STANDARDIZATION OF A RAPID AND INEXPENSIVE *IN-VITRO* CYTOTOXICITY ASSAY

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ABSTRACT

A large number of chemicals in our environment and in day-to-day life are not adequately tested for toxicity. Currently used toxicity tests have limitations such as, (a) *in-vitro* cell based assays depend on costly and specialized animal cell lines, (b) most assays are growth dependent and require chemicals, media and inventory for aseptic conditions and growth of cells (biosafety cabinets, CO_2 incubators, etc.), (c) *in-vivo* assays require laboratory animals leading to additional financial burden and ethical issues. In order to encourage even the smalltime manufacturers of harmful chemicals to test their products, preferably in-house, there is a need to develop rapid, reliable and low-cost cytotoxicity assay. In the present work, metabolically active primary cells from a potential target organ (liver) were obtained by macerating liver tissue taken from a local chicken/mutton shop. Interestingly, tissue preserved at 2-8^{0}C for up to 48 hr could be used in the assay. After adjusting cell density (OD_{576}= 0.5), cells were exposed to different test compounds such as textile dyes, festival color and other chemicals for up to 3 hr under ambient conditions. Cells were kept in plain DMEM medium (without fetal calf serum) to maintain controlled conditions for the cells. After incubation, viability of cells was determined by dye exclusion test using Trypan Blue. The assay was standardized by varying different factors viz. concentration of test chemicals, exposure time, etc., one at a time while keeping other parameters constant. Test compounds were classified as highly toxic if the LC50 values were below 2µg/ml, toxic if the LC_{50} values were between 2-10µg/ml, low toxic for LC_{50} values between 10-50µg/ml and non-toxic for LC_{50} values above 50µg/ml. It was found that viability of cells exposed to festival color and orange2 was similar to that of the control (84.7%) and LC_{50} values for these chemicals were more than 50µg/ml (non-toxic). On the other hand, lead acetate and mercuric chloride, which are well known for toxicity, could be classified as highly toxic (LC_{50} values <2µg/ml) with viability reduced to almost non-detectable levels. The statistical significance of these observations was evaluated by applying the student t-test. The proposed liver cell-based assay could be potentially useful for high throughput screening of samples and development of a toxicological database for risk assessment and protection of public. It must be highlighted that inclusion of different animal organs which are usually thrown away (not consumed) might be useful in toxicity studies; especially for studying mechanism of toxicity. In addition to less time duration, the proposed assay will result in reduced numbers of animals for confirmatory *in-vivo* tests.

Key Words : Chemicals, *In-vitro*, Cytotoxicity assay, Taxicological, Dyes, Chemicals

INTRODUCTION

Toxicology is the science of poisons and a specialized branch of science that deals with safety of humans from different forms of chemicals. Toxicity testing identifies hazards or adverse effects that might occur due to exposure to a chemical. This is important for environmental and public health decision making. Humans and animals can be exposed to both naturally occurring and man-made chemicals in a variety of ways- by mouth, skin contact, or inhalation[1].

