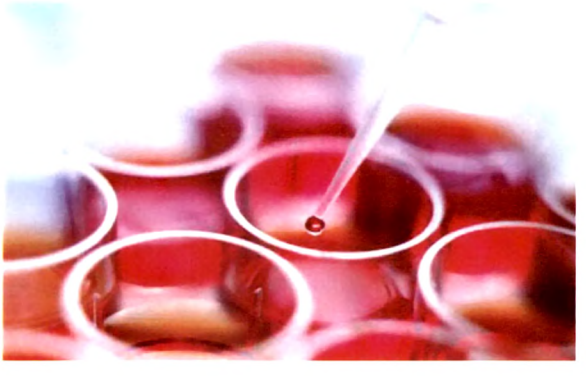
Chapter VII

Ex-vivo Studies



The *ex-vivo* study provides an insight into the probable *in-vivo* efficacy of the prepared drug delivery system. After the optimization and characterization of prepared formulations, the nanoparticles were intended for *in-vivo* characterization. However, prior to *in-vivo* studies, developed formulations should be evaluated for their efficacy *ex-vivo* to ascertain activity and further improvement in the system, if required. The aim of this study was to examine and evaluate influence of unconjugated and transferrin conjugated PLGA nanoparticles entrapping pioglitazone (PIO) and rosiglitazone (ROS) for hemolytic toxicity and *in vitro* cytoprotective activity. The cytoprotective activity of developed formulations was compared with drug solution on murine neuroblastoma (Neuro 2a) cells in presence of β-amyloid.

7.1 HEMOLYTIC TOXICITY STUDY

Whole human blood from healthy volunteers was collected in HiAnticlot blood collection vials (Himedia Labs, Mumbai, India). The red blood cells (RBC) were separated by centrifugation and resuspended in normal saline solution (10% hematocrit). One milliliter of RBC suspension was incubated with distilled water (taken as 100% hemolytic standard) and normal saline (taken as blank for spectrophotometric estimation). To 1 mL of RBC suspension taken in separate tubes, 1 mL of pioglitazone solution in 0.1% DMSO, pioglitazone loaded unconjugated (PIO-NP) and transferrin conjugated nanoparticulate (Tf-PIO-NP) formulations (equivalent to 1 mg/mL of drug) were added separately and volume made up to 10 mL with normal saline. The glass test tubes were allowed to stand for 1 h at 37°C with intermittent shaking and were then centrifuged for

15 min at 3000 rpm and the absorbance of supernatants was measured at 540 nm, which was used to estimate percent hemolysis using distilled water. Similar study of hemolytic activity of rosiglitazone solution in normal saline, rosiglitazone loaded unconjugated (ROS-NP) and transferrin conjugated nanoparticulate (Tf-ROS-NP) formulations was also performed. Percent hemolysis by each sample was calculated by using the following equation (Nahar et al., 2008):

$$\% \text{ Hemolysis} = \frac{AB_S}{AB_{100}} X\,100$$

Where AB_s is absorbance of the sample and AB_{100} is absorbance of the control (without formulation). The percent hemolytic toxicity is recorded in **Table 7.1**. The hemolytic toxicity of PIO in 0.1% DMSO solution was recorded after subtracting hemolytic toxicity of 0.1% DMSO.

7.2 MTT CYTOTOXICITY ASSAY

The cytoprotective activity is evaluated by assessment of viable cells after the treatment with drug or the formulation in presence of cytotoxic compound. Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. In the present study, the cell viability of the cell lines in presence of neurotoxic compound β-Amyloid(Aβ) was studied by using MTT assay method. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a simple, non-radioactive colorimetric assay used to determine the ability of viable cells to convert a soluble tetrazolium salt (MTT) into an insoluble formazan precipitate. MTT is a yellow, water-soluble salt that accepts electrons from

