CHAPTER 6

CELL LINE STUDIES

6.1 Histopathological Studies:

6.1.1 Method

Rat nasal tissue was obtained from the rats housed in the Pharmacology Department . The skin, as well as the soft tissues surrounding the nasal cavity, was removed. Then, the bony – framework of the nasal cavity including nasal septum were cut out. The nasal mucosa was pushed out from its cartilaginous attachments. Freshly isolated tissues were stored in phosphate buffer solution pH 6.4. Histopathological evaluation of rat nasal tissue was conducted after incubating the isolated tissue in PBS (pH= 7.4) with the optimized Galantamine loaded (2 mg/mL) or Bapineuzumab loaded nanoparticles (2mg/mL) for 2 hours. Control was also kept under similar condition, wherein saline solution was kept in contact with the nasal tissue for 2 hours. Tissue was fixed in 10% buffered formalin (pH= 7.4) and decalcified with 5% ethylenediaminetetraacetic acid. After preservation, nasal samples were directly dehydrated in a graded series of ethanol and embedded into paraffin wax. Five mm sections were cut with microtome. These were then stained with hematoxylin and eosin. Sections were examined under a light microscope (Primostar, Carl Zeiss, German) to detect any damage to the tissue by a pathologist blinded to the study at Unique Bio – Diagnostics Enterprises, Mumbai (Jiang, Cui, Fang, Wei, & Xi, 1995).

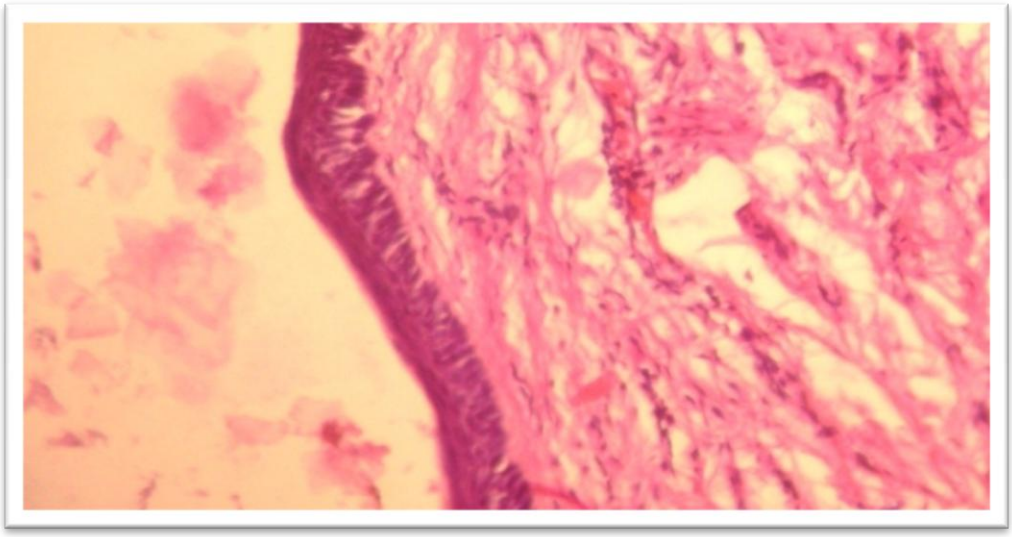


Figure 6.1: Muco ciliary lining after exposure to Galantamine loaded nanoparticles

