Chapter 8
In-vivo studies

8 In-vivo studies

8.1 Background

In-vivo is latin word meaning ‘within the living’ which deals with studies to determine effects of various biological agents by testing them on whole, living organisms like animals including humans. Although, there are some moral and economic issues in-vivo studies are highly favored as it has several advantages including (i) assist in innovation of new drugs and treatments (ii) ensure safety (iii) can give idea of effect of administered formulation on living body (iv) cell culture fail to predict toxic effects like cardiac arrest, rashes, tumors etc. while computer models fail to predict unknown variables which can be overcome by use of animal testing.

The in-vivo pharmacological study comprised of acute toxicity study, efficacy study using suitable disease model, distribution study and nasal ciliotoxicity study. Acute toxicity consists of dose dependent adverse or toxic effects, which may cause functional impairment or lesions, that occur within short period of time after dosage administration. This study is aimed to determine lethal dose (LD_{50}), safety and therapeutic index of the administered drug or drug product [1, 2]. Similarly, histological study of nasal mucosa in terms of nasal ciliotoxicity study was performed to assess whether administered formulation has any deteriorating effect on absorptive cells of nasal epithelia [3]. This study also predicted local and systemic adverse effects due to administered formulation and thereby predicting whether it is suitable for in-vivo administration. Additionally, absorption enhancers used in intranasal formulation have reports of affecting temporary/ permanent damage to nasal mucosa along with irritation and inflammation, which can be assessed by nasal ciliotoxicity study [4, 5]. Furthermore, distribution study was performed to estimate the delivery extent of administered formulations to brain [6]. Finally, efficacy of prepared drug product, at proposed dose, was estimated by phencyclidine induced rat model which resulted in schizophrenia like symptoms by augmenting NRG1 expression [7, 8].

All protocols and study described in the present work were approved by the Institutional Animal Ethical Committee (IAEC) of Pharmacy Department, The M. S. University of Baroda with registration number MSU/IAEC/2014-15/1441. The experimentation was carried out with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

8.2 Selection of animals

The selection of animal model and particular species was based on the nature of study. For acute toxicity study, swiss-albino mice of 6-8 weeks having weight of 25-30 gm were employed. For, nasal ciliotoxicity study, adult wistar rats of either sex having weight of 180-250 gm were employed. Likewise, for distribution study 12-14 weeks old wistar rats of either sex having weight of 180-250 gm were employed. Finally, efficacy study was performed in phencyclidine induced schizophrenic model by employing 1 week old male pups of wistar rats having weight of 10-15 gm.

8.3 Housing and feeding

Animals were housed in propylene cages in animal houses under controlled environmental conditions with temperature maintained at 22 ± 2 °C and relative humidity around 55 ± 5%. Three animals were housed per cage and exposed to artificial lighting with alternating 12 h light and 12 h dark condition. The animals were fed ad-libitum with standard rodent diet with free access to drinking water.

8.4 Preparation of animals

Animals were selected randomly, divided into respective groups and marked accordingly for suitable identification of the respective treatment groups. Animals were acclimatized in the laboratory conditions before initiating study.

8.5 Acute toxicity study

8.5.1 Preparation and administration of formulation

The lyophilized copolymers and conjugates were solubilized in suitable solvents and polymeric solution of desired concentration was prepared such that maximum desired concentration of polymers was obtained in 20 µl. Such prepared polymeric solutions were administered via. intranasal route to respective group of animals. For intranasal administration, 10 µl of prepared solution was administered to each nostril using micropipette attached to low density polyethylene tube with 0.1 mm internal diameter [9].

8.5.2 Main test

Before initiation of study, the animals were fasted overnight and then weight of each animal was recorded. The dose of polymeric solution was administered via. intranasal route as a single dose but if single dosing was not possible then the polymeric solution was administered in fractions over a period of 24 h. After dosage

administration animals were withheld from food for 2-3 h but if multiple dosing approach was employed then the duration was decided according to dosing interval.

