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CHAPTER 7 IN VITRO CYTOTOXICITY STUDIES

7.1 Effect of formulations on viability of nasal and neuronal cells

7.1.1 Introduction

While cytotoxicity testing is currently conducted using animals, studies published in recent years have shown a correlation between in vivo and in vitro acute toxicity. These studies suggest that in vitro methods may be helpful in predicting in vivo cytotoxicity. The use of cell culture in vitro as alternative to predict acute lethality in vivo has been under study for almost 50 years (1).Numerous demonstration of correlations between cytotoxicity in vitro and animal lethality *in vivo* exist (2, 3). Recently, several major international in vitro initiatives have been directed towards reducing the use of laboratory animals for acute toxicity testing (4, 5).

It is strongly suggested, however, that any proposed *in vitro* protocols incorporate the following conditions:

(a) Use a cell line that divides rapidly with doubling times of less than 30 hours under standard culture conditions, preferably with normal serum type e.g. calf serum , new born calf serum or serum free medium.

(b) Use only cells in the exponential phase of growth. Never use cells immediately after thawing them from frozen stock. Allow cell to grow one to two passages before they are used in cytotoxicity test.

(c) The chemical exposure period should be at least the duration of one cell cycle i.e. 24 to 72 h

(d) Initial seeding should be done at a density that allows rapid growth throughout the exposure period.

(e) Use appropriate positive and vehicle control materials for which cytotoxicity or lack of cytotoxicity has been well characterized by the performing laboratory experiments.

(f) Use solvents only at levels previously shown not to cause cytotoxicity to the cell system over the entire period of the assay.

(g) Use a measurement end point that is well established and has inter-laboratory reproducibility. Preference is given to end points that determine either cell proliferation or cell viability (e.g., NRU, MTT, XTT).Simple end points such as total protein content are not recommended, as they may under predict the toxicity of certain test chemical by staining dead cells.

(h) The protocol should be compatible with 24 well plates and apparatus such as spectrophotometer that allow a quick and precise measurement of the end point.

(i) Complete a detailed concentration response experiment using a progression factor that yields graded effect between no effect and total cytotoxicity. Any desired toxicity study measure can be derived from a well-designed concentration response experiments. Experiments that seek to detect only a marker concentration such as the highest tolerated dose or a lowest cytotoxicity dose are characterized by lack of information and a low level of accuracy.

Nasal absorption of drug is often studied using excised mucosal tissue. In order to avoid the limits of animal study scientist have developed cellular in-vitro models. As such there are many nasal cell available but, the selection of nasal cell line is selected based on the population doubling time (PDT) required for cell. Human nasal epithelial cell line i.e. RPMI 2650 was selected as *in- vitro* cell culture for study. Also studies were carried out on SH-SY5Y cell line estimate the neuroprotective effect of drug.

7.1.1.1 RPMI-2650 Cell line

Cell type: Human nasal septum squamous cell carcinoma

Origin: From the pleural effusion of a 52-year-old man with anaplastic squamous cell carcinoma of the nasal septum in 1962

Morphology: Adherent, epitheloid, very small cells growing in clusters

Medium: 90% MEM (with Earle's salts) + 10% FBS + 1x non-essential amino acids

Subculture: Split confluent culture 1:4 to 1:8 every 4-5 days using trypsin/EDTA; seed out at about 0.5-1.0 x 10^6 cells/25 cm^2

Incubation: At 37 °C with 5% CO_2

Doubling time: about 40-50 hours

Harvest: Cell harvest of about 0.6 x 10^6 cells/cm^2

Storage: Frozen with 70% medium, 20% FBS, 10% DMSO at about 2-4 x 10^6cells/ampoule

7.1.1.2 SH-SY5Y Cell line

Cell type: Human bone marrow neuroblastoma cells

Origin: From the bone marrow of JL Biedler in 1970.

Morphology: Epithelial cells having mixed characteristics of adherent as well as suspension type cells.

Medium: 90% MEM Eagle (with Earle's salts) + 10% FBS + 1X non-essential amino acids

Subculture: Split confluent culture 1:20 to 1:50 every 4-7 days using Trypsin/EDTA

Incubation: At 37 °C with 5% CO_2

Doubling time: About 48 hours

Storage: Frozen with Complete growth medium supplemented with 5% (v/v) DMSO at about 2-4 x 10^6cells/ampoule

7.1.1.3 MTT Assay

Traditionally cell growth and cell counting culture was done by counting stained viable cells. One of the approaches to count cells is Trypan blue staining which stains the cell membrane (and thus assume cell proliferation or death) but this method is not sensitive and cannot be adopted for high through put screening. Another approach is to use radioactive substances, but such method cannot be used because of its time consuming nature and use of radioactive substances.