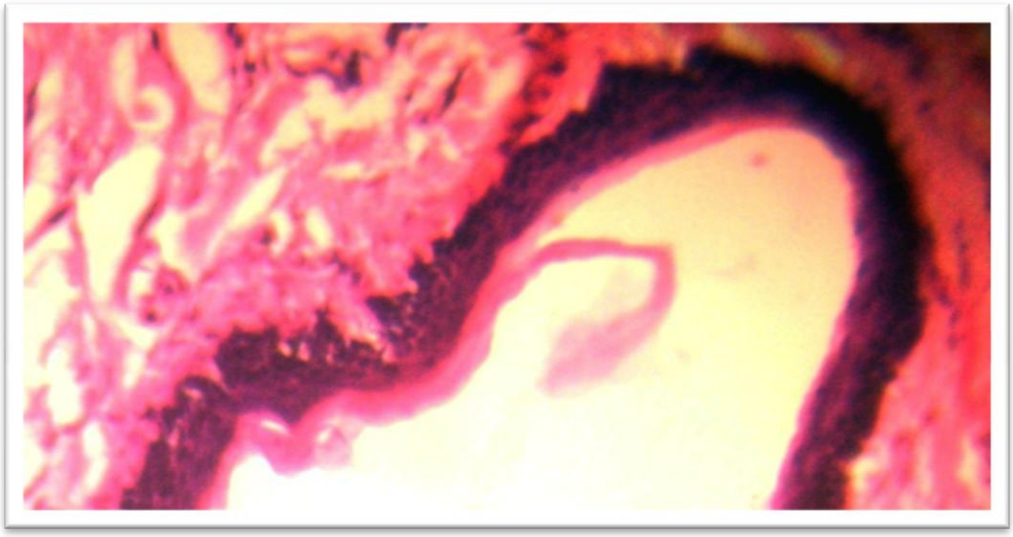


Figure 6.2: Muco ciliary lining after exposure to Bapineuzumab loaded nanoparticles

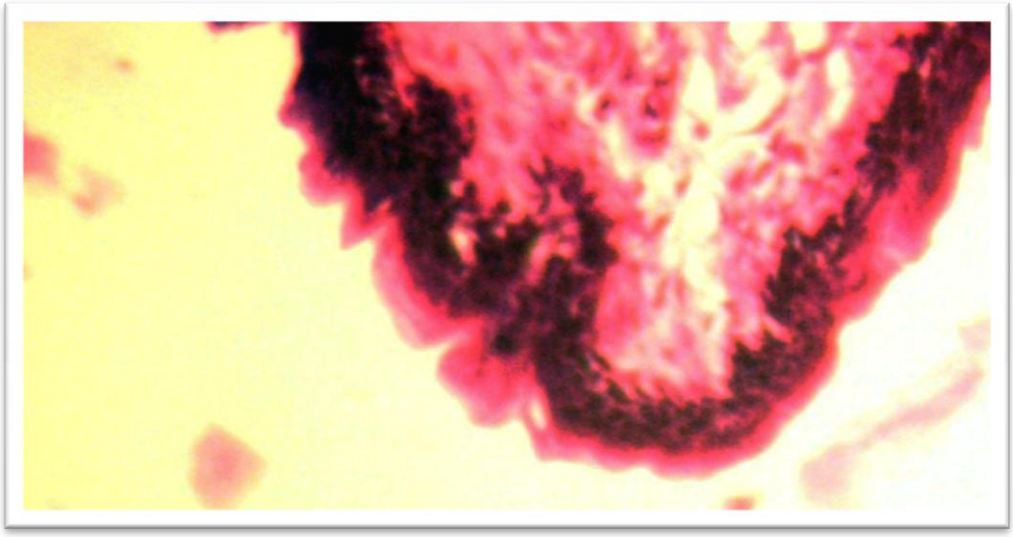


Figure 6.3: Control (exposed only to saline).

6.1.2 Results and discussions:

Figure 6.1 shows the tissue after its contact with Galantamine nanoparticles. In comparison to control as seen in Figure 6.3, it could be seen that no damage occurred to cilia and epithelial cells of the nasal mucosa. There was no evidence of cellular necrosis. The epithelial cells were also intact and not uprooted from the mucosal base.

Figure 6.2 depicts the image of tissue after its contact with Bapineuzumab nanoparticles. In comparison to control as can be seen in Figure 6.3, there was no significant change in the cellular structure in Figure 6.2. Normal upper respiratory epithelium varying from cuboidal to columnar appearance was observed and no abnormalities were detected.

Therefore, the formulations were non toxic to the nasal mucosa, indicating the nanoparticle formulation can be administered via the nasal route.

6.2 Cell Line Studies

At early stages of development, cell cultures are usually preferred to whole animal studies. Prediction of *in vivo* absorption based on *in vitro* methodology may help reduce the volume of necessary clinical investigations. Cell monolayers have been widely employed for studying the cellular uptake and cytotoxicity of delivery systems. They present many advantages, including easy to culture and studies can be performed within a controlled environment. In many cases a significant correlation between the studies performed on *in vitro* cell monolayers and *in vivo* human studies has been observed. Hence, *in vitro* studies can be used as predictive tools for estimating the fate and activity of the delivery system in the actual human body (Tavelin et al; 2003).