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Traditional chemical toxicity testing employs rodent bioassay that measures the effects of a limited exposure of an animal to a substance (acute toxicity) as well as repeated, long-term exposure (chronic toxicity). Such *in-vivo* tests, although most reliable, may involve nearly 900 animals for each chemical evaluation[2]. The skill intensive work involved in these studies, longer time duration, legal and ethical issues and public pressure to reduce use of animals for experimentation have intensified the use of alternative *in-vitro* test systems for chemical analyses. Moreover, the animal models do not always reflect the behavior of chemicals in the human body. *In-vitro* assays on the other hand make use of established animal and human cell-lines for determination of more specific endpoints such as cytotoxicity (ability to damage cells), mutagenicity (ability to cause changes in genetic material), carcinogenicity (ability to cause cancer), and teratogenicity (ability to cause birth defects). Some of the more popular *in-vitro* toxicity assays are the Lifetime Rodent Bioassay (LRB), Syrian Hamster Embryo (SHE) cells, *in-vitro* micronucleus (MN) assay using human lymphocytes, etc. It has been observed that the use of tests such as the AMES test, MLA (mouse lymphoma assay) and MN assay in a battery could increase the sensitivity of toxicity testing[2]. The range of alternative tests for toxicity testing that use the 3Rs (Reduction, Replacement and Refinement) approach is described by Bhanushali et. al[3] and advantages of *in-vitro* assays have been reported in the literature[3- 6]. Some of the advantages can be listed as follows: (i) availability of well characterized cell lines, (ii) reduced cost of testing, (ii) lesser test time, (iii) better control over experimental conditions, (iv) quantification and reproducibility of results, and (v) possibility of high throughput analysis. It has been reported that the LC_{50} ranking of the pharmacological compounds was comparable to the potency of the oral toxicity in rats with minor exceptions[7]. These features make *in-vitro* testing appropriate for preliminary screening of a large number of chemical compounds and drugs. Many researchers have performed toxicity testing of various test compounds using established cell lines. It needs to be mentioned here that the phrases often associated with cell line cultures, viz. reduced cost, cost effectiveness, speed, rapidity and less time are often misleading; a fact that is exemplified by some recent reports[8-10]. Moreover, the drawback of *in-vitro* cell culture based testing is the inability to predict the effect of a toxin on a living organism with its complex interaction of nervous, endocrine, immune, and hematopoietic systems. Moreover, the cells must show the differentiated characteristics as in the whole animal to be useful as a model test system[11]. *In-vitro* systems can predict the cellular and molecular effects of a drug or toxin, but only a human or animal can exhibit the complex physiological response of the whole organism, including signs and symptoms of injury.

Thus, neither *in-vivo* nor *in-vitro* tests are alone sufficient predictors of risk to the human life. A combination of these two approaches, where in, *in-vitro* tests are first used for screening out a large number of toxic compounds, will reduce the number of animals for experimentation to a great extent. The smaller number of low to non toxic compounds can be subsequently tested on animals. Although this approach appears rational, the presently used *in-vitro* assays are still cumbersome on account of the almost exacting requirements imposed by animal cell culture work. Some of the difficulties involved in the current *in-vitro* testing are: (i) cell lines are known to alter the gene expression and do not behave like *in-vivo* cells, (ii) specialized cell lines are very costly and are available in select few laboratories, (iii) it is very difficult and costly to maintain the cell lines for long durations of time, (iv) infrastructure and inventory for carrying out animal cell culture is cost intensive, (v) special skills are required for carrying out such work, (vi) the cost of getting the chemicals tested from authorized laboratories on a per sample basis is high, (vii) only a few specialized testing laboratories exist sporadically in developing nations, resulting in high cost of transportation of samples to the testing facility. The large number of chemicals in our environment, many of which have been insufficiently tested for toxicity, justifies the need for rapid and reliable in vitro testing to predict toxicity[12]. An

alternative approach, wherein, tissue or cells for assay can be directly taken from the meat yielding animals, could provide the desired types and numbers of cells without sacrificing animals especially for experimental purposes. Such an attempt was made by making use of primary culture of human hepatocytes that were derived from surgically removed liver tissue[13]. However, ethical issues and difficulty in getting sufficient material for screening might be major deterrents in such work. The advantages of using primary hepatocytes from the liver tissue for cytotoxicity studies have been discussed in detail by Grisham et. al.[14], wherein, the authors have also stated limitations of cultured hepatic cells for assessment of chemicals. Considering these factors, the present investigations were carried out using primary liver cells from chicken which were exposed to a number of common household and laboratory chemicals such as festival color, azodyes, laboratory dyes and laboratory chemicals, detergent, etc. for assessment of toxicity. Heavy metal salts were used as positive controls since these are well known for their toxic effects and some investigators have used metals for comparing different *in-vitro* cytotoxicity assays[15]. Although many researchers have tested toxicities of various molecules, perhaps this an only attempt at studying a wide range of chemicals encountered in day-to-life. However, due to limitations of scope of this work, a very large number of samples could not be included. Since the assay utilizes metabolically active primary cells taken in their "natural" state and at sufficient cell density, the cells need not be "grown" in an artificial environment eliminating the need for any high-cost inventory, chemicals, media and technical skills. With appropriate modifications, the assay might provide a versatile system for high throughput screening.