8.5.3 MTD determination

The swiss albino mice were randomly divided into respective groups for administration of copolymers/ conjugates such that 3 mice were allocated to each group. The study was performed by the fixed dose procedure as mentioned in OECD guidelines. Initial dose of all polymers, as decided by sighting study, was administered intranasally in each group and the animals were observed for 14 days for any signs of toxicity or mortality. The dose was escalated and administered to another group of animals when 2 or more mice survived per group. The study was continued till safety of the prepared carrier was validated (approximately 10 times the proposed dose). All the animals used in study were weighed before initiation and after completion of the study. Additionally, moribund animals or those showing signs of distress were euthanized.

8.6 Nasal ciliotoxicity study

Adult wistar rats were divided into groups such that each group contained three animals (n=3). All rats were anaesthetized using ketamine (100 mg/kg) and diazepam (5 mg/kg) and polymeric solution was administered intranasally. The polymeric solution was kept in contact of nasal mucosa for 6 h and later animals were sacrificed by anesthetic overdose. Then, nasal mucosa from sacrificed rats was isolated and kept overnight in formalin solution for fixation. Later, wax blocks of mucosa were prepared, stained using hematoxylin-eosin and examined under light microscopy. Nasal mucosa of rats treated with IPA was used as positive control while nasal mucosa of PBS (pH 7.4) treated rats was used as negative control.

8.7 Brain distribution study

Adult wistar rats were divided into groups such that each group contained three animals (n=3). All rats were anaesthetized using ketamine (100 mg/kg) and xylazine (20 mg/kg) and were given additional doses as needed throughout the experiment [10]. For intranasal administration, fluorescently labelled polyplexes or lipopolyplex (Cy5-siRNA conjugated formulations) were delivered with micropipette attached to low density polyethylene tube with 0.1 mm internal diameter. To determine the distribution in brain tissue after intranasal administration, it was harvested 2 h after intranasal administration. Later on, this was washed with phosphate-buffered saline (PBS) and

homogenized for 3 min at 12,000 rpm in lysis buffer (1 mM EDTA, 0.05% Triton X-100, 0.1% Tween-80, 2 M NaCl, and 0.1 M Tris-HCl) using a homogenizer. Subsequently, the homogenates were centrifuged and fluorescence intensity in the brain tissue supernatants was detected using fluorimeter [11].

8.8 Efficacy study

3 male pups of wistar rats were allocated to each group for efficacy study. Schizophrenia was induced in pups by intraperitoneal administration of phencyclidine (10 mg/kg) on postnatal day 7, 9 and 11. After washout period of phencyclidine (7 days) the pups were treated with the prepared formulations and saline. At postnatal day 70, pups were sacrificed and brain homogenate was utilized to estimate NRG1 protein levels by western blot technique [12, 13]. Additionally, gene expression levels were estimated for each group using polymerase chain reaction (RT-PCR).

8.8.1 Western blot

8.8.1.1 Preparation of lysate

- The brain tissue was dissected and 300 µl ice-cold lysis buffer, composition of which is depicted in Table 8. 1, was added to 5 mg tissue. This was homogenized with electric homogenizer and rinsed twice with 200 µl ice-cold lysis buffer.
- This was then kept under constant agitation for 2 h at 4 °C by placing it on orbital shaker.
- The resulting homogenate was centrifuged at 12000 rpm for 20 min maintaining temperature at 4 °C. the supernatant was collected in fresh microcentrifuge tubes kept on ice and the remaining pellet was discarded.

Table 8. 1: Composition of lysis buffer (NP-40 buffer)

Reagents	**Composition**
Sodium chloride	150 mM
Triton X-100	0.1%
Tris-HCl, pH 8.0	50 mM

8.8.1.2 Sample preparation

- The lysate from each sample was analyzed for protein concentration using Bradford reagent.
- According to protein concentration, sample volume for each lysate (equivalent to 30 µg protein concentration) was taken in microcentrifuge tube and equal volume of 2x laemmli buffer (Table 8. 2) was added.

- The resulting mixture was boiled at 95 °C for 5 min to reduce and denature the sample proteins.