Also, *in vitro* cytotoxicity study data are now being considered by various regulatory agencies like Environment Protection Agency (EPA), National Institute of Health (NIH), National Institute of Cancer (NIC), Food and Drugs Administration (FDA). This necessitates the evaluation of cytotoxicity study of any formulation to be administered through any route.

Nanoparticles have high therapeutic benefits but also the potentially unpredictable and adverse consequences of human exposure thereto. In this context, nanoparticle toxicity refers to the ability of the particles to adversely affect the normal physiology as well as to directly interrupt the normal structure of organs and tissues of humans and animals. It is widely accepted that toxicity depends on physiochemical parameters such as particle size, shape, surface charge and chemistry, composition, and subsequent nanoparticles stability. The exact underlying mechanism is as yet

unknown. However, recent literature (Bahadar, Maqbool, Niaz, & Abdollahi, 2016) suggests cytotoxicity to be related to oxidative stress and pro-inflammatory gene activation. For nanoparticles, the administered dose, route of administration and extent of tissue distribution are important parameters in nano-cytotoxicity. Typically, cell-based toxicity studies use increasing doses of the nanoparticles in order to observe dose-related cellular or tissular toxicity. Such dose response correlations are the basis for determining safe limits of particle concentrations for *in vivo* administration. Despite the theoretically brilliant logic, animal and human studies have taught us differently and highlighted the issue of the feasibility of correlating organ toxicity with the pre-determined dose; there exists a widely acknowledged problem of extrapolating *in vitro* concentrations into *in vivo* scenarios which can be subdivided into two points; firstly, it has yet to be determined how efficiently any administered nanoparticles dose is reaching the target tissue and secondly, nanoparticles can induce biochemical changes in vivo which may have gone unnoticed in isolated cell based studies. With the potentially disastrous consequences in mind, new ways of predicting as yet unpredictable, nondosage-dependent actions of NPs in vivo must be sought. Apart from the dosing issue, another, so far underexposed area of nanotoxicity relates to the route of particle administration which may also, quite independently from the dose, influence toxicity in an adverse fashion. It is sensible to assume that biodistribution, accumulation, metabolism and excretion of nanoparticles will differ depending on the route of administration as will its toxicity (Yildirimer, Thanh, Loizidou, & Seifalian, 2011).

Therefore, taking the above mentioned facts into consideration it was essential to evaluate the toxicity and cellular uptake for the developed formulations

6.3 Cellular uptake and toxicity studies for nose to brain nanoparticles

The blood brain barrier (BBB) is impermeable to most drugs, impeding the establishment of novel neuroprotective therapies and strategies for many neurological diseases. Intranasal administration offers an alternative path for efficient drug delivery into the CNS. The anatomical structures involved in the transport of intranasally

administered drugs into the CNS include the trigeminal nerve, olfactory nerve and the rostral migratory stream (RMS) (Scranton et al., 2011) .

The nanoparticles designed for nose to brain delivery, need to take one of the above mentioned three routes to be successfully delivered to the brain. The routes involve neurons, hence to understand the uptake *in vitro*, the cell lines used generally are of neuronal origin.

Various cell lines of neuronal origin have been utilized to study cellular uptake. The choice of cell line also depends upon the disorder to be targeted.

Z Liu et al (Liu et al., 2013) studied cytotoxcity and cellular uptake of lactoferrin modified neuroprotective nanoparticles for Alzheimer's disease employing the 16HBE14o-cells. This is a bronchial epithelial cell line, which forms an ideal cell culture model to understand cellular uptake. The study revealed time-dependent, temperature- dependent and concentration-dependent mode of cellular uptake of modified nanoparticles. The mode of cellular uptake was endocytosis.

A Mathew et al (Mathew et al., 2012) studied cellular uptake on GI-1 glioma cell line. This was a human glioblastoma cell line. In this study, the cellular uptake was observed after 4 hours, using flow cytometry.

MS Mufamadi et al (Mufamadi et al., 2013) studied cellular uptake of galantamine nanoliposomes using the PC-12 cell line. It is a cell line derived from apheocromocytoma of the rat adrenal medulla, that have an embryonic origin from the neural crest that has a mixture of neuroblastic cells and eosinophilic cells. The cellular uptake was studied at 0, 2, 4, 6, 8 and 24 h time intervals. The uptake was observed through confocal microscopy after tagging the nanoparticles with an appropriate dye.

