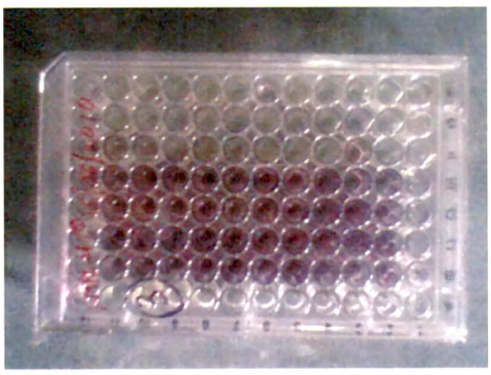
Chapter 8

In-vitro cell line Study



8.1. Introduction

There are many types of different nasal epithelial cell lines available. The criteria considered for the selection of cell lines were Population doubling time (PDT). The commercially available human nasal epithelial cell line RPMI 2650 was chosen for the *in vitro* cell culture experiments. This cell line was isolated from a squamous cell carcinoma of the nasal septum (Moore et al., 1964).

RPMI-2650 Cell line

Cell line: RPMI-2650

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 106 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 10^6 cells/ampoule

Proliferation Assay

Traditionally, the determination of cell growth is done by counting viable cells after staining with a vital dye. Several approaches have been used in the past. Trypan blue staining is a simple way to evaluate cell membrane integrity (and thus assume cell proliferation or death) but the method is not sensitive and cannot be adapted for high through put screening. Measuring the uptake of radioactive substances, usually tritium-labeled thymidine, is accurate but it is also time-consuming and involves handling of radioactive substances. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption max is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells

(Figure 8.1). When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve. Solutions of MTT solubilized in tissue culture media or balanced salt solutions, without phenol red, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance. The use of the MTT method does have limitations influenced by: (1) the physiological state of cells and (2) variance in mitochondrial dehydrogenase activity in different cell types. Nevertheless, the MTT method of cell determination is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors and other cell growth promoting reagents, cytotoxicity studies, and in the derivation of cell growth curves. The MTT method of cell determination is most useful when cultures are prepared in multiwell plates. For best results, cell numbers should be determined during log growth stage. Each test should include a blank containing complete culture medium without cells. Thiazolyl Blue Tetrazolium Bromide (MTT) Storage temperature 2-8 °C.

Preparation Instructions: MTT is soluble in water (10 mg/ml), ethanol (20 mg/ml) and is also soluble in buffered salt solutions and culture media (5 mg/ml). Reconstituted MTT solution is stable for at least 6 months when stored at -0°C. Storage at 4°C for more than four days will result in decomposition and will yield erroneous results.

MTT Solution: 5 mg/ml MTT in phosphate buffer saline (pH 7.4). Solution must be filter sterilized after adding MTT.

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

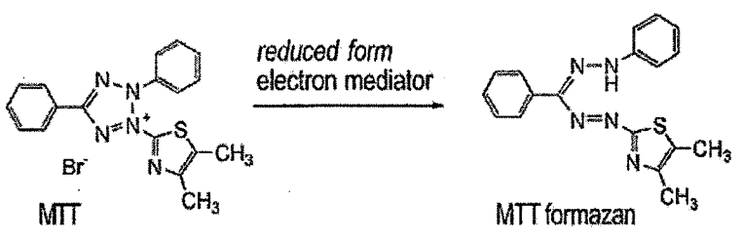


Figure 8.1: Reduction of MTT using mitochondrial reductase

Table 8.1: Materials and equipments

Material	Source
Water (Double distilled)	Prepared in laboratory by distillation
RPMI-2650 cell line	NCCS, Pune, India
Minimum Essential Medium Eagle Medium (MEM)	Himedia, Mumbai, India
N-[2-hydroxyethyl] piperazine- N'-[2-ethanesulphonic acid] (HEPES)	Himedia, Mumbai, India
Hank's balanced salt solution	Himedia, Mumbai, India
Dimethyl Sulfoxide	Merck, Mumbai, India
Triton X-100	S.D.Fine chemicals, Mumbai, India
Sodium hydroxide	S.D.Fine chemicals, Mumbai, India
MTT	Himedia, Mumbai, India
Nuclepore Polycarbonate membrane 0.2, 0.45 and 2 µm 25mm	Milipore, Whatman, USA.
Equipments	**Source/Make**
Calibrated pipettes of 1.0 ml, 5.0 ml and 10.0 ml, volumetric flasks of 10 ml, 25 ml, 50 ml and 100 ml capacity, Funnels (i.d. 5.0 cm), beakers (250 ml) and other requisite glasswares	Tarsons Ltd., Mumbai
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
Micropipette	Brand, Germany
Analytical balance	AX 120, EL 8300, Shimadzu Corp., Japan
pH meter	Pico$^+$ Labindia, Mumbai, India
Media Bottles 250ml, 500ml, 1000ml	Durga glasswares Ltd, Baroda
Microtitre plate reader	ELISA reader