Table 8. 2: Composition of 2x Laemmli buffer

Reagents	**Composition**
SDS	4%
2-mercaptoethanol	10%
Glycerol	20%
Bromophenol blue	0.004%
Tris-HCl	0.125 M
Adjust pH to 6.8	

8.8.1.3 Loading and running gel

- The sample prepared was loaded in wells of SDS-PAGE gel – 12% resolving gel and 4% stacking gel, along with 15-20 µl molecular weight marker in first well.
- This SDS-PAGE was performed using Mini-Protean electrophoresis system (Biorad, USA) at 100 V till dye front reaches ~1 cm above the bottom of gel.

8.8.1.4 Transfer proteins on membrane

- The bands formed on SDS-PAGE gel were transferred on nitrocellulose membrane using Mini-Trans Blot electrophoretic transfer cell (Biorad, USA).
- The stack was assembled as depicted in Figure 8. 1.

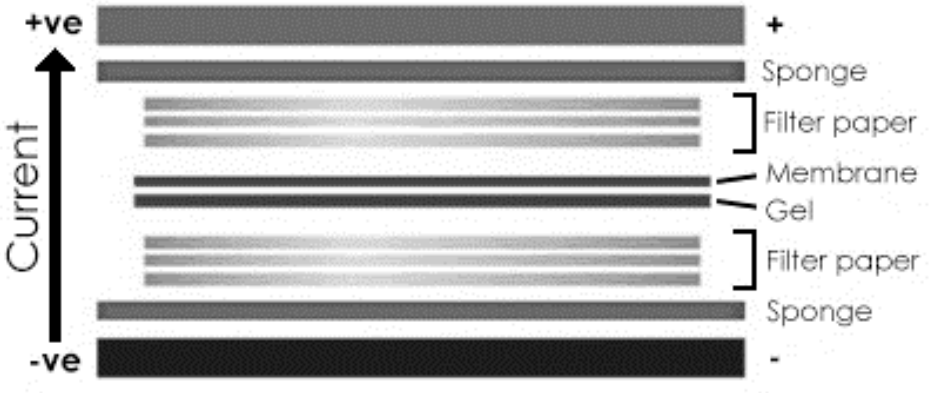

Figure 8. 1: Representation of gel stack for transfer of bands on membrane

- The protein bands were transferred at 100 V for 1.5 h using 2 l ice cold transfer buffer, the composition of which is described in Table 8. 3.
- The entire process was carried out in cold condition and thus gel packs were used to regulate the temperature of buffer.
- The completion of transfer of protein bands was assured by Ponceau S staining.

Table 8. 3: Composition of transfer buffer

Reagents	**Composition**
Tris base	25 mM
Glycine	190 mM
Methanol	20%
Adjust pH to 8.3	

8.8.1.5 Antibody staining

- The nitrocellulose membrane after transfer, was blocked using 5-10 ml 5% bovine serum albumin solution, viz. used as blocking buffer, at 25 °C for 1 h on gel rocker (L1-GR-E-100, GeNei, India).
- The blot was incubated with appropriately diluted NRG1 isoform-10 antibody (Signalway antibody, USA) in blocking buffer for 12 h at 4 °C on gel rocker.
- Later, the blot was washed 4 times, 15 min each, with PBST buffer (0.1% Tween-20 in phosphate buffer saline pH=7.4) on gel rocker.
- Subsequently, the blot was incubated with appropriately diluted anti-rabbit IgG antibody (Sigma Aldrich, USA) in blocking buffer at 25 °C for 1 h on gel rocker.
- Furthermore, the blot was washed 4 times, 15 min each, with PBST buffer and 3 times, 15 min each, with PBS (pH=7.4) buffer on gel rocker.
- The resultant blot was incubated with Clarity ECL western blotting substrate (Biorad, USA) for 5 min in dark.
- This was immediately observed for chemiluminescence in Alliance 4.7 western blot imaging system (Uvitec, Cambridge, UK).
- The blot was stripped using stripping buffer (Table 8. 4) for 5-10 min at 25 °C. This was washed twice with PBS (pH=7.4) buffer for 10 min and twice with TBST buffer (Table 8. 5) for 5 min.
- The blot thus obtained was reprobed for β-actin which was used as loading control.