However, out of all the various alternatives available for cell line studies, the IMR – 32 cell line can be considered best suited for Alzheimer's disease (Rao & Kisaalita, 2002). The IMR – 32 cell line is a human neuroblastoma cell line (IMR-32) which can mimic large projections of the human cerebral cortex and under certain tissue culture conditions, forms intracellular fibrillary material, commonly observed in brains of patients affected with Alzheimer's disease.

The aim of this study was to evaluate the quantitative cellular uptake of Galantamine loaded nanoparticles and Bapineuzumab loaded NPs in IMR 32 cell lines. Tolerability

and safety of Galantamine loaded nanoparticles and Bapineuzumab loaded nanoparticles were assessed by cytotoxicity studies on IMR 32 cell lines.

6.4 Materials

Galantamine hydrobromide was kindly gifted by SPARC, Vadodara, India. Bapineuzumab was purchased from Arihant Traders (India). IMR – 32 cells were purchased from NCCS, Pune, INDIA. Minimum Essential Medium (MEM) supplemented with 10% FBS, 0.2% sodium bicarbonate, penicillin-streptomycin solution, Trypsin-EDTA solution, Fetal bovine serum (FBS) and Earle's Balanced Salt Solution were purchased from Himedia, Mumbai, India. 6 and 96 well plates were purchased from Costar, Corning, USA. MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye was purchased from Sigma Aldrich, Germany.

6.5 Methods

6.5.1 Cell Culture

IMR - 32 cells (NCCS, Pune) of passages between 60 and 64 were used for *in vitro* cellular uptake studies. IMR – 32 cells were cultured in $25cm^2$ tissue culture flasks. Minimum Essential Medium with 2mM L- Glutamine with Earle's Balanced Salt Solution supplemented with 1mM sodium pyruvate, 1.5 g/L of sodium bicarbonate and 0.1 mM Nonessential Amino acids was used as culture medium. Cells were cultured as a monolayer in 10 mL medium at 37◦C in a humidified atmosphere containing 5% CO_2 and medium was replenished every alternate day (Zhou et al., 2005)

6.5.2 Cytotoxicity Assays

Fresh MTT reagent was prepared. MTT reagent is available as a yellow colored powder. A 5mg/mL solution made in phosphate buffer saline (pH = 7.4) was used for the study. The *in vitro* cytotoxicity of Galantamine hydrobromide loaded nanoparticles, Bapineuzumab loaded nanoparticles, plain Bovine Serum Albumin solution, and plain drug and for antibody solution were done.

The cells were cultured in 96-well plates at a seeding density of 1.0×10^4 cells/well for 24 and 48 h. The drug or antibody formulation was taken in the initial concentration of 10 mg/mL which was diluted to 1 mg/mL with MEM culture media. This was serially

diluted with MEM culture media to different concentrations. Plain drug or antibody solutions were prepared for the highest concentration in the series. Experiments were initiated by replacing the culture medium in each well with 100µl of sample solutions (10, 20, 40, 80, 100, 150, 200, 250, 300 µg/ml) at 37 ◦C in the CO_2 incubator. After 24 and 48 h of incubation, the medium was removed and 150 µl of MTT reagent (1 mg/ml) in the serum-free medium was added to each well. The plates were then incubated at 37 ◦C for another 4 h. At the end of the incubation period, the medium was removed and the intracellular formazan was solubilised with 150µl DMSO and quantified by reading the absorbance at 595 nm on a micro-plate multi-detection instrument, using SYNERGY-HT multiwall plate reader (Bio-Tek, USA) using Gen5 software (Bio-Tek, USA).

The medium treated cells were used as controls. Percentage of cell viability was calculated based on the absorbance measured relative to the absorbance of cells exposed to the negative control.

6.5.3 Qualitative Cellular Uptake by Flow Cytometry

Flow cytometric detection of Galantamine hydrobromide and Bapineuzumab nanoparticles were carried out according to the method of Suzuki et al. (2007) using light scattering principles.

$1.0X10^5$cells/well were seeded in 6-well cell culture plates. After 24 h of seeding, the cells were exposed to Galantamine hydrobromide and Bapineuzumab nanoparticles (100, 150, 200, 250 and 300 µg/ml) for 6 and 24 hr. After exposure, the culture medium containing nanoparticles was removed and the cells were harvested using 0.05% trypsin. Cells were then centrifuged at 250g for 5 min (Remi Instruments, India). The supernatant was discarded and the pellet was re-suspended in 0.5 ml of PBS. The uptake of particles was determined by flow cytometer (FACSCalibur™, BD BioSciences, San Jose, CA, USA) equipped with a 488 nm laser.