8.2. Methods

8.2.1. *In-vitro* cytotoxicity Studies

The cytotoxicity of the chitosan/thiolated chitosan/trimethyl chitosan and drug loaded chitosan/thiolated chitosan/trimethyl chitosan NPs was performed on the differentiated RPMI 2650 cells (human nasal septum squamous cell carcinoma). RPMI 2650 cells were seeded at a density $5x10^3$ cells per well into 96-well culture plates in Eagle's Minimum Essential *Medium* (MEM) (The MEM was supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml penicillin and streptomycin) and then incubated for two days at 37 °C in 95% air and 5% CO_2. 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 was used as vehicle for preparation of formulations. The cells were exposing to HBSS-HEPES buffer, soluble chitosan, soluble thiolated chitosan, soluble trimethyl chitosan and drug loaded chitosan, thiolated chitosan, trimethyl chitosan NPs in HBSS-HEPES with different concentration and incubated at 37 °C for 2 hrs. Thereafter, the NPs formulations and polymers were removed and gently washed the cells with HBSS-HEPES buffer and replaced by 100 µl MEM and 20 µl of (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (MTT 5mg/ml) and cells were incubated for at 37 °C for 2 hrs. To dissolve formazane crystal the MTT solution was removed and replace with 100 µl of DMSO per well and kept in incubator for 10 minutes at 37 °C in 95% air and 5% CO_2 and afterwards, 15 minutes at 6 °C, before analysis. Finally, the absorbance was measure at 570 nm with the reference filter of 655 nm using ELISA reader. The data were statistically analyzed using one-way analysis of variance (ANOVA) (Maryam et al., 2006). Figure 8.2 shows the Cell viability study of polymers and NPs formulations and results are tabulated in Table 8.2 and Table 8.3 respectively.

8.2.2. Permeability studies of chitosan/thiolated chitosan/trimethyl chitosan NPs using RPMI 2650 cell monolayer

TEER measurement and permeation of TZ/CBZ loaded chitosan/thiolated chitosan/trimethyl chitosan NPs

TEER measurement

RPMI 2650 cells were seeded at a density $5x10^5$ cells per well into 12-well culture plates in Eagle's Minimum Essential *Medium* (MEM) supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml penicillin and streptomycin. The cells were incubating for two days at 37 °C in 95% air and 5% CO_2. Trans-epithelial electrical resistance of RPMI 2650 cells across cell monolayer was measured at 24-hour intervals until the cells form confluent monolayer using Millicell® ERS meter (Millipore, Bedford, Massachusetts, USA). Cell monolayer TEER was measure by subtracting the background resistances of blank RPMI 2650 cells. TEER readings were increase subsequently that indicates the cells were forming tight junction and forms close packing. The value of TEER at t = 0 hours time were taken as 100 % (initial values) and the TEER values at each sampling time (0, 30, 60, 150 minutes)

point were expressed as percent of initial values. After 150 minutes of incubation, the formulations were replaced by HBSS-HEPES buffer to determine the recovery of TEER to its initial value (Shuhua et al., 2008, Maryam et al., 2006, Shah et al., 2008).

Permeation studies

Before starting of permeation experiment first the culture media was removed from the both apical (AP) and basolateral (BL) sides of the cell monolayers and washed the cell monolayer with fresh Hank's balanced salt solution (HBSS) buffered with 30 mM N-[2-hydroxyethyl] piperazine- N'-[2-ethanesulphonic acid] (HEPES) (37 ± 1°C) and replace with phosphate buffer saline and kept equilibrated for 30 minutes. TEER measurement and permeation study was carried out on the AP to BL direction by adding TZ/CBZ loaded thiolated chitosan/trimethyl chitosan/chitosan NPs (equivalent to 2 mg/ml of drug) and TZ/CBZ solution (2 mg/ml) on the AP side and 2.5 ml of phosphate buffer saline (pH 7.4) on BL side. Collect 200 µL of sample from the receiver (BL) side at different time intervals (0, 30, 60, 150 minutes) and replaced with 2.5 ml of fresh (37 ± 1°C) phosphate buffer saline (pH 7.4) for maintenance of sink conditions. Collected sample were immediately analysed for the drug quantification using HPLC. Permeation studies were carried out by placing the well plates on a calibrated shaker at 50 rpm at 37°C (Shuhua et al., 2008, Maryam et al., 2006, Shah et al., 2008).