Table 8. 4: Composition of stripping buffer

Reagents	**Composition**
Glycine	1.5%
SDS	0.1%
Tween 20	1%
Adjust pH to 2.2	

Table 8. 5: Composition of TBST buffer

Reagents	**Composition**
Tris base	20 mM
Sodium chloride	150 mM
Tween 20	0.1%
Adjust pH to 7.6	

8.8.2 Gene expression study

Gene expression study from the isolated brain tissue was performed by RT-PCR as per the procedure described previously in Section 6.8.2.

8.9 Result and discussion

8.9.1 Acute toxicity study

Acute toxicity study, performed on swiss albino mice, was done to prove safety profile of developed copolymers and conjugates in-vivo. All the synthesized copolymers and conjugate including bPEI-HA copolymer, bPEI-Lf conjugate, bPEI-Chi copolymer, biodegradable bPEI-Chi copolymer, were administered initially at dose equivalent to 2.5 nmole conjugated siRNA. The animals, when observed for 14 days, showed no signs of morbidity or mortality. Additionally, there was no alteration in initial and final weight of mice thus proving safety of the polymers at administered dose. Later, the dose was escalated up to 10 times the initial dose and similarly no toxicity was observed. Furthermore, optimized formulation of polyplex and lipopolyplex was administered at the highest safe dose of copolymers and conjugates. Conjugation of siRNA at the maximum dose didn't have any significant effect on toxicity profile of the polymers. The results concluded that the synthesized formulations were safe, up to 10 times the therapeutic dose, in the animals tested.

8.9.2 Nasal ciliotoxicity study

The result of nasal ciliotoxicity study performed on wistar rats, when observed under optical microscope, is demonstrated in .