oxidized substances or reduced at the ubiquinone and cytochrome b and c site of the mitochondrial activity. The dehydrogenase enzymes present in metabolically active cells are able to convert this dye into a water-insoluble dark blue formazan by reductive cleavage of tetrazolium ring. The formazan crystals are then dissolved in an organic solvent such as dimethylsulphoxide (DMSO) and the resultant amount of formazan in the solution is quantified by spectrophotometrically measuring the absorbance of solution at 570 nm on a microtitre plate reader (www.atcc.org). The resultant value is related to the number of living cells (de Souza et al., 2004) as the MTT reagent yields low background absorbance value in the absence of viable cells (Skehan et al., 1990). This technique has many advantages and is used to measure cytotoxicity, proliferation or viability. It measures the cell proliferation rate and conversely, the reduction in cell viability, when metabolic events lead to apoptosis or necrosis. Beside it is rapid, versatile, quantitative and highly reproducible, with a low intra-test variation between data points (±15% SD), and hence it is useful in a large-scale drug-screening program. Moreover, the test can also be used for floating cells such as leukemias and small cell lung carcinoma and always allows sufficient time for cell replication, drug-induced cell death, loss of enzymatic activity, which generates the formazan product, the MTT substrate (Mosmann, 1983).

Cell Culture

Principle:

Cells in the exponential phase of growth are exposed to a well reported neurotoxic compound Aβ. The duration of exposure is determined as the time required for maximal damage to occur. After removal of the neurotoxic compound, the number of surviving cells is determined indirectly by MTT dye reduction. The

amount of MTT-formazan produced can be determined spectrophotometrically once the MTT formazan has been dissolved in a suitable solvent.

Materials:

Sterile

- Growth medium (MEM with 2mM L-Glutamine, 1mM Sodium pyruvate, NEAA and 1.5 gms/litre Sodium bicarbonate) (HiMedia, Mumbai, India).
- Fetal bovine serum (FBS)
- Sterile antibiotic solution 100X (10,000 units penicillin and 10 mg streptomycin per ml in 0.9 % normal saline)
- Trypsin (0.25% + EDTA, 1 mM, in PBSA)
- MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 50 mg/mL, filter sterilized (HiMedia, Mumbai, India)
- Sorensen's glycine buffer (0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with 1 M NaOH)
- Microtitration plates (96 well flat bottom; Tarsons, Mumbai, India)
- Pipettor tips in an autoclavable tip box
- Petri dishes (non-TC-treated), 5 cm and 9 cm as reservoir
- Falcon tubes, 30 mL and 50 mL

Nonsterile:

- Plastic box (clear polystyrene, to hold plates)
- Multichannel pipettor (HiMedia, Mumbai, India)
- Dimethyl sulfoxide (DMSO)
- ELISA plate reader (BioRad, 680 XR Microplate Reader)
- Inverted microscope (Olympus CKX 41)

Procedure:

The mouse neuroblastoma (neuro 2a) cells obtained from National Centre for Cell Science, Pune were grown as monolayers in MEM medium with 2mM L-Glutamine, 1mM sodium pyruvate, NEAA and 1.5 g/L sodium bicarbonate (supplemented with 10% heat inactivated fetal bovine serum (FBS), 10000 $IUmL^{-1}$ penicillin and 100 mg mL^{-1} streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C. The medium was replaced with fresh medium every alternate day. A subconfluent monolayer culture was trypsinized using 0.25% trypsin solution leaving it for 1 minute. When the cells have detached, harvesting media was diluted with fresh culture media containing 10% FBS to neutralize trypsin followed by centrifugation for 5 min at 200 g to pelletize the cells which were subsequently resuspended in growth medium and counted by trypan blue method in Neubeur chamber using inverted microscope (Olympus CKX 41, with camera attachment). Cells were plated in 96-well flat bottom microtitration plates (Tarsons, Mumbai) at the concentration of $7x10^3$ cells/mL and incubated for 24 hrs. Subsequently, formulations (PIO solution, ROS solution, PIO-NP and Tf-PIO-NP, ROS-NP and Tf-ROS-NP), were diluted to various concentrations in culture medium. Then 24 hrs after seeding, cells were incubated in fresh culture media with or without $A\beta_{1-40}$ (2.5 µM/mL) and different concentrations of drugs either in solution or nanoparticulate formulations. The formulations were added to each well in triplicate, at the concentration in a decreasing manner equivalent to 5 µM/mL to 0.5 µM/mL of drugs. At the end of exposure period, i.e. after 48 hrs, the media was replaced with 200 µL of fresh medium and 50 µL of MTT solution was added to each well, the plates were wrapped in aluminum foil and again incubated at 37±1°C for 4 hrs to allow the viable cells to reduce the MTT into purple formazan crystals. The MTT solution was prepared by dissolving 0.3