TEER value and Apparent permeability coefficient (P_{app}) of all the formulations were calculated using Equation 1 and results were tabulated in recorded in Tables 8.4 to Table 8.6.

$$P_{app} = [(dQ/dt) * (V_R/A*C_0)] \qquad \text{Equation--------I}$$

Where,

P_{app}- Apparent permeability co-efficient (cm/s)

dQ/dt- Cumulative flux in the AP to BL direction (µg/s)

V_R - Volume of the receptor compartment (cm^3)

A- Diffusion area of the monolayer (3.8 cm^2)

C_0- initial concentration applied on the AP side (µg/s)

The data were statistically analyzed using one-way analysis of variance (ANOVA) and differences between groups at were found to be $p < 0.05$.

8.3. Results and discussion

8.3.1. *In-vitro* cytotoxicity studies

The cytotoxicity assay was conducted using MTT assay and the cytotoxicity of the chitosan/thiolated chitosan/trimethyl chitosan and drug loaded chitosan/thiolated chitosan/trimethyl chitosan NPs was performing on the differentiated RPMI 2650 cells (human nasal septum squamous cell carcinoma). Influence of chitosan/thiolated chitosan/trimethyl chitosan and drug loaded chitosan/thiolated chitosan/trimethyl chitosan NPs on the RPMI 2650 nasal epithelial cells (human nasal septum squamous cell carcinoma) is shown in Figure 8.2 & Figure 8.3. The toxicity of TC NPs suspension (40 mg/ml), TMC NPs suspension (40mg/ml), soluble TC (20 mg/ml) and soluble TMC (20 mg/ml) were found to be non significant ($p < 0.05$); which may be due the covalent and reversible binding of polymer with the nasal epithelial cells. We observed a decrease in cell viability when cells were incubated with soluble TC and soluble TMC at relatively high concentration ($p < 0.001$). The Triton X 100 (50 microlitere/ml) was choose as positive control. To illustrate the safety of TC and TMC, its effect on the cell viability was directly compared with that of Triton X 100. A substantial decrease of the cell viability was observed after incubation with Triton X 100 compared to HBSS-HEPES ($p < 0.001$). Chitosan NPs suspension (10 mg/ml) and soluble chitosan (5 mg/ml) showed less cell viability compared to TC NPs suspension (40 mg/ml), TMC NPs suspension (40mg/ml), soluble TC (20 mg/ml) and soluble TMC (20 mg/ml). The possible explanation is the non-covalent and irreversible binding of polymer with the nasal epithelial cells; which leads to more cytotoxicity of chitosan and chitosan NPs compared to modified chitosan and its NPs. Soluble chitosan also shows the less cell viability compared to the chitosan suspension. The high cell viability of TC NPs and TMC NPs may be explained by increased solubility of chitosan after thiolation/trimethylation at physiological pH resulting in to quicker removal from the site of application but at the same time forming intimate contact with the mucosae due to covalent linkage. Hence, trans-mucosal drug delivery will be improved due to required retention of thiolated chitosan/trimethyl chitosan NPs on nasal mucosae but due to complete removal from the mucosae through mucociliary clearance improves safety which is otherwise problem with the chitosan and is a contributing factor to its nasal mucosa toxicity.

The differences between the cytotoxicity of TC, TMC and Triton X 100 may be due to differences in chemistry, surface charge, 3D structure of the polymer backbone,

molecular weight distribution, etc., all of which may affect the interactions of the polymers with cell membranes, and thus their toxicity (Maryam et al., 2006). Figure 8.2 and Figure 8.3 shows the cell viability study of polymers and NPs formulations and results are tabulated in Table 8.2 and Table 8.3 respectively.