6.6 Results and discussion

Cytotoxicity of Galantamine hydrobromide and Bapineuzumab were assessed in human neuroblastoma cells (IMR 32) for 24 and 48 hr, respectively. In the MTT assay, a significant ($p<0.05$) reduction in the enzymatic activity of succinate dehydrogenase was observed upto 67 and 65% (relative to 100% of control) at 250 and 300 µg/ml of Galantamine hydrobromide nanoparticles exposure after 48 hr in IMR 32 cells. However, after 24 hr exposure, no significant cytotoxicity was observed. Moreover, no statistically significant cytotoxicity was observed in Bapineuzumab nanoparticles after 24 and 48 hr exposure.

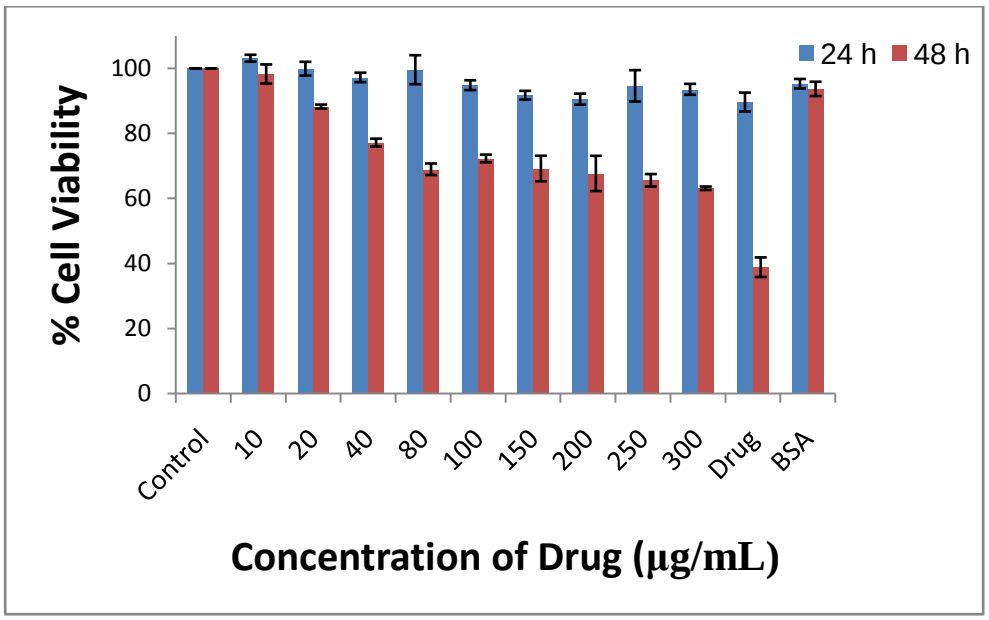
The results can be seen in Table 6.1 and Table 6.2 respectively. The graphical representation of the same can be seen in Figure 6.4 and Figure 6.5.

Table 6.1: *In vitro* cytotoxicity of Galantamine hydrobromide loaded nanoparticles at 24 and 48 h

Concentration (µg/mL)	**% cell viability at 24 h**	**% cell viability at 48 h**
Control	100	100
10	103.11±2.38	98.25±6.58
20	99.88±4.75	88.22±1.37
40	97.20±3.2	77.14±2.72
80	99.58±9.98	68.96±3.98
100	94.81±3.37	72.29±2.62
150	91.73±3.00	69.19±8.77
200	90.52±3.81	67.67±12.11
250	94.62±10.78	65.65±4.29
300	93.54±3.75	76.50±1.18
Drug solution (300 µg/mL)	89.63±6.48	38.83±6.74
Bovine Serum Albumin (300 µg/mL)	95.27±3.26	93.68±4.93