AIMS AND OBJECTIVES

The present work aims to provide an alternative toxicity assay to circumvent the problems encountered in the use of established cell-lines. The need for an alternative assay stems from the fact that currently used *in-vitro* tests are too cumbersome, costly and time and skill intensive. Thus, the main objective of the work was to develop a rapid, easy, cost-effective and ethically acceptable assay for *in-vitro* cytotoxicity testing without the need to "grow" cells in an artificial environment. The research work was also aimed at creating a sense of awareness and interest among the small-time chemical manufacturers to take initiative in "on-site toxicity testing" to provide safe chemicals to the society.

MATERIAL AND METHODS

Assay system

(i) Primary explant tissue: Fresh liver tissue (about 5g) was procured from a local chicken/mutton shop in sterile screw capped glass container and transported to the laboratory under cold conditions. The tissue was frozen at -20^0C for a maximum period of 48 hr prior to any experimentation. Metabolically active primary cells were obtained by thawing the tissue and then dissecting out and macerating the innermost 1mm3 piece of the tissue using sterilized dissecting tools. Filter sterilized plain DMEM medium (4 ml) was used during maceration to keep the cells moist and in an isotonic environment. The OD_{576} was adjusted to a desired level (0.5 OD units) by dilution of the cell suspension with plain DMEM if required. The suspension (100 µl) was added to 1ml plain DMEM in the wells of a 24-well cell culture plate.

Determination of viability of liver tissue: Frozen liver tissue was thawed at different time intervals from 24hr to 96hr, macerated and tested for cell viability using the Trypan Blue assay as described later. The percent viability was calculated with reference to viability count at zero hour (fresh tissue).

Test compounds : A range of different types of test compounds that are routinely encountered either in the laboratory or household setting were used in the assay. The compounds included azo-dyes such as malachite green, acid black, orange[2] and golden yellow, hair dye (Godrej natural black), detergent powder (Rin Shakti), lime (from a local market), festival color and shendur color (from a local market), and laboratory chemicals such as acrylamide, acridine orange

and ethidium bromide (Hi Media Laboratories, Mumbai). Salts of heavy metals such as mercury, lead and cadmium were used as positive controls for toxicity. Stock solutions (1mg/ml) of the test compounds and heavy metal controls were prepared in double distilled water. These were suitably diluted directly in the assay system to get the desired working concentrations.

Standardization of parameters for cytotoxicity assay: Different parameters were tested individually by varying each parameter and keeping others constant. All experiments were performed in triplicates with appropriate controls. The significance of the results, i.e. the difference in cell viability in case of control and test compounds was evaluated for significance by using the statistical student t-test.

(i) Initial cell density: Macerated liver cells at different starting cell densities (OD_{576}= 0.1 to 1) were observed under inverted microscope. The OD of cell suspension was adjusted on the basis of the ability to count cells and differentiate the viable and non-viable cells. Cells at a suitable cell density (100µl) were then added to 1ml DMEM in each well of the 24-well culture plate.

(ii) Period of exposure of cells to the test compounds: Cells at suitable density in the 24-well plates were exposed to a fixed concentration (50 µg/ml) of the test compounds for varying periods of time such as 1 hr, 2hr and 3 hr. Wells without addition of test compound served as negative controls. The plates were incubated in a CO_2 incubator set at 5% CO_2 and 37^0C temperature. Humidity was effected by placing a sterile distilled water tray in the incubator. After the exposure times were over, viable cell count was determined by Trypan Blue dye exclusion assay. Briefly, equal volumes of cell suspension and Trypan Blue solution (0.02% in PBS) were mixed and incubated for 10 min in an Eppendorf tube at room temperature. Unstained viable cells were counted under high power objective of an inverted microscope using an improved Neubauer's chamber (WBC squares). The experiment was carried out in triplicates. Cell viability was expressed as percent viability, i.e. number of viable cells per 100 cells. Any toxicity of the test compounds was interpreted on the basis of reduction in cell viability which was more than three standard deviations.