Table 8.2: Cell viability of different molecular weight chitosan thiolated chitosan, trimethyl chitosan, HBSS-HEPES and Triton-X 100 (100 mictoliter/2 ml)

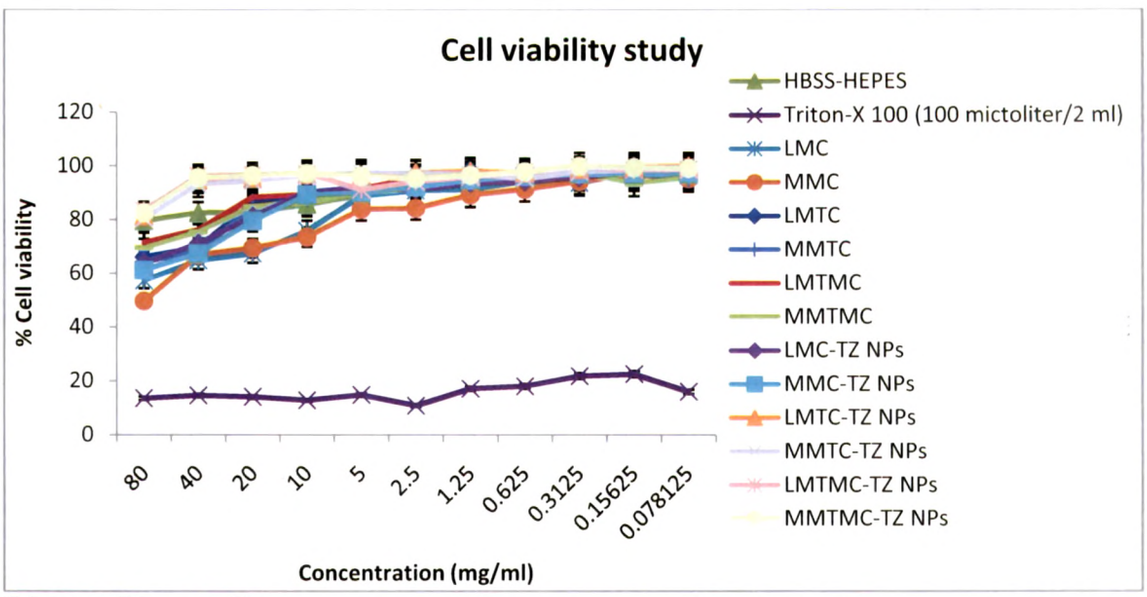
Concentration (mg/ml)	Cell Viability (%) ± SD (n=3)							
	HBSS-HEPES	Triton-X 100 (100 mictoliter/2 ml)	LMC	MMC	LMTC	MMTC	LMTMC	MMTMC
80	79.45±2.3	13.54±0.67	57.34±1.2	49.65±2.1	66.12±3.2	64.32±3.8	71.54±1.2	69.43±1.3
40	82.45±8.9	14.62±0.11	64.76±2.1	66.89±3.2	69.65±4.9	68.45±2.4	76.21±3.2	75.43±1.9
20	82.70±9.5	14.08±0.56	67.32±1.4	69.43±3.9	86.34±4.1	84.31±1.9	88.44±1.2	84.89±3.2
10	85.59±5.8	12.82±0.34	75.87±1.6	73.54±2.4	88.89±3.3	88.12±3.2	89.43±4.3	86.43±4.1
5	89.02±3.7	14.80±0.33	88.76±3.2	83.89±2.7	91.45±3.9	90.54±4.1	91.67±2.9	90.32±3.2
2.5	92.81±6.4	10.83±0.44	90.88±2.6	84.23±1.8	95.78±4.3	94.32±3.8	95.33±4.3	91.54±2.8
1.25	96.78±6.2	17.15±0.56	91.23±1.9	89.21±2.9	98.23±5.6	95.45±4.9	96.45±3.4	95.32±5.3
0.625	97.43±3.2	18.05±0.45	94.21±3.2	91.34±3.1	96.96±3.9	94.32±5.2	97.45±4.1	96.11±4.1
0,3125	96.78±5.2	21.84±0.34	93.67±3.9	94.22±3.6	97.88±5.3	96.54±4.3	97.12±3.9	97.43±3.9
0.15625	95.65±5.4	22.57±0.98	97.56±4.3	96.78±2.8	99.94±4.1	97.12±4.1	98.32±4.3	93.45±3.2
0.078125	98.40±4.3	16.03±1.1	96.34±2.4	95.23±3.4	98.78±4.3	95.34±3.9	99.12±5.4	95.45±4.7

LMC- Low molecular weight chitosan
MMC-Medium molecular weight chitosan
LMTC- Low molecular weight thiolated chitosan
MMTC-Medium molecular weight thiolated chitosan
LMTMC- Low molecular weight trimethyl chitosan
MMTMC-Medium molecular weight trimethyl chitosan