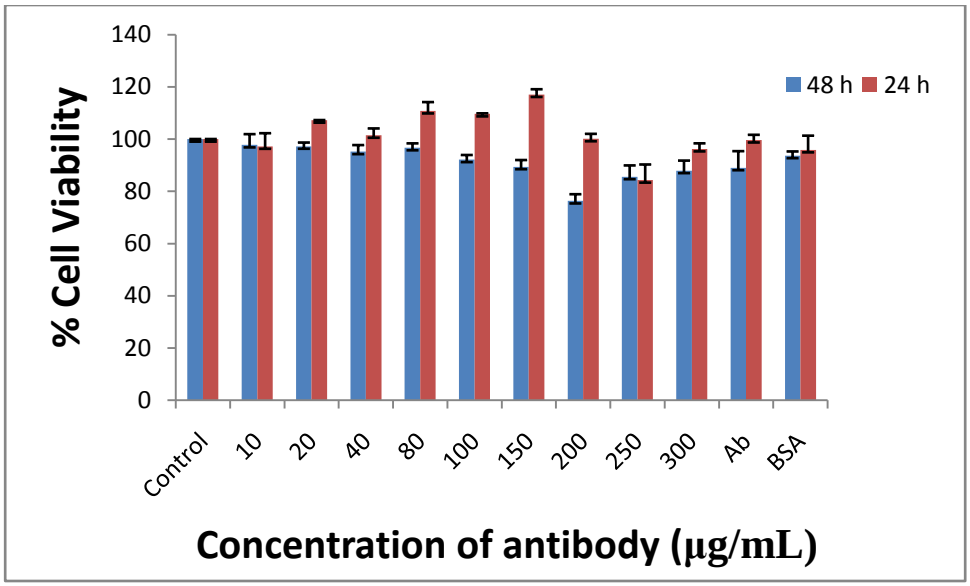
Table 6.2: *In vitro* cytotoxicity of Bapineuzumab loaded nanoparticles at 24 and 48 h

Concentration (µg/mL)	**% cell viability at 24 h**	**% cell viability at 48 h**
Control	100	100
10	97.25±11.13	97.88±9.98
20	107.23±0.25	97.27±3.39
40	101.49±5.74	95.21±6.06
80	110.12±7.45	96.68±4.06
100	109.67±0.36	92.15±4.24
150	117.11±4.39	89.43±6.17
200	100.14±4.12	76.33±6.20
250	84.31±13.29	85.61±10.49
300	96.26±4.68	87.94±9.31
Antibody solution (300 µg/mL)	99.68±4.34	89.05±15.42
Bovine Serum Albumin (300 µg/mL)	95.90±12.05	93.67±3.85



The Data are expressed as mean ± SEM from three independent experiments. *p<0.05 when compared to control.

Figure 6.4: Cytotoxicity studies of Galantamine hydrobromide nanoparticles at 24 and 48 h respectively.



The Data are expressed as mean ± SEM from three independent experiments. *p<0.05 when compared to control.

Figure 6.5: Cytotoxicity studies of Bapineuzumab nanoparticles at 24 and 48 h respectively.

Cytotoxicity studies of the formulations were done to assess the Mitochondrial activity of the cell which in turn indicates the cell viability after its exposure to the formulations. MTT assay aided in understanding the safety / tolerability of formulation by the viable cells. This is a quantitiative colorimetric method, based on the reduction of a yellow tetrazolium salt to insoluble purple formazan crystals by the mitochondrial dehydrogenases of viable cells.

For Galantamine hydrobromide nanoparticles (Table 6.1), there were no significant decrease in cell viability at 24 hours over the entire range of concentration studied that is from 10—300 µg/mL. However, for the drug solution there was a slight decrease in viability of cells to 89 %, which could be due to the high concentration of plain drug solution that was in contact with the cells. At 48 hours, the % viability decreased to 70% till100 µg/mL. Here it is important to note that, the time period of exposure of formulation to cells was 48 hour.

For Bapineuzumab nanoparticles (Table 6.2), good viability was noted in comparison to control. The pure antibody solution also showed good viability of cells. This is an expected result, as antibody would be less toxic to cells in comparison to any drug