Table 7.1: Hemolytic profile of PIO & ROS and their formulations

Formulation code	% Hemolysis
PIO-S	4.2 ±0.8
ROS-S	5.1 ±0.9
Blank PLGA NP	1.3±0.2
PIO-NP	2.4 ±0.4
ROS-NP	2.6 ±0.5
Tf-PIO-NP	1.9±0.3
Tf-ROS-NP	2.1±0.2

* Hemolysis (%) produced by 1 mg/mL formulations in RBC (10% hematocrit) after incubation for 1 h., Values represent mean ± S.D. (n=3)

Table 7.2: Cytoprotective study of various PIO & ROS formulations after 48 h incubation with β-Amyloid

Formulation code ↓	% Viable cells		
Drug concentration →	0.5 μM	1.25 μM	5.0 μM
Control 1 (without β-Amyloid)	100		
Control 2 (with 2.5 μM/mL Aβ)	77.1±3.6		
PIO-S	41.4±1.3	57.0±2.4	67.7±2.3
ROS-S	46.0±1.5	60.1±2.9	81.7±3.0
PIO-NP	61.7±2.1	62.5±1.8	85.8±3.7
ROS-NP	86.9±3.9	90.3±3.8	107.2±2.8
Tf-PIO-NP	60.7±2.6	64.3±2.2	86.8±3.4
Tf-ROS-NP	79.9±3.8	100.5±3.5	138.5±2.1

Values represent mean ± S.D. (n=3)

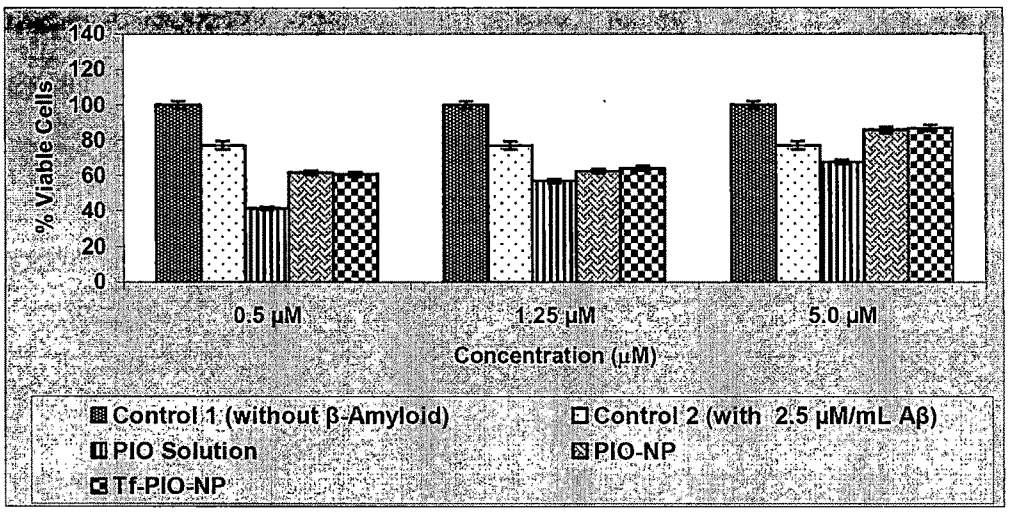


Fig. 7.1: Cytoprotective activity of various Pioglitazone formulations (concentration equivalent to 0.5 µM, 1.25 µM and 5.0 µM) after 48 h incubation with β-Amyloid

Each bar represent mean±S.D. (n=3)