Table 8.3: Cell viability of different molecular weight chitosan/thiolated chitosan/trimethyl chitosan NPs

Concentration (mg/ml)	Cell Viability (%) ± SD (n=3)											
	LMC-TZ NPs	MMC-TZ NPs	LMTC-TZ NPs	MMTC-TZ NPs	LMTMC-TZ NPs	MMTMC-TZ NPs	LMC-CBZ NPs	MMC-CBZ NPs	LMTC-CBZ NPs	MMTC-CBZ NPs	LMTMC-CBZ	MMTMC-CBZ
80	63.89±1.5	61.23±1.9	81.34±3.3	80.32±2.4	83.44±1.3	82.56±2.8	65.32±2.5	65.67±2.2	78.43±2.2	74.89±2.4	79.43±1.9	75.78±1.6
40	71.23±2.3	67.45±2.9	94.78±3.9	93.12±3.1	96.33±2.3	95.67±3.1	67.34±1.9	68.89±1.9	85.45±2.8	84.33±1.8	87.90±3.2	86.98±3.1
20	81.32±3.1	79.54±3.2	95.32±3.9	94.32±2.1	96.77±2.8	96.43±3.4	77.45±2.6	75.34±2.5	87.45±3.2	86.43±2.3	89.43±2.9	89.34±2.9
10	90.34±3.5	89.31±2.9	96.89±4.1	96.34±2.5	97.23±3.2	97.22±2.6	84.34±3.1	81.23±4.3	90.32±3.4	88.43±3.6	91.43±3.1	91.45±3.2
5	91.98±2.3	90.11±3.1	96.55±4.4	97.45±4.3	91.23±3.9	96.11±2.9	86.75±2.9	87.34±2.7	92.12±3.8	89.55±2.9	93.21±4.5	94.32±4.1
2.5	91.22±4.3	92.34±3.8	97.45±5.3	97.21±3.9	94.23±3.4	95.34±3.1	89.21±4.2	89.99±2.9	84.89±3.7	92.89±4.3	95.32±3.7	92.89±4.1
1.25	93.56±3.6	94.88±3.9	97.99±3.9	96.89±3.1	95.96±3.9	96.54±3.5	91.23±3.8	91.23±3.4	95.44±5.2	95.78±3.9	96.09±4.3	95.78±2.9
0.625	94.21±2.5	96.32±4.2	97.45±4.4	95.34±2.6	97.89±4.1	97.89±3.9	94.22±2.7	91.12±4.2	96.43±3.9	96.33±2.7	98.67±4.1	98.34±3.8
0.3125	95.66±4.2	97.12±4.3	98.67±5.3	97.89±3.5	99.78±2.9	99.91±4.1	96.98±3.3	94.33±3.6	97.90±4.4	98.45±4.9	98.99±5.8	98.56±4.1
0.15625	97.89±2.9	96.89±3.6	99.61±5.9	98.44±4.1	98.45±3.2	99.43±5.3	97.33±3.9	96.10±4.3	98.43±3.6	99.32±4.1	97.56±3.2	99.12±4.9
0.078125	96.89±3.6	97.10±4.3	99.89±5.1	98.99±4.3	98.67±3.8	99.12±3.5	96.45±4.1	96.32±3.8	99.21±4.2	97.56±4.8	98.40±4.2	98.43±3.7