Enzyme reductase present in the mitochondria of the live cell reduce MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a Tetrazolium) to purple Formazan (**Figure 7.1**). The absorbance of purple colored cell suspension is quantified spectrophotometrically. The absorption maximum of the cell suspension depends upon the solvent employed. The crystals are usually dissolved in DMSO and the resultant purple color is measured at a wavelength of 570 nm by ELISA plate reader. Reduction take place only in live cells and thus the relation can be related to the number of viable (living) cells. So, when a comparison is carried out between the absorbance of untreated control cells and treated cells, the toxicity of formulation can be deduced, through the production of a dose-response curve.

The method is associated with limitations such as: (1) the physiological state of cells and (2) efficacy of mitochondrial dehydrogenase to reduce MTT in different cell types. This method is mainly helpful when cultures are prepared in multiwell plates. For best results, cell counting should be carried out during log growth stage. For comparison a blank well containing culture medium without cells must be kept. MTT must be stored at 2-8°C. Despite these, ease of method allows for the better preparation of the

Preparation of MTT: Solubility of MTT in water is (10 mg/ml), in ethanol (20 mg/ml) and is also soluble in buffered salt solutions and culture media (5 mg/ml).At -20^{o}C reconstituted MTT solution is stable for at least 6 months and at 4°C if it is kept for more than four days it will lead to decomposition and will give erroneous results.

MTT Solution: The MTT solution (5 mg/ml) is prepared in phosphate buffer saline (pH 7.4). Solution is filtered through 0.22µm sterile filter after adding MTT.

MTT Solvent: 4 mM HCl, 0.1% Nondet P-40 (NP40) all in isopropanol.

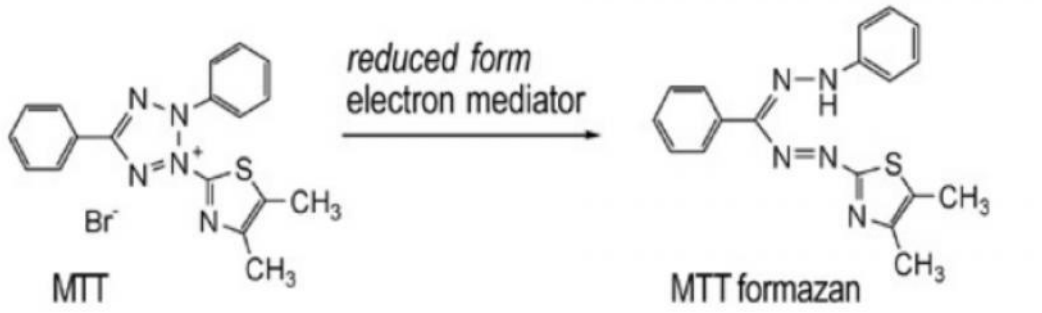

Figure 7.1 Reduction of MTT using mitochondrial reductase

Table 7.1: Material and Equipment list

Sr. No	Material/chemicals	Source
1.	Water (Triple distilled)	Prepared in laboratory by distillation
2.	RPMI-2650 cell line	NCCS, Pune, India
3.	SY SY5Y cell line	NCCS, Pune, India
4.	Dulbecco's modified F-12Eagle's medium	Himedia, Mumbai, India
5.	Minimum Essential Medium Eagle (MEM)	Himedia, Mumbai, India
6.	N-[2-hydroxyethyl] piperazine- N'-[2-ethanesulphonic acid] (HEPES)	Himedia, Mumbai, India
7.	Hank's balanced salt solution	Himedia, Mumbai, India
8.	Dimethyl Sulfoxide(DMSO)	Merck, Mumbai, India
9.	Triton X-100	S.D.Fine chemicals, Mumbai, India
10.	Sodium hydroxide (NaOH)	S.D.Fine chemicals, Mumbai, India
11.	MTT	Himedia, Mumbai, India
12.	Millipore Polycarbonate membrane	Millipore, Whatman, USA.
	Equipment	**Source/Make**
13.	Calibrated micropipettes of 10 µl, 100 µl, 200 µl, 1.0 ml, 5.0 ml and 10.0 ml, volumetric flasks of 10 ml, 25 ml, 50 ml and 100 ml capacity, beakers (250 ml), micro tips	Tarsons Ltd., Mumbai
14.	Tissue culture flasks (T 75, T25), 96-well plates, 24-well plates, 35mm PD serological pipettes 1.0 ml, 5.0 ml and 10.0 ml	Tarsons Ltd., Mumbai
15.	Analytical balance	AX 120, EL 8300, Shimadzu Corp., Japan
16.	pH meter	Pico+ Labindia, Mumbai, India
17.	Media Bottles 250ml, 500ml, 1000ml	Durga glass wares Ltd, Baroda
18.	Microtitre plate reader	ELISA reader

7.1.2 Cell line subculture

7.1.2.1 Protocol for RPMI 2650 cell line subculture

Volumes used in this protocol were for 75 cm^2 flasks.