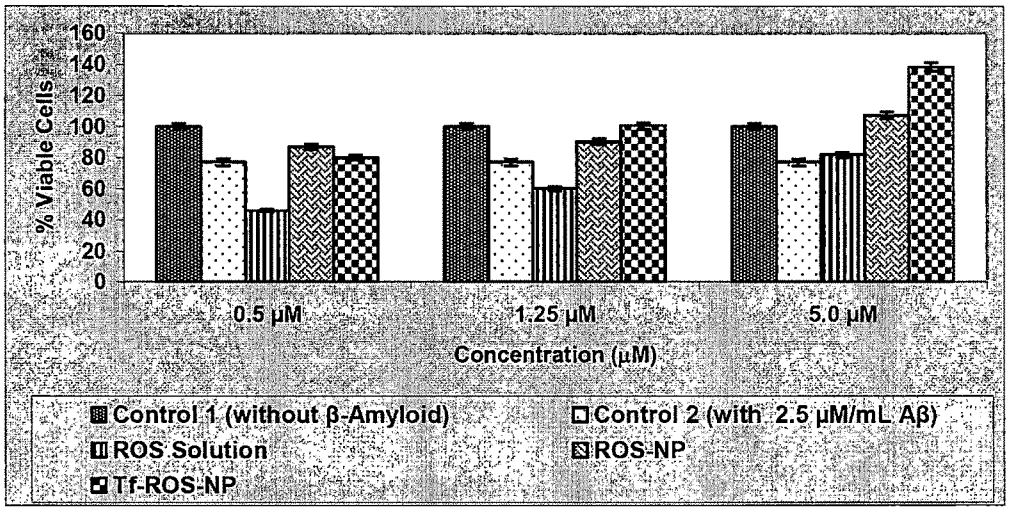


Fig. 7.2: Cytoprotective activity of various Rosiglitazone formulations (concentration equivalent to 0.5 µM, 1.25 µM and 5.0 µM) after 48 h incubation with β-Amyloid

Each bar represent mean±S.D. (n=3)

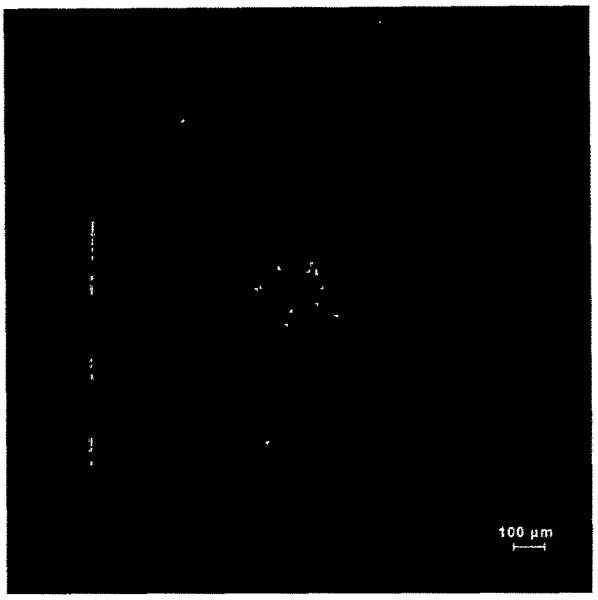


Fig. 7.3: Photomicrograph of neuro-2a cells after 48 hrs incubation with PIO-S (equivalent to 5 µm of Pioglitazone)

Fig. 7.4: Photomicrograph of neuro-2a cells after 48 hrs incubation with PIO-NP (equivalent to 5 µm of Pioglitazone)

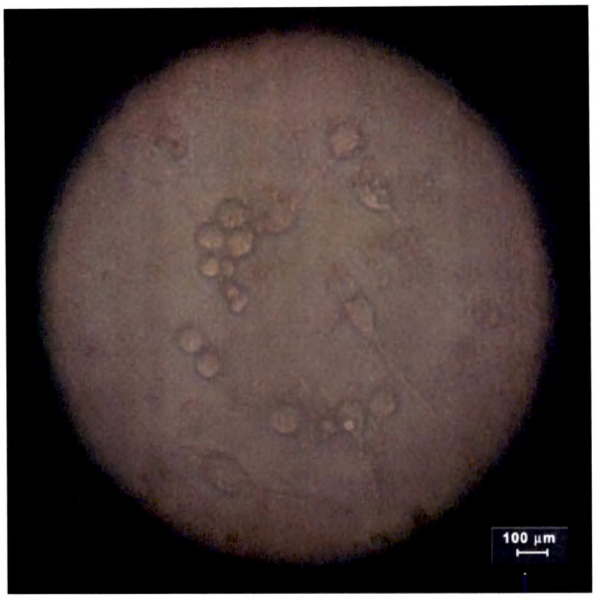


Fig. 7.5: Photomicrograph of neuro-2a cells after 48 hrs incubation with Tf-PIO-NP (equivalent to 5 µm of Pioglitazone)