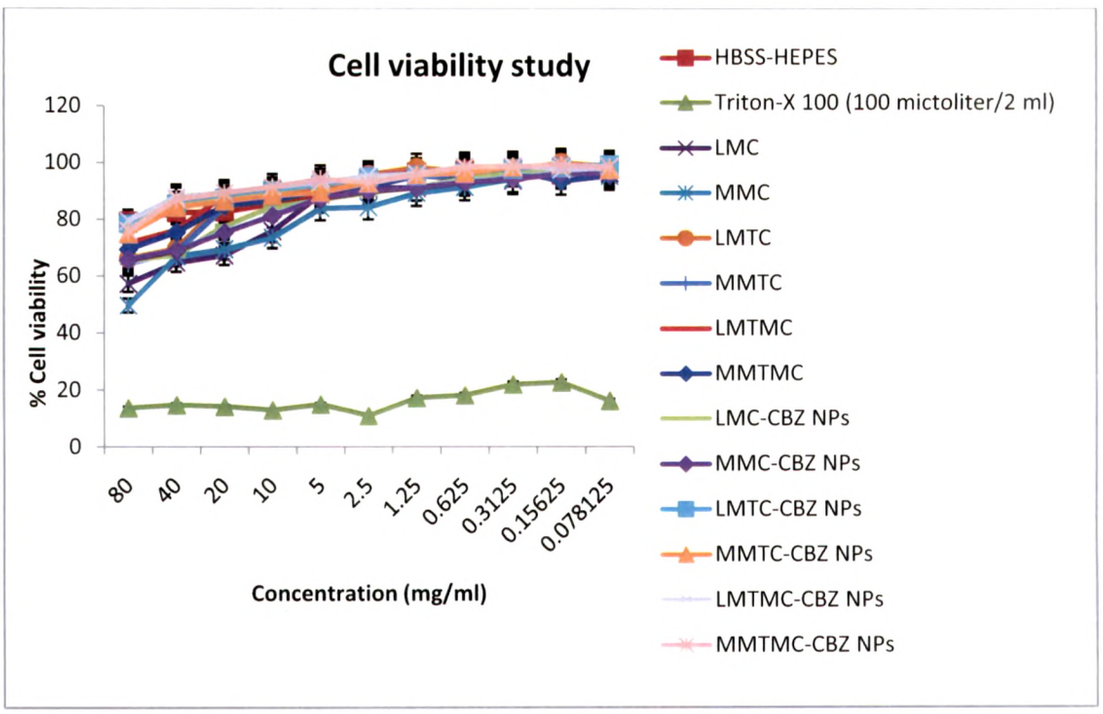
LMC-TZ NPs- Low molecular weight Tizanidine HCl loaded chitosan nanoparticles
LMC-CBZ NPs- Low molecular weight Cyclobenzaprine HCl loaded chitosan nanoparticles
MMC-TZ NPs-Medium molecular weight Tizanidine HCl loaded chitosan nanoparticles
MMC-CBZ NPs-Medium molecular weight Cyclobenzaprine HCl loaded chitosan nanoparticles
LMTC-TZ NPs- Low molecular weight Tizanidine HCl loaded thiolated chitosan nanoparticles
LMTC-CBZ NPs- Low molecular weight Cyclobenzaprine HCl loaded thiolated chitosan nanoparticles
MMTC-TZ NPs- Medium molecular weight Tizanidine HCl loaded thiolated chitosan nanoparticles
MMTC-CBZ NPs- Medium molecular weight Cyclobenzaprine HCl loaded thiolated chitosan nanoparticles
LMTMC-TZ NPs- Low molecular weight Tizanidine HCl loaded trimethyl chitosan nanoparticles
LMTMC-CBZ NPs- Low molecular weight Cyclobenzaprine HCl loaded trimethyl chitosan nanoparticles
MMTMC-TZ NPs- Medium molecular weight Tizanidine HCl loaded trimethyl chitosan nanoparticles
MMTMC-CBZ NPs- Medium molecular weight Cyclobenzaprine HCl loaded trimethyl chitosan nanoparticles



(Mean ± S.D., n = 3)

LMC- Low molecular weight chitosan
MMC- Medium molecular weight chitosan
LMTC- Low molecular weight thiolated chitosan
MMTC- Medium molecular weight thiolated chitosan
LMTMC- Low molecular weight trimethyl chitosan
MMTMC- Medium molecular weight trimethyl chitosan
LMC-TZ NPs- Low molecular weight Tizanidine HCl loaded chitosan nanoparticles
MMC-TZ NPs-Medium molecular weight Tizanidine HCl loaded chitosan nanoparticles
LMTC-TZ NPs- Low molecular weight Tizanidine HCl loaded thiolated chitosan nanoparticles
MMTC-TZ NPs- Medium molecular weight Tizanidine HCl loaded thiolated chitosan nanoparticles
LMTMC-TZ NPs- Low molecular weight Tizanidine HCl loaded trimethyl chitosan nanoparticles
MMTMC-TZ NPs- Medium molecular weight Tizanidine HCl loaded trimethyl chitosan nanoparticles

Figure 8.2: Cell viability study of chitosan, thiolated chitosan trimethyl chitosan and its tizanidine HCl loaded NPs formulations.