(iii) Concentration of test compounds: Test compounds were added to cell suspensions at varying concentrations such as 2, 5, 10 and 50 µg/ml and the interaction of the compound with the cells was allowed to occur for 2 hr in a CO_2 incubator. Other experimental parameters were maintained as described in the above experiment.

(iv) Effect of temperature on toxicity of test compounds: Cells exposed to test compounds were incubated at different temperatures (4^0C, 15^0C, 27^0C and 37^0C) in order to determine whether temperature effects may alter the results of toxicity testing. Each test was carried out in triplicate and appropriate controls were kept.

(v) Determination of Lethal Concentration for 50% cell death (LC_{50} values): The plot of concentration of test compounds versus cell viability obtained in the above experiment was used for computation of the LC_{50} values. The LC_{50} value was used as an index for the degree of toxicity of the test compound and expressed in terms of concentration of test compound that resulted in 50% reduction in the cell viability.

RESULTS AND DISCUSSION

Determination of viability of liver tissue: Prior to any experimentation on the primary explants tissue, it was necessary to determine whether the cells from the tissue remain viable until the duration of the experiment. Cell death resulting from the exposure to test compounds and that occurring naturally should be clearly differentiated to prevent any serious error in the experimental results. It is evident from **Table 1** that the percent viability of liver cells was not drastically affected when the tissue was stored at -20^0C for up to 3 days. The loss in viability during this period was to the extent of only 5%. In most situations, it may be assumed that the explant tissue/s will be consumed in 1-2 days. Since the tissue samples may be available easily, it might not be necessary to store it beyond 3 days. Moreover, it is important to highlight that a small $1mm^3$ piece of tissue may be used for carrying analysis of more than 30

samples taken in duplicates. It should therefore be possible to carry out hundreds of analyses with few gram tissue. This is perhaps the most distinctive advantage of using a fresh explant tissue for toxicological studies. On the other hand, use of cell lines involves extensive work in maintenance and growth of cells prior to actual toxicity studies. These activities are often time consuming as well as resource intensive.

Table 1 : Viability of frozen liver tissue as a function of time (days) tested by Trypan Blue assay. Liver tissue was thawed each subsequent day and cell viability under ambient conditions was determined at hourly intervals

Number of days frozen	Time after thawing(hr)	Viability (%)	Mean viability (%)
Day 1	0	88.83	85.03
	1	86.9	
	3	82.92	
	6	81.46	
Day 2	0	83.21	82.18
	1	83.76	
	3	81.53	
	6	80.23	
Day 3	0	81.67	80.15
	1	80.14	
	3	79.52	
	6	79.25	

Standardization of parameters for cytotoxicity assay:

(i) Initial cell density: Cells suspensions obtained by maceration of tissues usually have a very high cell density. High density could have few important implications in toxicity studies, viz. inability of the cells to be observed under the microscope due to overcrowding, non-uniform interaction with the test compound leading to inconsistent results and masking effects that may alter cellular response to the test compounds. In view of these factors, it was decided to determine the cell density that is neither too high nor too low and that will permit better analysis of cell viability by the dye exclusion assay. It was found that the OD of 0.5 units permitted proper observations to be made, hence cell density was fixed as OD576=0.5.