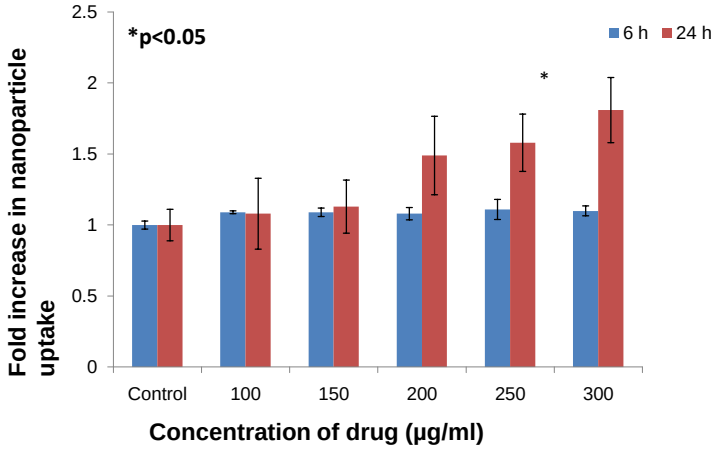
solution. At 48 hours, the cells were significantly viable for the entire range of concentration of exposed formulation.

In comparison to control, which had no nanoparticles in contact with the cell line, the nanoparticles demonstrated time- dependent and concentration – dependent uptake.

There was a two – fold increase in uptake at 24 h for a concentration of 300 µg/mL Galantamine hydrobromide loaded nanoparticles. This observation can be extrapolated to its in vivo performance after being administered through the nasal route. The nanoparticles after reaching the target area, for example the olfactory lobe; would be taken up by the neuronal cells and quantity of nanoparticles would increase by two fold at 24 h. The possible pathway could be by endocytosis. Their small diameter potentially allows nanoparticles to be transported transcellularly through olfactory neurones to the brain via the various endocytic pathways of sustentacular or neuronal cells in the olfactory membrane (A. Mistry, S. Stolnik, & L. Illum, 2009).

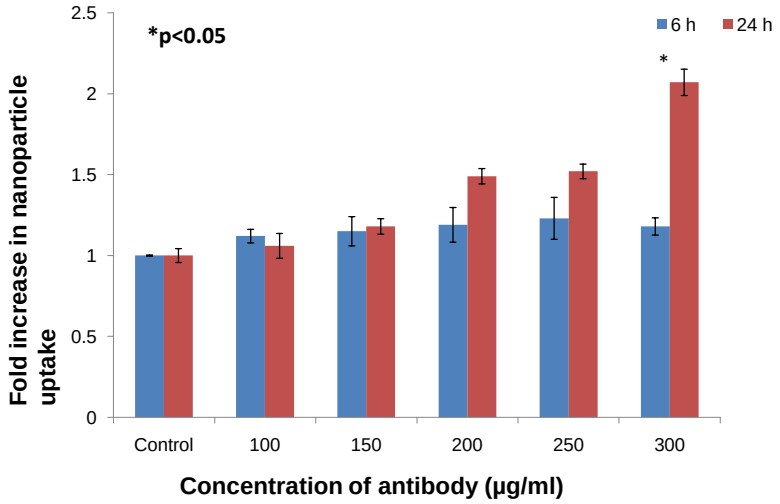
Similar observations were seen for Bapineuzumab loaded nanoparticles.

The graphical representation for uptake can be seen in Figure 6.6 and Figure 6.7 respectively.



The Data are expressed as means ± SEM from three independent experiments.
***p<0.05 when compared to control.**

Figure 6.6: Cellular uptake using flow cytometry for Galantamine hydrobromide nanoparticles.



The Data are expressed as means ± SEM from three independent experiments.
***p<0.05 when compared to control.**

Figure 6.7: Cellular uptake using flow cytometry for Bapineuzumab nanoparticles.