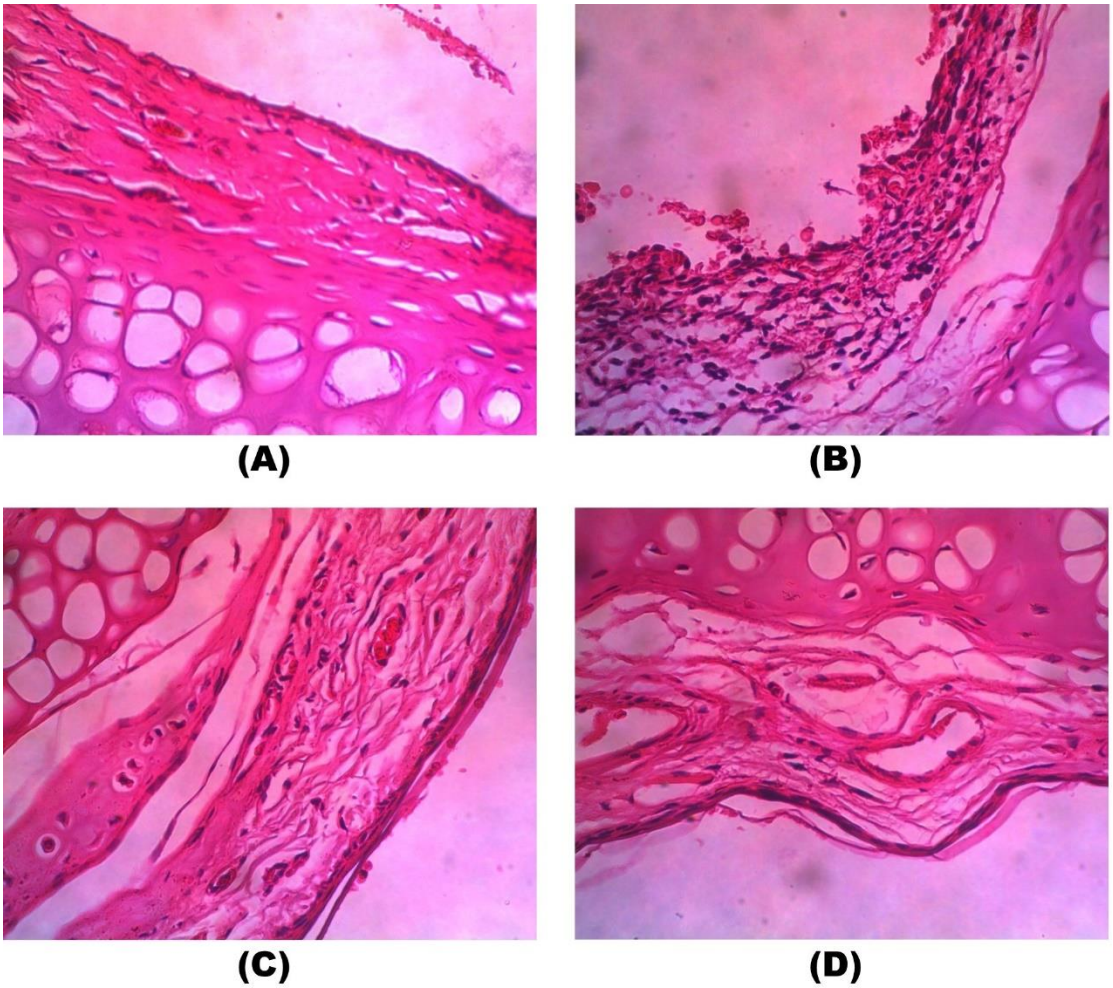

Figure 8. 2: Nasal ciliotoxicity study showing (A) PBS pH 7.4 (B) Isopropyl alcohol (C) Polyplex and (D) Lipopolyplex treated rat nasal mucosa.

The above results demonstrated that treatment of PBS pH 7.4 did not have any effect on homogeneity of epithelial layer which was used as normal control while isopropyl alcohol (B) caused complete disruption of cilia and epidermal tissue along with deformed deeper tissues that was considered as negative control. Optical microscopy of polyplex (C) and lipopolyplex (D) treated nasal mucosa showed similar characteristics as normal control indicating safety of the developed formulations and supporting data obtained using acute toxicity study. Additionally, there was

insignificant inflammation of nasal mucosa treated with optimized formulations signifying biocompatibility of developed formulations [14].

8.9.3 Brain distribution study

The result of brain distribution study determined by performing fluorimetry on brain homogenate is summarized in Table 8. 6 and represented graphically in Figure 8. 3.

Table 8. 6: % brain distribution of different formulations

Formulation	% brain distribution Mean ± SD
Naked siRNA	35.99 ± 3.04
Biodegradable bPEI-Chi polyplex	89.53 ± 2.09
Lipopolyplex	87.60 ± 2.66

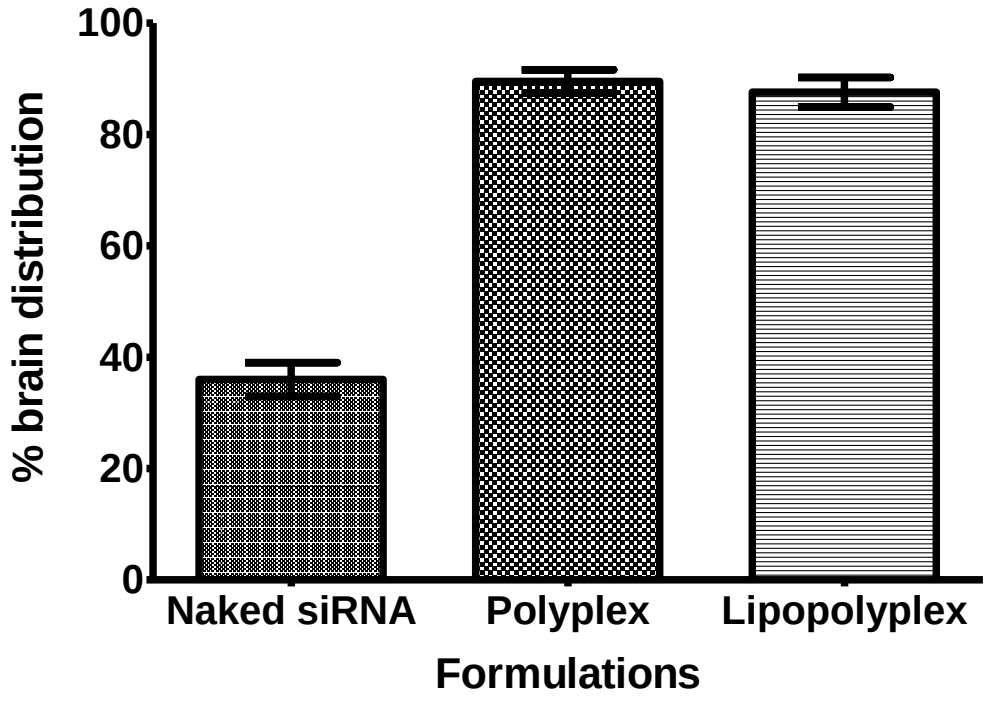

Figure 8. 3: Graphical representation of % brain distribution of different formulations