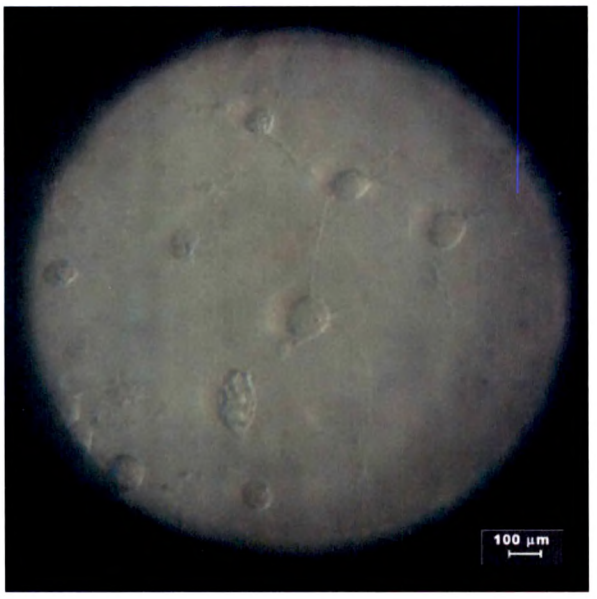


Fig. 7.6: Photomicrograph of neuro-2a cells after 48 hrs incubation with ROS-S (equivalent to 5 µm of Rosiglitazone)

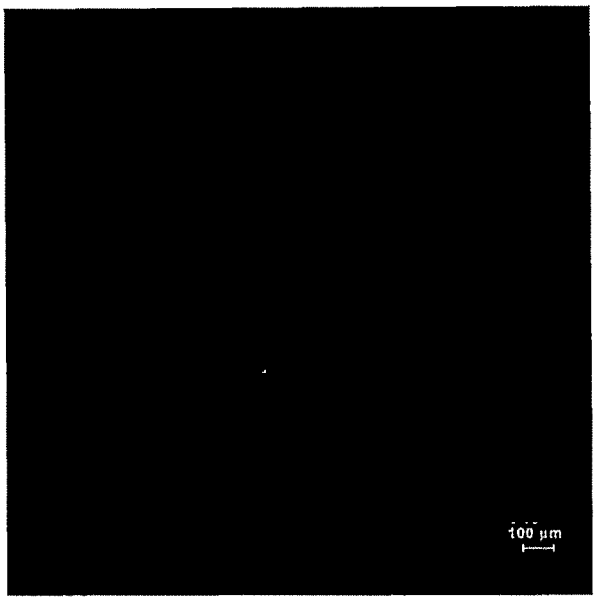


Fig. 7.7: Photomicrograph of neuro-2a cells after 48 hrs incubation with ROS-NP (equivalent to 5 µm of Rosiglitazone)

Fig. 7.8: Photomicrograph of neuro-2a cells after 48 hrs incubation with Tf-ROS-NP (equivalent to 5 µm of Rosiglitazone)

7.3 RESULTS AND DISCUSSION

Hemolytic toxicity

The hemolytic toxicity study of PIO-S, ROS-S, blank PLGA NP's, PIO-NP, ROS-NP, Tf-PIO-NP and Tf-ROS-NP was performed to assess the toxicity of drugs/formulations to RBC's on administration of formulations. The observations are recorded in **Table 7.1**. All the nanoparticulate formulations have shown an average hemolytic toxicity of 1 to 3 %. The hemolytic toxicity of PIO-S (after subtraction of solvent toxicity) and ROS-S was found to be 4.2±0.8% and 5.1±0.9%, respectively. The hemolytic toxicity of blank PLGA NP's was found to be 1.3±0.2% suggesting the better biocompatibility of the blank polymeric formulation and signifying the suitability of prepared formulation for the delivery of drugs which is also desired for an ideal drug delivery application. The PIO-NP was found to have 2.4±0.4% hemolytic toxicity which was higher than blank PLGA NP's and this may be due to the presence of surface adsorbed PIO on the nanoparticulate formulations but was low as compared to PIO-S. Hence encapsulation of PIO in nanoparticulate formulation has significantly suppressed its hemolytic toxicity. Similarly, ROS-NP was found to have 2.6±0.5% hemolytic toxicity. It may also be attributed to the adsorbed ROS on the surface of nanoparticulate formulation. Lowest haemo-toxicity was observed with Tf-PIO-NP (1.9±0.3%) and Tf-ROS-NP (2.1±0.2%) and this may be ascribed to the synergistic effect of encapsulation of drug in the nanoparticles and a stearic double barrier provided by transferrin conjugation that further minimized the release of drug and its interaction with RBC's. Also, during transferrin conjugation process, the surface adsorbed drug was released which prevented burst release of drug and hence transferrin conjugated formulations produced minimum haemo-toxicity.