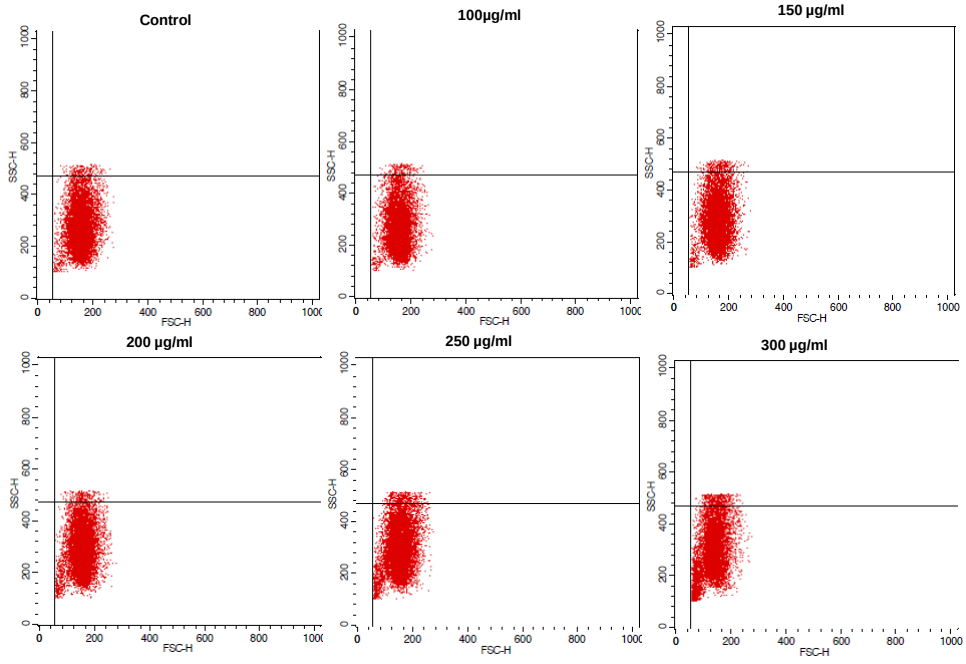


Figure 6.8: Scatter plot for cellular uptake at 24 hr for Galantamine hydrobromide nanoparticles.

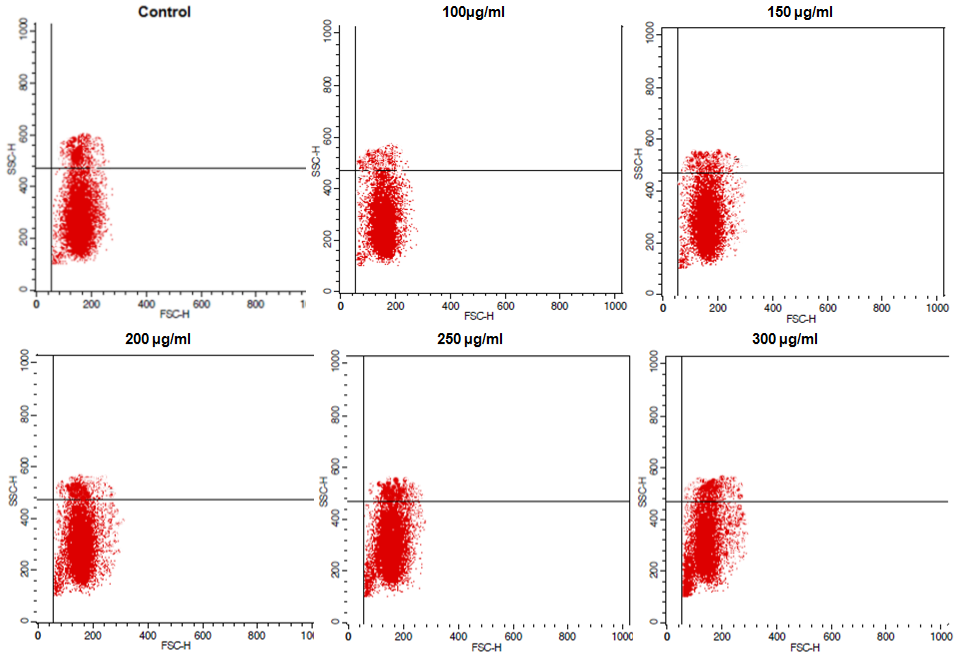


Figure 6.9: Scatter plot for cellular uptake at 24 hr for Bapineuzumab nanoparticles.

Fig 6.8 and fig 6.9 also denotes the cellular uptake of nanoparticles for Galantamine hydrobromide and Bapineuzumab respectively at 24 hr. The flow cytometry by Fluorescence Assisted Cell Sorter (FACS), involves simultaneous measurement of multiple physical characteristics of a single cell as the cell suspension flows through a measuring device. The light scatter is used to measure the intrinsic size and granularity of the cell. Two types of scattering is executed for studying the cellular uptake. Side scatter on the cell surface and forward scatter for the internal structure of the cells, wherein either of the parameter is a representative for uptake of nanoparticles in cells (Suzuki, Toyooka, & Ibuki, 2007). The forward scatter value is progressively increasing with an increase in concentration of nanoparticles (Fig 6.8 and Fig 6.9). This shows an increase in uptake of nanoparticles with an increase in concentration of the nanoparticles kept in contact with the cells. (Suzuki et al., 2007)

6.7 References

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