1. Culture medium from the grown cell was removed and discarded.

2. Cells were rinsed with 0.25% w/v Trypsin-0.53mM EDTA solution to remove all traces of serum, which contains trypsin inhibitor.

3. 2.0 to 3.0 mL of Trypsin-EDTA solution was added to flask. Cells were observed under an inverted microscope until cell layer was dispersed (usually within 5 to 15

minutes). **Note:** To avoid clumping, agitation was avoided which could be the case if flast is hit or shook while waiting for the cells to detach.

4. 6.0 to 8.0 mL of complete growth medium was added and cells were aspirated by gently pipetting. Appropriate aliquots of the cell suspension was added to new culture vessels and incubated at 37°C.

Sub-cultivation Ratio: Recommended sub-cultivation ratio of 1:2 to 1:4 was used for sub-culturing.

Medium Renewal: Medium was renewed in each flask 2 to 3 times per week

7.1.2.2 Protocol for SH SY5Y cell line sub-culture (6, 7)

a. These cells form a floating cluster of neuroblasts, but mostly these cells grow as adherent cells under the conditions described in this protocol.
b. Sub-culturing was carried out at 60-80% confluency; before trypsinization, complete growth media was removed and cells were washed with 1X PBS twice.
c. The cells were incubated with 3 ml of 0.05% Trypsin-EDTA in culture flask) for 5 minutes at 37°C.
d. The added Trypsin was inactivated by adding 500 ul of FBS, and the cells were triturated using sterile tips and micro pipette.
e. The cells were centrifuged in sterile falcon tubes at 1000 rpm for 5 min and washed twice with 10ml 1X PBS each time.
f. A sample of cells was stained with Trypan blue and counting was done using Neuber slide.
g. The cells were seeded ($1x10^6$ cells/mL) in fresh culture media and incubated at 37°C in 5% CO_2 incubator.
h. After 3-4 days, the cells become 60-80% confluent and were used for MTT assay and cell uptake study.
i. A stock solution of viable cells was preserved in 10% DMSO containing growth medium at -70°C.

7.1.2.3 Protocol for MTT assay

This study was aimed to determine the effect of the drug solution, blank micelle (Poloxamer 188/TPGS/mPEG-DSPE), drug loaded micelle (Nicergoline / Poloxamer F127/TPGS/mPEG-

DSPE) and drug loaded peptide conjugated micelle (Nicergoline / Poloxamer F 127/TPGS/mPEG-DSPE/Peptide-PEG-DSPE) on cell viability. Initially, the cells from tissue culture flask were detached using Trypsin-EDTA solution and counted by Neubauers chamber using Trypan blue as an indicator. The stock solution of both RPMI 2650 cells and SH-SY5Y cells was suitably diluted such that it yielded desired number of cells in 100 µl. The obtained RPMI 2650 cells dispersion was seeded at a density $5x10^3$ cells per well into 96-well culture plates in *Eagle's Minimum Essential Medium* (MEM) supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml penicillin and streptomycin and then incubated for two days at 37°C in humidified atmosphere containing 5% CO_2.

In case of SH SY5Y, cells were seeded into 96-well culture plates at a density 3 x 10^4 cells per well in Dulbecco's modified F-12 Eagle's medium (DMEM/Nutrient Mixture F-12 HAM with 2.5mM L-Glutamine, 15mM HEPES buffer, 0.5mM Sodium pyruvate, 1.2g/l Sodium Bicarbonate) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and then incubated for two days at 37°C in humidified atmosphere containing 5% CO_2.

After incubation cells were treated with different formulations like drug solution, blank micelle, drug loaded micelle and conjugated micelle suspended in Hank's balanced salt solution (HBSS) buffered with 30 mM N-[2-hydroxyethyl] piperazine- N'-[2-ethanesulphonic acid] (HEPES), adjusted with 0.1 M NaOH up to pH 7.2 having Nicergoline concentration equivalent to 0.2, 0.4, 0.6, 0.8 µM.

After 24 hr incubation with formulations, 20 µl of MTT solution was added to each well and allowed to incubate for 4 hr. Subsequently, MTT solution was removed and cells were washed three times with PBS. Afterwards, DMSO was added to solubilize the formazan crystals formed by reaction with MTT and obtain purple color. This was determined spectrophotometrically using ELISA plate reader at 570 nm with a reference filter of 620 nm. In this study, 0.1% Triton X-100 was used as positive control while HBSS-HEPES media was used as negative control.

Calculation of cell viability was done using following equation:

$$\text{Cell viability (\%)} = \frac{A570 \text{ of treated cells}}{A570 \text{ of control cells}} \times 100.$$

A graph of % cell viability vs. formulation concentration was plotted and data were analyzed statistically using one-way analysis of variance (ANOVA).