(Mean ± S.D., *n* = 3)
LMC- Low molecular weight chitosan
MMC- Medium molecular weight chitosan
LMTC- Low molecular weight thiolated chitosan
MMTC- Medium molecular weight thiolated chitosan
LMTMC- Low molecular weight trimethyl chitosan
MMTMC- Medium molecular weight trimethyl chitosan
LMC-CBZ NPs- Low molecular weight Cyclobenzaprine HCl loaded chitosan nanoparticles
MMC-CBZ NPs-Medium molecular weight Cyclobenzaprine HCl loaded chitosan nanoparticles
LMTC-CBZ NPs- Low molecular weight Cyclobenzaprine HCl loaded thiolated chitosan nanoparticles
MMTC-CBZ NPs- Medium molecular weight Cyclobenzaprine HCl loaded thiolated chitosan nanoparticles
LMTMC-CBZ NPs- Low molecular weight Cyclobenzaprine HCl loaded trimethyl chitosan nanoparticles
MMTMC-CBZ NPs- Medium molecular weight Cyclobenzaprine HCl loaded trimethyl chitosan nanoparticles.

Figure 8.3: Cell viability study of chitosan, thiolated chitosan trimethyl chitosan and its cyclobenzaprine HCl loaded NPs formulations

8.3.2. TEER measurement and permeation of TZ/CBZ loaded chitosan/thiolated chitosan/trimethyl chitosan NPs

Transepitheial electrical resistance was measured for TZ/CBZ formulations at different time intervals. In TZ/CBZ solution sample showed negligible change in the initial TEER value during the incubation of cells up to 150 minutes. In case of TZ/CBZ loaded chitosan/thiolated chitosan/trimethyl chitosan NPs, the TEER value was decrease in short time (30 minutes) and further decrease more at 150 minutes. TZ/CBZ loaded thiolated chitosan/trimethyl chitosan NPs showed a significant ($p < .05$) reduction in initial TEER values than the TZ/CBZ solution and TZ/CBZ loaded chitosan NPs. TEER value of TZ/CBZ formulations were recorded in Table 8.4 & Table 8.5.

Table 8.4: TEER Values of RPMI 2650 Cell monolayer at different time intervals

Time	TEER value ($\Omega.cm^2$)*						
	TZ solution	LMC-TZ NPs	MMC-TZ NPs	LMTC-TZ NPs	MMTC-TZ NPs	LMTMC-TZ NPs	MMTMC-TZ NPs
30 minutes	98.32±5.3	56.34±3.7	62.78±2.9	42.87±3.1	47.90±2.8	43.33±2.1	45.21±3.8
60 minutes	89.45±6.3	50.98±2.3	57.32±2.1	37.89±2.4	43.87±1.6	35.09±1.4	41.62±1.9
150 minutes	78.34±4.1	46.21±2.2	49.43±1.5	31.32±1.7	34.89±1.4	29.78± 1.1	38.76±1.7
Recovery after 150 minutes	96.87±3.2	59.01±3.8	61.11±4.2	96.57±5.1	96.89±4.9	97.43±4.8	97.65±4.9

*TEER values are expressed as percent of initial values
($p < 0.05$), **M ± SD (n = 3)**

Table 8.5: TEER Values of RPMI 2650 Cell monolayer at different time intervals

Time	TEER value ($\Omega.cm^2$)*						
	CBZ solution	LMC-CBZ NPs	MMC-CBZ NPs	LMTC-CBZ NPs	MMTC-CBZ NPs	LMTMC-CBZ NPs	MMTMC - CBZ NPs
30 minutes	97.86±4.7	54.21±2.8	65.32±3.8	43.44±2.9	46.12±2.9	45.77±3.2	47.11±3.1
60 minutes	85.87±4.1	51.56±2.8	59.98±4.1	39.21±1.9	40.12±3.1	37.43±2.1	40.32±1.3
150 minutes	79.43±3.4	48.32±3.2	51.23±2.5	30.43± 1.3	32.65±3.1	29.43± 2.1	32.21.±1.9
Recovery after 150 minutes	96.11±4.2	61.55±4.1	58.65±3.9	97.51±4.9	97.88±5.1	96.32±5.2	96.98±5.6

*TEER values are expressed as percent of initial values
($p < 0.05$), **M ± SD (n = 3)**

Permeation of TZ/CBZ across the RPMI 2650 cell monolayer determined in AP to BL direction. P_{app} of TZ/CBZ solution/chitosan/thiolated chitosan/trimethyl chitosan NPs was measured and recorded in the Table 8.6. P_{app} of TZ and CBZ solution was found to be 0.674±0.032 x 10^6 cm/second and 0.596±0.029 x 10^6 cm/second respectively. TZ/CBZ loaded thiolated chitosan NPs and trimethyl chitosan NPs shows the significant ($p < 0.05$) highest P_{app} value than TZ/CBZ loaded chitosan NPs and TZ/CBZ solution across the RPMI 2650 cell monolayer. P_{app} of TZ loaded thiolated chitosan NPs, trimethyl chitosan NPs and chitosan NPs was found to be 29-fold, 31-fold and 13-fold higher than the higher than the TZ solution respectively. P_{app} of CBZ loaded thiolated chitosan NPs, trimethyl chitosan NPs and chitosan NPs was found to be 33-fold, 37-fold and 18-fold higher than the higher than the CBZ solution respectively. Table 8.6 shows the Apparent Permeability coefficient (P_{app}) of TZ/CBZ formulations across the RPMI 2650 cell monolayer.