Additionally, the blank PLGA NP's showed negligible hemolysis suggesting that polymer was not responsible for hemolysis and it was mainly due to drug (PIO/ROS).

It can be concluded from this study that transferrin anchored PLGA NP's are safer for use as an intravenous carrier of the drugs.

MTT Cytotoxicity studies

The β-amyloid, a sticky self-adhesive peptide tend to polymerize and form β-sheet rich fibrillar aggregates which eventually give rise to the senile plaques, a pathological hallmark of Alzheimer's disease (AD) (Tierney et al., 1988). It is generally believed that the fibrillar Aβ and/or the processes leading to its formation are neurotoxic, inducing neuronal cells death in culture (Lorenzo and Yankner, 1994) and neuronal loss *in vivo* (Geula et al., 1998). Thus, it is not surprising that agents that antagonize the toxicity and/or attenuate the formation of the toxic Aβ species are being actively pursued as potential intervention therapies for AD. In this regard, the toxic effect of Aβ on cultured cortical neurons or other Aβ-sensitive neuronal or non-neuronal cells or cell lines (Gschwind and Huber, 1995; Xu et al., 2001; Yankner et al., 1990) can be used as a primary screening assay in the search for potential leads.

The *ex vivo* cytoprotective activity of the optimized nanocarrier systems against cytotoxic Aβ was assessed on mouse neuroblastoma (neuro-2a) cells. The media was not changed during the 48 hrs exposure period. Normal cell growth was monitored in the control group in which neither of the Aβ or drug formulations (drug solution or drug loaded nanoparticles) were added. The drug amount from all the formulations was adjusted to be the same as that of drug solution.

The results of cytoprotective studies performed in neuro-2a brain cells by MTT assay are recorded in **Table 7.2** and shown graphically in **Fig. 7.1 - 7.2**. To facilitate the basis of comparison, drug solution, drug loaded unconjugated nanoparticles and transferrin conjugated nanoparticles were added at same concentration of drugs.

From the initial experiments, it was found that as compared to control (untreated cells), there was no significant cytotoxicity observed from the blank PLGA nanoparticles and transferrin conjugated blank nanoparticles in the study and hence, their toxicity was not considered for the study.

In the presence of Aβ, the cell viability was drastically reduced in the wells of control group (77.1±3.6%). When cells were incubated with Aβ along with PIO solution (in DMSO), PIO-NP and Tf-PIO-NP, at a concentration of 5.0μM/ml, the percent cell viability was found to be 67.7±2.3% (after subtraction of solvent toxicity), 85.8±3.7% and 86.8±3.4%, respectively. Similarly, in presence of Aβ, ROS solution (in PBS pH 7.4), ROS-NP and Tf-ROS-NP, at a concentration of 5.0μM/ml, showed percent viability of 81.7±3.0%, 107.2±2.8% and 138.5±2.1%, respectively after 48 h of incubation. The data clearly suggested that drug loaded nanoparticulate formulations have significantly decreased the cell death mediated by Aβ and this might be due to the cell protective action of drugs (PIO and ROS) against Aβ induced cell death. Nanoparticulate formulations, both unconjugated and transferrin conjugated nanoparticles, were found to have higher cytoprotective activity as compared to corresponding drug solutions as very high percent cell viability was observed. Maximum cytoprotective activity was observed in case of transferrin conjugated nanoparticles. This may be attributed to the presence of transferrin on the NP's surface which possibly facilitated greater internalization of NP's and hence drug, via its receptors

present on the surface of neuroblastoma cells. Augmented entry in the cells followed by sustained release of drug might be the rational for enhanced % cell survival. These results were in agreement with the findings of earlier reports (Gan and Feng, 2010).

The free drugs have shown cytotoxic effect at low concentration (0.5 μM/ml) while cytoprotective activity was obtained at high concentration (5 μM/ml).