7.1.3 Results and discussion

MTT assay was used to study the cytotoxicity of formulation. The cytotoxicity study was done for drug solution, blank micelle, drug loaded micelle and peptide conjugated micelle on the differentiated RPMI 2650cells (human nasal septum squamous cell carcinoma) as well as SH-SY5Y cells. The results of cytotoxicity study of different formulations including drug solution, blank micelle, drug loaded micelle and peptide conjugated micelle on the RPMI 2650 nasal epithelial cells (human nasal septum squamous cell carcinoma) is depicted in **Table 7.2 and Figure 7.2.**

Table 7.2 Relative cell viability of different formulations in RPMI 2650 cell line

Concentration in µM	**% Cell viability (RPMI 2650) ± SD (n=3)**					
	Triton-X 100 (100 µl /2 ml)	**HBSS-HEPES**	**Drug Solution (DS)**	**Blank micelle (BM)**	**Drug loaded micelle (DM)**	**Peptide Conjugated Micelle (PCM)**
0.2	9.61± 0.11	98.24 ± 3.3	98.48 ± 2.6	92.45 ± 3.6	95.51 ± 4.1	96.93 ± 5.4
0.4			97.85 ± 1.3	90.85 ± 2.8	93.81 ± 4.3	94.04 ± 1.9
0.6			97.45 ± 3.0	88.14 ± 2.1	91.27 ± 3.9	92.65 ± 3.5
0.8			97.14 ± 1.6	83.88 ± 5.3	90.72 ± 5.3	91.84 ± 2.7

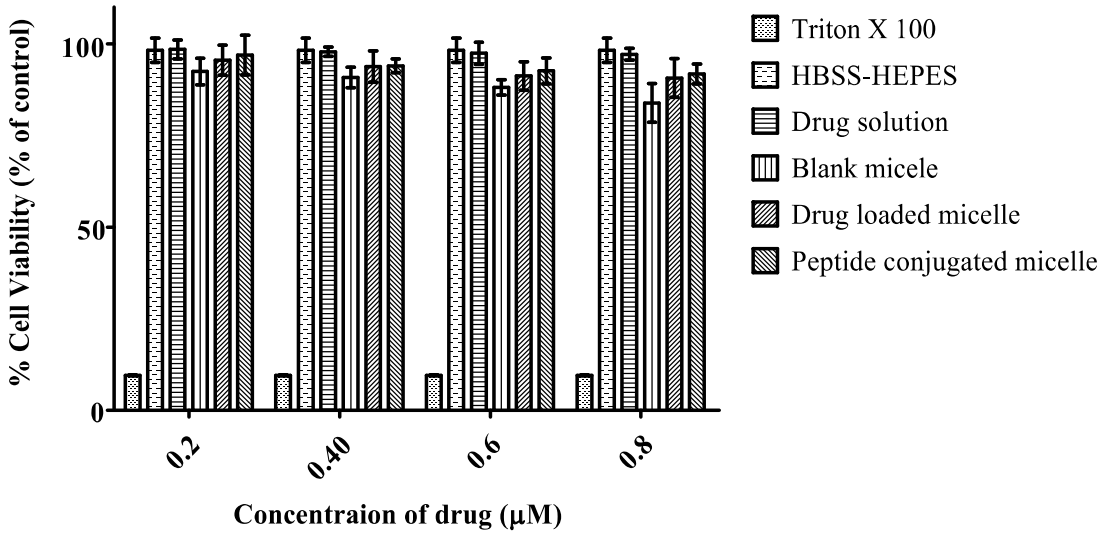

Figure 7.2 Effect of different formulations on cell viability of RPMI 2650 cell line

The results of cytotoxicity study of different formulations on the SHSY5Y cells are depicted in **Table 7.3** and **Figure 7.3.**

Table 7.3: Relative Cell viability of different formulations in SHSY5Y cell line

Concentration in µM	**% Cell viability (SHSY5Y)** ± SD (n=3)					
	Triton-X 100 (100 µl /2 ml)	**HBSS-HEPES**	**Drug Solution (DS)**	**Blank micelle (BM)**	**Drug loaded micelle (DM)**	**Peptide Conjugated Micelle (PCM)**
0.2	10.13 ± 1.22	98.93 ± 4.9	98.57 ± 3.6	96.45 ±3.9	97.84 ± 2.5	98.27 ± 3.6
0.4			97.34 ± 2.9	93.85 ± 4.2	96.30 ± 2.7	96.84 ± 2.8
0.6			97.37 ± 3.8	90.14 ± 3.7	94.77 ± 3.7	95.35 ± 3.1
0.8			97.10 ± 2.5	84.56 ± 4.1	92.92 ± 2.7	93.84 ± 4.4

Concentration of triton X 100 used was 100 µL/2 mL and that of blank micelle was the blank micelle dose required for the administration of the equivalent amount of drug delivered by the drug loaded micelle. As demonstrated from results, it was clear that triton X-100, when allowed to incubate with RPMI 2650 cells, resulted in maximum cell cytotoxicity and resultant % cell viability obtained using triton X-100 was 9.61 ± 0.11 % at concentration of 50 µl/ml. Moreover, HBSS-HEPES buffer when used as negative control with RPMI 2650

cells resulted in negligible cytotoxicity of cells i.e. % cell viability 98.24 ± 3.3 %. The % cell viability of RPMI 2650 cells after incubation with different formulations like drug solution, blank micelles, drug loaded micelles and peptide conjugated micelles at maximum concentration i.e. 0.8 µM was about 97.14 ± 1.6, 83.88 ± 5.3, 90.72 ± 5.3, 91.84 ± 2.7 respectively.

