytotoxic effect on macrophages as determined by MTT assay.

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