As shown in **Fig. 7.1 - 7.2**, among the drug solutions tested only ROS solution (at concentration of 5μM/ml) significantly protected neuro-2a cells from the toxic effect of Aβ. ROS solution increased the % cell survival and more than 81% of neuro-2a cells survived on adding 5μM/ml of ROS after 48-h incubation with 2.5μM/mL of Aβ. The cytoprotective effect appeared to be concentration-dependent and became significant at the highest concentration of ROS tested i.e. 5 μM/ml. However in the same range of concentration tested, PIO solution exhibited no effect on Aβ-induced cell death, and the overall cell survival was reduced to ~68%.

For both the drugs, PIO and ROS, the cytoprotective action of the drug loaded PLGA nanoparticles and transferrin conjugated PLGA nanoparticles formulation was significantly higher ($p<0.05$) than their corresponding drug solutions at equimolar concentrations. Again, the cytoprotective action was found to be dose dependent, as evident from the results that with increase in drug amount from formulations (0.5 μM to 5.0 μM), the cell viability increased, indicating an increase in the cytoprotective activity of the drug loaded nanoparticles at higher dose.

As shown in **Table 7.2**, after 48 hrs, at all concentrations of PIO, though the nanoparticulate formulations (both unconjugated and Tf-conjugated) showed

higher cytoprotective activity than equimolar drug solution, the difference between unconjugated and conjugated nanoparticles was insignificant ($p>0.05$). At concentrations of 0.5 μM and 1.25 μM, the viability of neuro-2a cells increased in dose dependent manner in case of PIO solution. But at the same concentrations, PIO-NP formulations (both unconjugated and Tf-conjugated) showed insignificant difference in cell viability. Conjugation of transferrin to NP's had not been able to affect neuronal cell viability in presence of Aβ at 0.5 μM and 1.25 μM of PIO.

At a concentration of 1.25 μM tested for ROS, the transferrin conjugated nanoparticles showed greater cytoprotective activity (100.5±3.5) than drug solution (60.1±2.9) and unconjugated nanoparticles (90.3±3.8) and this value was equivalent to control group 1 (i.e. without Aβ). But at higher concentration of ROS, i.e. at 5 μM, the ROS-NP and Tf-ROS-NP showed much higher cytoprotective activity (above 100%) than drug solution and probably this may be due to the cell proliferative activity. After 48 hrs, both ROS-NP and Tf-ROS-NP produced significantly higher cell viability than corresponding drug solution at all concentrations.

Neuro-2a cells and other neuronal cells are reported to express efflux transporters like P-gp and BCRP which may be responsible for low responsiveness of these cells to free drug solution (Lamprechet and Benoit, 2006). PIO and ROS being substrates to P-gp (Abe et al., 1994) could have been exocytosed by P-gp and hence their intracellular levels might have been reduced leading to lower cytoprotective activity of drug solutions as compared to nanoparticulate formulations. The nanoparticulate formulations acted as drug reservoir and released the drug in a sustained manner that reduced the exocytosis of drug thereby giving more cytoprotective activity.

The mouse neuro 2a cells have been reported to show over expression of the transferrin receptors (Recht L et al., 1997, Eavarone D et al., 2000) and this over expression of transferrin receptors leads to selective receptor mediated endocytosis of the transferrin conjugated nanoparticles and thereby resulting in greater intracellular delivery than the unconjugated nanoparticle and drug solution. In the present study, the comparative superior cytoprotective activity along with proliferative effect from transferrin conjugated nanoparticles could be because of enhanced intracellular uptake of transferrin conjugated nanoparticles via transferrin receptors as compared to unconjugated nanoparticles and drug solution which finally led to the enhanced intracellular levels of drug available after internalization of the transferrin conjugated nanoparticles (Qian et al., 2002, Sahoo and Labhsetwar, 2005). Further, the Tf receptor has the ability to recycle within minutes after endocytosis. Single Tf receptor can lead to transport of multiple number of Tf conjugated nanoparticles for greater intracellular drug delivery (Qian et al., 2002, Sahoo et al., 2004). Alternatively, as reported by Sahoo and Labhshetwar, Tf conjugated nanoparticles could have reduced exocytosis than the unconjugated nanoparticles because of difference in uptake pathway between conjugated and unconjugated NPs (Sahoo and Labhshetwar, 2005). Additionally, *in vitro* release study (as discussed in **Section 5.4.8**) indicated that 50% and 20% PIO was released from unconjugated and Tf-conjugated nanoparticles respectively in 3 days. For ROS, the release was found to be 58% and 35% for unconjugated and Tf-conjugated nanoparticles respectively, in 3 days. This indicated the sustained release property of nanoparticles. The greater intracellular availability of transferrin conjugated nanoparticles combined with the sustained release has led to the superior cytoprotective activity.