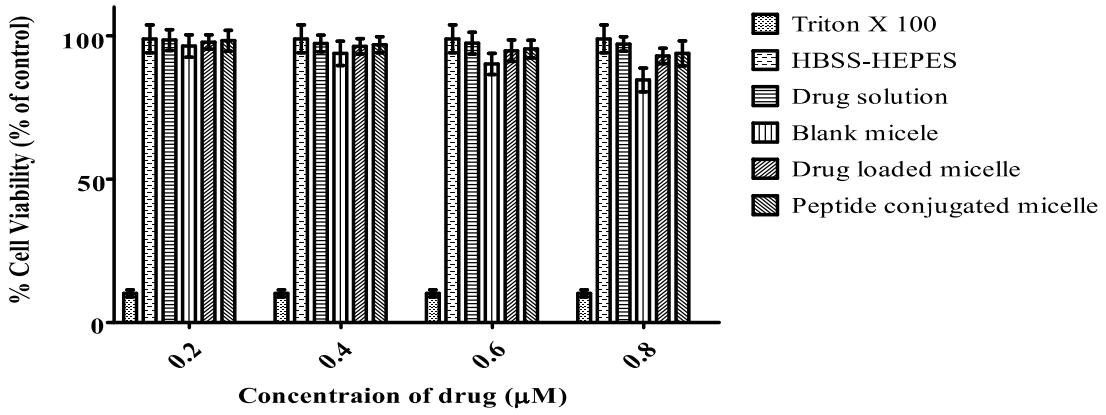

Figure 7.3 Effect of different formulations on cell viability of SHSY5Y cell line.

Concentration of triton X 100 used was 100 µL/2 mL and that of blank micelle was the blank micelle dose required for the administration of the equivalent amount of drug delivered by the drug loaded micelle. Additionally, results obtained from SHSY5Y cell line demonstrated that triton X-100, when used as positive control gave maximum cell inhibition giving % cell viability of about 10.13 ± 1.22 %. Moreover, HBSS-HEPES buffer when used as negative control with SHSY5Y cells resulted in % cell viability of approximately 98.93 ± 4.9%. The % cell viability of SHSY5Y cells after incubation with different formulations like drug solution, blank micelles, drug loaded micelles and peptide conjugated micelles at maximum concentration i.e. 0.8 µM was about 97.14 ± 2.5, 84.56 ± 4.1, 92.92 ± 2.7, 93.84 ± 4.4 % respectively. The data of both cell lines was evaluated statistically by one way ANOVA using Graphpad Prism (version 5.0). The results of statistical analysis showed that there was significant difference in cytotoxicity of triton X-100 and other formulations like drug solution, blank micelles, drug loaded micelles and peptide conjugated micelles ($p < 0.05$) in both cell lines. Moreover, a typical trend was observed in cytotoxicity of different formulations i.e. blank carrier showed cytotoxicity to both cells types because of presence of surfactant in formulation. Triton act as cytotoxic material by affecting the cell membrane integrity, whereas Pluronic prevent this toxicity by resealing the membrane. Pluronic

attenuated the loss of membrane integrity in vivo and in vitro suggest membrane targeting mechanism may be involved in its neuroprotective effects. It was reported that Pluronic did not show apparent cytotoxicity to cells (8). In one of the study it was also shown that the cytotoxicity of Pluronic /TPGS empty micelles was higher than that of Pluronic empty micelles (9). Hence, Pluronic plays an important role of stabilizing the micelle as well as reducing the cytotoxicity of micelle formulation. As well as TPGS has its unique apoptosis-inducing properties via the generation of reactive oxygen species (ROS) and the generated ROS could damage DNA, proteins, and fatty acids in cells, resulting in apoptotic cell death (10). Yet, marginal cytotoxicity was observed with blank formulation i.e. % cell viability of blank micelle at highest concentration (0.8 µM) was 83.88 ± 5.3 % and 84.56 ± 4.1 % respectively in RPMI 2650 and SH-SY5Y cells. After addition of drug in formulation there was reduction in cytotoxicity of formulation due to protective effect of drug (11, 12). Furthermore, peptide conjugated carriers showed higher neuroprotection than non-conjugated carriers due to their higher uptake in the cells. There was unpredicted behaviour of drug solution as it gave maximum cell viability compared to other formulations. This was attributed to absence of any surfactant or other toxic components in drug solution.