(ii) Period of exposure of cells to the test compounds: The period of exposure of cells to the test compounds could be regarded as the most important parameter, since it dictates the duration of the cytotoxicity assay and hence its acceptability. For standardization purposes, a concentration of 50 µg/ml of the test compounds was arbitrarily fixed in order to determine the period of exposure. It was observed that some test compounds such as the heavy metals caused a significant reduction in cells numbers in a short time (less than 1 hr); whereas, for other compounds, even a long duration of more than 3 hr was ineffective in reducing the cell population (data not shown). It was found that exposure of 2hr was sufficient to reveal toxicity of most compounds and there was no significant change in the 2 hr and 3 hr values. However, the difference between the 1 hr and 2 hr viabilities was quite impressive, indicating that 2 hr exposure was necessary to reveal the toxicity. In the case of heavy metals, since there was almost complete loss of cells at 2 hr exposure, it was thought worthwhile to reduce the concentration of test compounds and extend the duration of the exposure. Hence, the next experiment on

standardization of dose of compounds was necessary. The use of lower concentration of test compounds may be advantageous, especially for some high-cost chemicals or drugs. The data in **Table 2** shows that 2 hr exposure duration could provide reliable data about toxicity of the various test compounds.

(iii) Concentration of test compounds: It was important to select any one or two levels of concentration of the test compounds in order to minimize the experimental setup. When a concentration range of 2-50 µg/ml was studied, it was found that the concentration of 10 µg/ml could give the most reliable results **(Table 2)**. Although at 50 µg/ml levels, the test substances showed the highest toxicity effects, the difference in the cell viability at 10 and 50 µg/ml levels was not very significant. The use of 10 µg/ml concentration of the compounds would however, significantly reduce the amount of chemicals used for testing purposes. Compounds such as Malachite green and acid black which did not show significant toxicity in 1 hr exposure did show the toxic effect in 2 hr when exposed to concentration of 10 µg/ml. Moreover, compounds that did not show toxicity at 10µg/ml level were not significantly toxic even at levels of 50µg/ml. Hence for preliminary screening of test compounds, a 10 µg/ml concentration of compounds (or drugs molecules) and a 2 hr exposure may be considered optimal for toxicity testing. Under these conditions it may be possible to calculate the EC_{50} values for classifying the compounds in grades of toxicity.

(iv) Effect of temperature on toxicity of test compounds: Temperature is a well-known factor that affects many biological processes. In the present studies, it was hypothesized that

Table 2 : Effect of duration of exposure and concentration of test compounds on the viability of liver cells.

Test compound and conc. (µg/ml)		% viability		Test compound and conc. (µg/ml)		% viability	
		1 hr	2 hr			1 hr	2 hr
Mercuric chloride	2	56.9	1.95	Acridine orange	2	81	79.44
	5	40	ND		5	80.4	79.88
	10	31.37	ND		10	78.2	77.5
	50	7.84	ND		50	77.4	74
Lead acetate	2	72.91	1.47	Lime	2	77.4	76.92
	5	41.17	ND		5	76	76.51
	10	5.55	ND		10	77.9	76.21
	50	0.00	ND		50	77.1	75.97
Malachite green	2	80.4	79.51	Hair dye	2	77.2	76.82
	5	79.7	78.91		5	76.7	76.02
	10	76.33	67.64		10	76.3	76.07
	50	76.1	52.28		50	75.6	75.62
Acid Black 210	2	79.08	78.65	Shendur	2	80	80.24
	5	78.08	73.33		5	80.6	80.12
	10	76.8	40.15		10	78.1	79.87
	50	75.3	30		50	80.3	79.16
Orange 2	2	80	79.44	Detergent	2	79.11	78.43
	5	79.59	77.3		5	78.91	78.34
	10	78.16	77.16		10	78.52	77.63
	50	76.43	74.44		50	78.48	77.33
Festival color	2	81.6	80.2	Control	00	86.5	83.00
	5	81.04	80.53				
	10	80.9	80.26				
	50	80.9	80.12				

different temperatures might alter the toxicity levels of different compounds. A higher temperature, it was thought, would enhance sensitivity of the assay by inducing more cell death in presence of a toxic compound. However, contrary to the belief, it was observed that cells exposed to test compounds at different temperatures did not show significant differences in the toxicity levels. The maximum difference that was observed (7%) was in the case of Acid Black stain where cells exposed to the chemical at 37^0C showed a lower viability (64%) compared to cells exposed at 4^0C (71%). Moreover, the difference between cell toxicity at room temperature (27^0C) and 4^0C was only about 4% (data not shown). These results suggest that it might not be necessary to invest more on the maintenance of temperature in the *in-vitro* toxicity tests, and reflects yet another advantage of the system.