Although, nanocarriers escape through endosomes, a major fraction of it probably remains in the recycling endosomes and undergoes rapid exocytosis. The fraction of nanocarriers that escapes the endosomes, however may remain inside the cell and release the drug slowly, thus sustaining the intracellular drug levels. The greater proliferative effect of ligand coupled nanocarriers was observed which could be due to greater intracellular uptake of the coupled nanocarriers via transferrin receptors than that of uncoupled nanocarriers. This greater retention could be the result of different pathway of their uptake i.e., via transferrin receptors mediated endocytosis as compared to a non-specific endocytic pathway of uptake for uncoupled nanocarriers. Since, transferrin receptor is recycled after endoctyosis of the carrier appended nanocarriers, multiple intracellular deliveries of ligand coupled nanocarriers through use of a single receptor occurs, thus leading to increased drug delivery. Moreover, slow intracellular release of the drug from the ligand appended nanocarriers localized inside the cells is expected to sustain the drug effect, and hence increase its overall therapeutic efficacy.

At a concentration of 0.5μM/ml, the higher effectiveness of ROS-NP as compared to Tf-ROS-NP might be due to the differences in easy higher availability of drug due to faster release of drug from ROS-NP as compared to Tf-ROS-NP. However, the drugs entrapped transferrin conjugated nanocarriers were found to be more cytoprotective than unconjugated nanocarriers at higher drug concentrations tested.

Thus, the phenomenon appeared to correspond reasonably well to the cellular uptake efficiency and drug release properties.

The blank nanocarriers (without drug(s)) showed no significant toxicity to neuro-2a cells which implies that these nanocarriers are non-toxic to the cells.

Hence, it can be concluded that these nanocarriers could be used as a safe drug delivery system with no significant cytotoxicity load.

It may also be concluded that the transferrin coupled nanoparticles possess better internalizing ability than unconjugated NP formulations.

However, recently it has been shown that a major fraction of the internalized NPs undergoes rapid exocytosis (Panyam and Labhasetwar, 2003). This seems to occur because of their inefficient escape from the endosomal compartment to the cytoplasmic compartment during their transit through the cell. It was hypothesized that Tf-conjugated NPs could have a different intracellular sorting pathway following their uptake via TfR than that of unconjugated NPs via nonspecific endocytosis. This difference in the uptake and sorting pathways of conjugated and unconjugated NPs could influence the intracellular retention of NPs as well as the therapeutic efficacy of the encapsulated agent (Sahoo and Labhasetwar; 2005).

Furthermore, in an appropriate animal model or human host, higher percent loadings that would achieve optimum release over much longer period of time, may be possible. Delivery of drugs by means of nanoparticulate formulations was able to reduce the hemolytic toxicity of Pioglitazone and Rosiglitazone. This is an important property that will be necessary for extended studies involving the use of these formulations to treat brain disorders in appropriate animal models and eventually in humans.

7.4 CONCLUSION

The blank PLGA nanoparticles demonstrated no cytotoxicity on mouse neuroblastoma cells suggesting the use of PLGA nanoparticles as safe carriers for delivery of antidiabetic agents. ROS formulations demonstrated better

cytoprotective activity than PIO formulations. The higher cytoprotective activity of the Tf conjugated nanoparticles was confirmed by microscopy. The formulations of PIO showed less cytoprotective effect as compared to those of ROS. This may be because, the two drugs (ROS and PIO) have been tested on neuro-2a cell line in equimolar concentrations for all formulations. The anti-diabetic dose of PIO is 15 mg and that of ROS is 1.5 mg. Hence, the dose of PIO required to sensitize PPAR-γ receptors is 10 times as compared to ROS. Therefore, PIO was not able to produce equivalent cytoprotective activity on neuro-2a cells in presence of β-amyloid as compared to ROS. Hence, it can be concluded that nanoparticles are suitable carriers to deliver ROS and PIO for higher and prolonged cytoprotective effect on neuroblastoma cells in presence of β-amyloid which is responsible for Alzheimer's disease.

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