7.1.4 Conclusions

To illustrate the safety of micelle formulations and its effect on the cell viability, it was directly compared with that of Triton X 100. A substantial decrease in the cell viability was observed after incubation with Triton X 100 compared to HBSS-HEPES, blank micelle, drug solution, drug loaded micelle and peptide conjugated micelle ($p < 0.001$). *In-vitro* cytotoxicity study demonstrated that the higher concentration of blank micelle has less viable cell in RPMI 2650 cells and SH SY5Y in comparison to drug solution and HBSS-HEPES. The drug loaded micelle had higher cell viability than the blank micelle suggesting neuroprotective effect of drug and membrane sealing activity of Pluronic protecting cells. This proves safety of formulation for the nose to brain delivery.

7.2 Effect of formulations on H_2O_2 induced oxidative stress

7.2.1 Introduction

Nicergoline is used for variety of symptoms and complications of brain diseases and disorders(13). It improves the cerebral energy metabolism and rectify age related reduction of choline acetyl transferase and muscarinic cholinergic receptor activities (14). Moreover, the protective effects of Nicergoline against ischemic brain damage due to the inhibition of lipid peroxide formationare reported (15).

Decrease in oxygen supply to neuronal cell due to reduced blood flow to brain in cerebral ischemia leads to oxidative stress. The harm is considered to be interceded by reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2). The human neuroblastoma SH-SY5Y cell line is widely used as a model cell system for studying oxidative stress-induced neuronal cell death. SH-SY5Y cells can be differentiated using retinoic acid (RA) with characteristics similar to neurons (16).

H_2O_2-induced cell death involves apoptosis and necrosis in a concentration dependent manner (17), and mitochondrial membrane depolarization (18).

7.2.2 Determination of IC 50 value of H_2O_2 induced oxidative stress

Procedure:

1. Cells were plated in 96-well culture plate at densities of 6 X 10^3cells per well and maintained in Dulbecco's modified F-12 Eagle's medium at 37^{o}C under humidified atmosphere having 5% CO_2.
2. Plated cells were grown for 2 days and then treated with concentrations of H_2O_2 ranging from 0 to 10 mM (0, 10, 30, 50, 100, 200, 300 µM and 1, 5, 10 mM).
3. The plates were incubated for 24 h at 37^{o}C under humidified atmosphere having 5% CO_2.
4. After H_2O_2 challenge, cell death was determined by MTT assay as described previously.
5. The toxicity of different concentrations of hydrogen peroxide was determined and the IC-50 value of H_2O_2 was used as the control to determine the protective action of micelle.

7.2.3 Neuroprotective effect of Nicergoline micelle in H_2O_2 induced oxidative stress

Procedure:

1) 3 X 10^4 cell per well were seeded and incubated for 24 hr in CO_2 incubator.

2) After complete cell adherence, cells were treated with 0.2, 0.4, 0.6, 0.8 µmole of Nicergoline suspension, drug loaded micelles and peptide conjugated micelles for 4 h.
3) The cells were washed with PBS (pH 7.4) to remove traces of drug formulations.
4) Then each well was exposed to 40 µM of H_2O_2 for 24 hrs.
5) Cell death was assessed 24 hr after H_2O_2 treatment by using modified MTT Assay.

7.2.4 Results and discussion

Table 7.2 Hydrogen Peroxide Toxicity Study

H_2O_2 Concentration (µM)	**Log_{10} Concentration**	**% Cell Death (n=3)**	
		Mean	**SD**
5	0.70	5.39	2.66
10	1.00	11.23	3.35
20	1.30	23.13	3.13
30	1.48	39.00	7.83
40	1.60	52.76	2.67
50	1.70	60.69	3.97
60	1.78	75.40	5.76
70	1.85	88.14	3.21
90	1.95	97.74	6.98