(iv) Determination of EC50 values: When the data in **Table 2** was plotted graphically with concentration of test compounds on X-axis and cell viability on Y-axis, the results could be extrapolated to find out dose of compounds required to reduce viability of cells to 50% **(Fig. 1a** and **Fig. 1b)**. This EC50 value could serve as an index for classifying the test compounds as being toxic or non-toxic. Thus, from these data, LC50 values for mercury chloride and lead acetate were below 2µg/ml and these were classified as being highly toxic. The compounds showing LC50 values in the range of 2-10 µg/ml, viz. could be regarded as being toxic. However, none of the compounds tested in the present investiga-

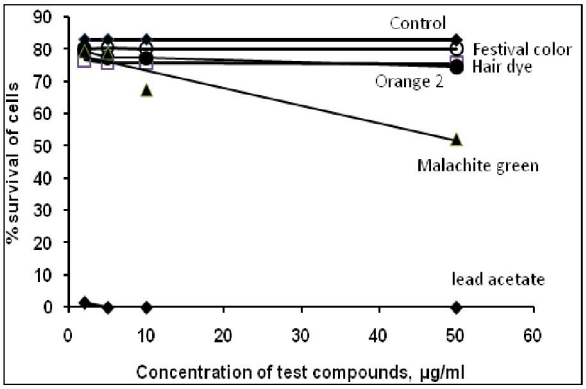

Fig. 1a : Plot of concentration of test compounds versus cell survival for determination of LC_{50} values of festival color, hair dye, orange 2, malachite green and lead acetate

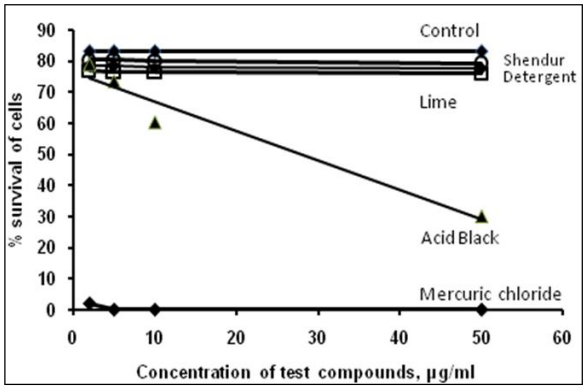

Fig. 1b. Plot of concentration of test compounds versus cell survival for determination of LC_{50} values of shendur, detergent powder, lime, acid black and mercuric chloride

tions fell in this class. The compounds such as malachite green and acid black with LC50 values between 10-50 µg/ml were classified as low toxic; while the test compounds orange 2, acridine orange, hair dye, shendur, detergent powder and festival color with LC50 values above 50 µg/ml were regarded as non-toxic.

CONCLUSION

Traditional methods of toxicity testing employ 24-48 hr assays that include growth of cell lines *in-vitro* and the acute toxicity tests *in-vivo*. The present 2 hr assay makes use of natural tissue containing a relatively homogenous cell population that yields a cell suspension of a sufficient cell density. The requirements for aseptic techniques and sterile labware are completely eliminated and hence, no special skills are required for conducting the assay. The assay may find many applications where high throughput analyses is required, viz. evaluation of growth factors/nutrients, discovering novel antibiotics and anti-cancer drugs, evaluating toxic effects of environmental pollutants, etc. The uniqueness of this assay lies in the fact that it

may also be used by the consumers of various chemicals either in the household or work settings.

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