Table 8.6: Apparent Permeability Coefficients (P_{app}) of TZ/CBZ formulations across the RPMI 2650 cell monolayer

Formulations	**Apparent Permeability Coefficients (*Papp*) X 10^{-6} cm/sec**
TZ solution	0.674±0.032
CBZ solution	0.596±0.029
LMC-TZ NPs	9.43±0.367
MMC-TZ NPs	8.67±0.495
LMTC-TZ NPs	19.76±0.678
MMTC-TZ NPs	18.43±0.564
LMTMC-TZ NPs	21.43±0.453
MMTMC-TZ NPs	19.42±0.543
LMC-CBZ NPs	10.98±0.204
MMC- CBZ NPs	8.99±0.361
LMTC- CBZ NPs	20.11±0.289
MMTC- CBZ NPs	18.54±0.356
LMTMC- CBZ NPs	22.43±0.498
MMTMC- CBZ NPs	20.78±0.324

($p < 0.05$)

M ± SD (n = 3)

Recovery after 150 minutes of incubation of TZ/CBZ loaded chitosan NPs formulation shows the irreversible decrease in initial TEER due to destruction of the tight junctions of the RPMI 2650 cell monolayer. These results might be associated with the higher cytotoxicity of chitosan NPs than the thiolated chitosan NPs and

trimethyl chitosan NPs (Maryam et al., 2006). Thiolated chitosan/Trimethyl chitosan NPs have an ability to decrease the initial TEER value, which can be explain by the possible interactions between the positive surface charge with the anionic components of the glycoproteins on the surface of the nasal epithelial or to the negative charges of the interior of the tight junction.

8.4. Conclusions

In-vitro cytotoxicity study demonstrated that the soluble chitosan, thiolated chitosan and trimethyl chitosan shows less cell viability than its NPs formulations on RPMI 2650 cells suggesting the use of these NPs for safe delivery of tizanidine HCl and cyclobenzaprine HCl by intranasal route. The soluble chitosan and chitosan NPs had lower cell viability than the soluble thiolated chitosan, soluble trimethyl chitosan and its NPs formulations also suggesting that the modified derivatives of chitosan are safe for the intranasal delivery. To illustrate the safety of thiolated chitosan and trimethyl chitosan and its effect on the cell viability was directly compared with that of Triton X 100. A substantial decrease of the cell viability was observed after incubation with Triton X 100 compared to HBSS-HEPES ($p < 0.001$). Permeability study on RPMI 2650 cell monolayer suggest that the high drug permeation with trimethyl chitosan, and thiolated chitosan NPs than the chitosan and drug solution (Both drug tizanidine HCl and cyclobenzaprine HCl). Hence, the trimethyl chitosan and thiolated chitosan derivatives were able to significantly decrease the initial TEER at pH values present in the nose. This ability of chitosan derivatives to decrease the initial TEER can be explained by the possible interactions of their positive surface charge with the anionic components of the glycoproteins on the surface of the epithelial cells or the fixed negative charges of the interior of the tight junction.

8.5. References

Bai, S., Yang, T., Thomas J., Abbruscato, F.A. (2008). Evaluation of human nasal RPMI 2650 cells grown at an air–liquid interface as a model for nasal drug transport studies. Journal of Pharmaceutical Sciences, 97(3), 1165–1178.

Maryam, A., Stefan, G.R., Gerrit, B., Hans, E.J., Wim, E.H., Wim, J. (2006). Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system. J Controlled Release, 111(1-2), 107 – 116.

Moore, G.E., Sandberg, A.A. (1964). Studies of a human tumor cell line with a diploid karyotype. Cancer, 17, (2), 170–175.

Shah, P., Jogani, V., Mishra, P., Mishra, A.K., Bagchi, T., Misra, A. (2008). In vitro assessment of acyclovir permeation across cell monolayers in the presence of absorption enhancers. Drug Dev Ind Pharm., 34(3), 279-288.