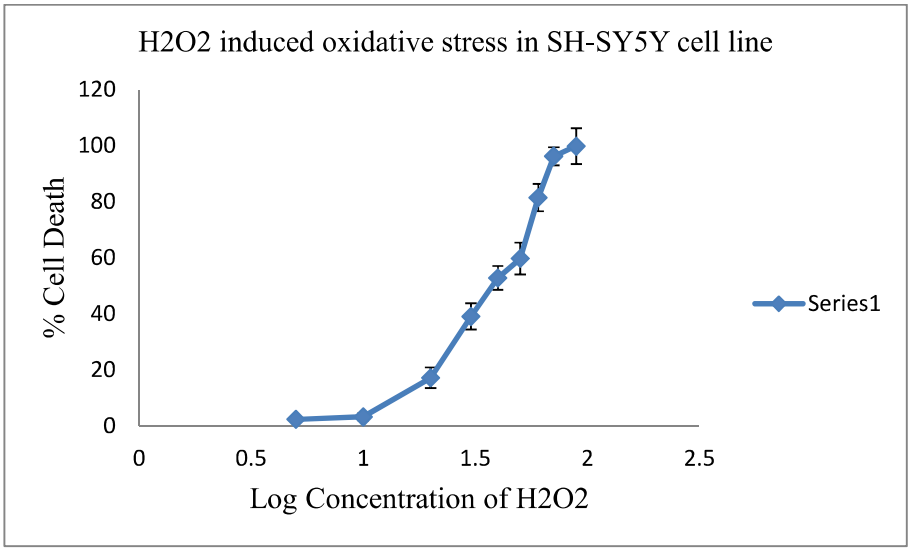

Figure 7.4 H_2O_2-induced cytotoxicity on SH SY5Y cells

Cultured cells were incubated with various amounts of H_2O_2 (5 to 90 µM) for 24 h. From results (**Table 7.4** and **Figure 7.4**) it was observed that, exposure of cells to 34.89 µM H_2O_2 for 24 h resulted in approximately 50% cell cytotoxicity in comparison to control cells.

Therefore, 40 µM concentration of H_2O_2 was chosen for incubation of SH-SY5Y cells for 24 h to induce cell death in all subsequent experiments so as to get sensitive and unbiased data.

Survival of SH-SY5Y cells exposed to H_2O_2 in the absence and presence of Nicergoline solution, Nicergoline loaded micelle and peptide conjugated Nicergoline micelle was evaluated using (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (MTT) assay. The results of neuroprotective study are depicted in **Table 7.3** and neuroprotection observed at each drug concentration levels is discussed separately in the subsequent section.

Table 7.3 Data of Neuroprotective study

Nicergoline (NCG) Concentration	% Cell viability							
	H_2O_2 40 µM Control		H_2O_2 40 µM + Drug Solution		H_2O_2 40 µM + Drug loaded micelle		H_2O_2 40 µM + Peptide conjugated drug loaded micelle	
0.2 µM			53.87	2.66	53.74	2.13	55.98	2.11
0.4 µM	50.03	3.72	54.98	1.59	64.73	2.89	68.01	5.61
0.6 µM			53.25	2.63	67.74	1.55	85.43	2.72
0.8 µM			55.84	2.88	69.83	4.71	97.12	3.01

The H_2O_2 concentration of 40 µM was selected based on the IC 50 value obtained as discussed previously. In control 50.03 % cell viability was observed after treatment with 40 µM of H_2O_2.

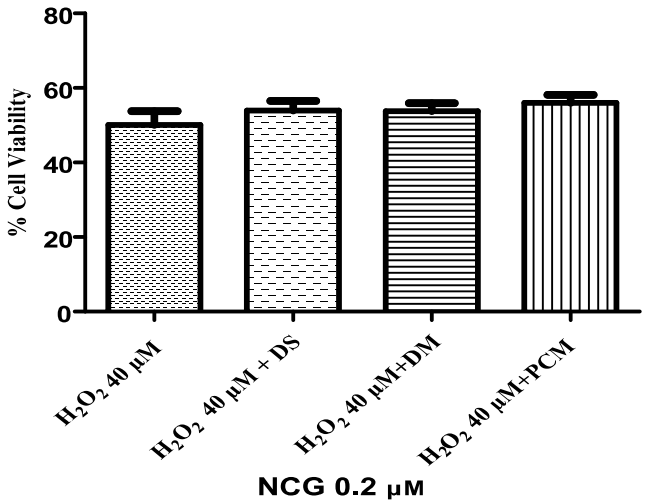

Figure 7.5 Effect of NCG 0.2 µM on cell viability

At 0.2 µM Nicergoline concentration cell viability was found to be 53.77 ± 2.66, 53.74 ± 2.13, 55.98 ± 2.11 for drug solution, drug micelle and peptide conjugated micelle,

respectively (**Figure 7.5**). After applying one way ANOVA to the data, it was found that at 0.2 µM Nicergoline concentration, no significant difference in cell viability was observed in Drug Solution, Drug loaded micelle and Peptide conjugated drug loaded micelle.

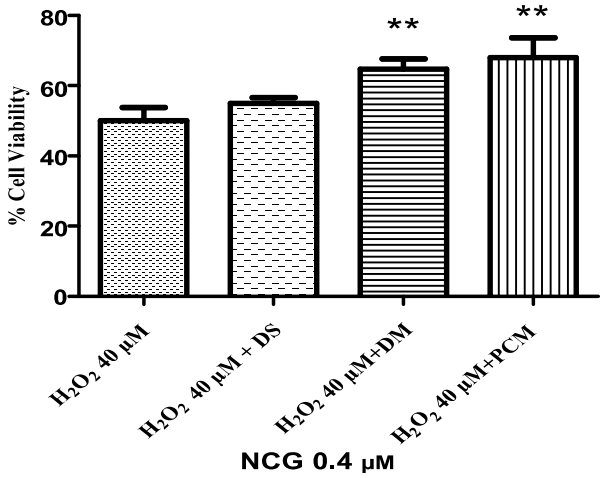

Figure 7.6 Effect of NCG 0.4 µM on cell viability

(= p<0.05 and *** = p<0.0001)**

At 0.4 µM Nicergoline concentration cell viability was found to be 54.98 ± 1.59, 64.73 ± 2.89, 68.01 ± 5.61for drug solution, drug micelle and peptide conjugated micelle respectively (**Figure 7.6**). After applying one way ANOVA to the data it was found that a significant reduction in cell death was observed in case of cell treated with drug micelle ($p < 0.05$)and peptide conjugated drug loaded micelle formulation ($p < 0.05$). No significant difference in cell viability was observed in case of Drug Solution.

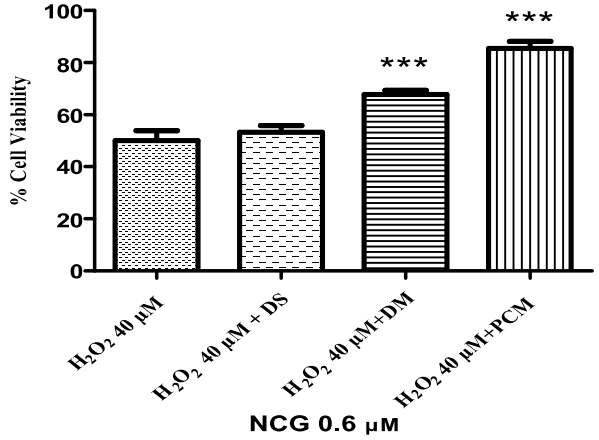

Figure 7.7 Effect of NCG 0.6 µM on cell viability

(= p<0.05 and *** = p<0.0001)** At 0.6 µM Nicergoline concentration cell viability was found to be 53.25 ± 2.63, 64.74 ± 1.55, 85.43 ± 2.72 for drug solution, drug micelle and peptide conjugated micelle respectively (**Figure 7.7**). After applying one way ANOVA to the data it was found that a significant reduction in cell death was observed in case cell treated with drug micelle ($p < 0.0001$) and peptide conjugated drug loaded micelle ($p < 0.0001$) formulation and no significant difference was found in case of drug solution.

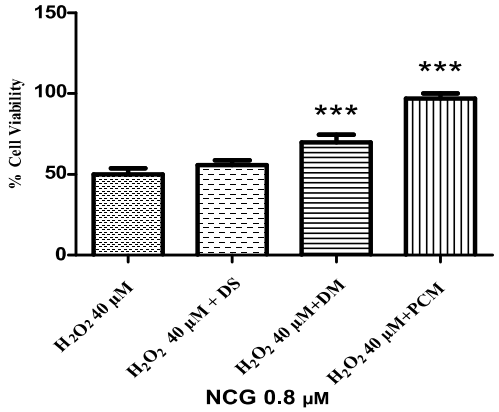

Figure 7.8 Effect of NCG 0.8 µM on cell viability

(= p<0.05 and *** = p<0.0001)**

At 0.8 µM Nicergoline concentration cell viability was found to be 55.84 ± 2.88, 69.83 ± 4.71, 97.12 ± 3.01 for drug solution, drug micelle and peptide conjugated micelle respectively (**Figure 7.8**). After applying one way ANOVA to the data it was found that a significant reduction in cell death was observed in case cell treated with drug micelle ($p < 0.0001$) and peptide conjugated drug loaded micelle ($p < 0.0001$) formulation and no significant difference was found in case of drug solution.

7.2.5 Conclusion

In this *in vitro* neuroprotective efficacy study, H_2O_2 treated SH-SY5Y cells were incubated with 0.5mM MTT at 37°C for 30 min, followed by measurement of absorbance at 450 nm. As it can be seen, on exposure to 40 µMH_2O_2 decreased cell viability to 50.03 ± 3.72.

In H_2O_2 challenge study it can be seen that pre-treatment with 0.6 and 0.8 µM nicergoline loaded micelle and peptide conjugated drug loaded micelle significantly increased cell viability compared to drug suspension. The results of the current study demonstrated that Nicergoline solution alone had no significant effect on cell survival but, Nicergoline at the

concentration of 0.4, 0.6 and 0.8 µMin the form of micelle formulation were able to counteract the deleterious effect of 40µM H_2O_2.

The positive results demonstrating neuroprotection in the abovementioned formulations may be attributed to resultant improvement of micelle permeability through SH-SY5Y cell membrane. Hence, one can conclude that the permeability of Nicergoline can be increased by incorporating it in micelle formulation.

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