The result concluded that naked siRNA when delivered through nasal route showed insignificant delivery to the target site which might be attributed to their hydrophilicity with net negative surface charge and higher molecular weight, both of which impede its brain distribution [15]. Additionally, naked siRNA has very low half-life, around 10 min, due to its degradation by nucleases. Even after its endocytosis, it is uptaken by lysosomes where it is cleaved resulting in loss of its therapeutic action [16-

19]. Conversely, there was drastic improvement in brain uptake of siRNA when administered in form of polyplex or lipopolyplex which was around 2-fold than naked siRNA. Such improved uptake might be due to augmented endosomal escape preventing its hydrolytic degradation from lysosomal enzymes. Furthermore, such formulation enhances cellular uptake of siRNA and its release and thereby facilitate its accumulation in cytosol. All these factors together contribute in improved brain distribution of conjugated siRNA as compared to naked siRNA [20].

8.9.4 Efficacy study

8.9.4.1 Western blot

The result of western blot, used to estimate NRG1 protein levels and thereby determining efficacy of administered formulations, is shown in Figure 8. 4, represented graphically in Figure 8. 5 and summarized in Table 8. 7.

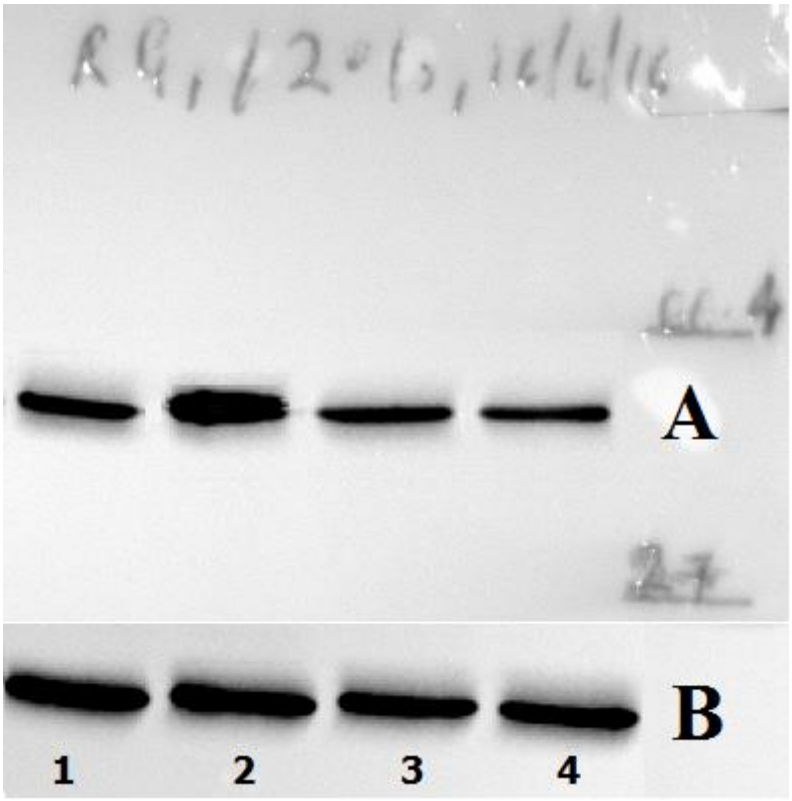

Figure 8. 4: NRG1 expression measured by western blot. Here (A) represent NRG1 expression while (B) represent expression of housekeeping β-actin gene.
Lane 1: Normal control; Lane 2: Diseased control; Lane 3: Polyplex treated; Lane 4: Lipopolyplex treated

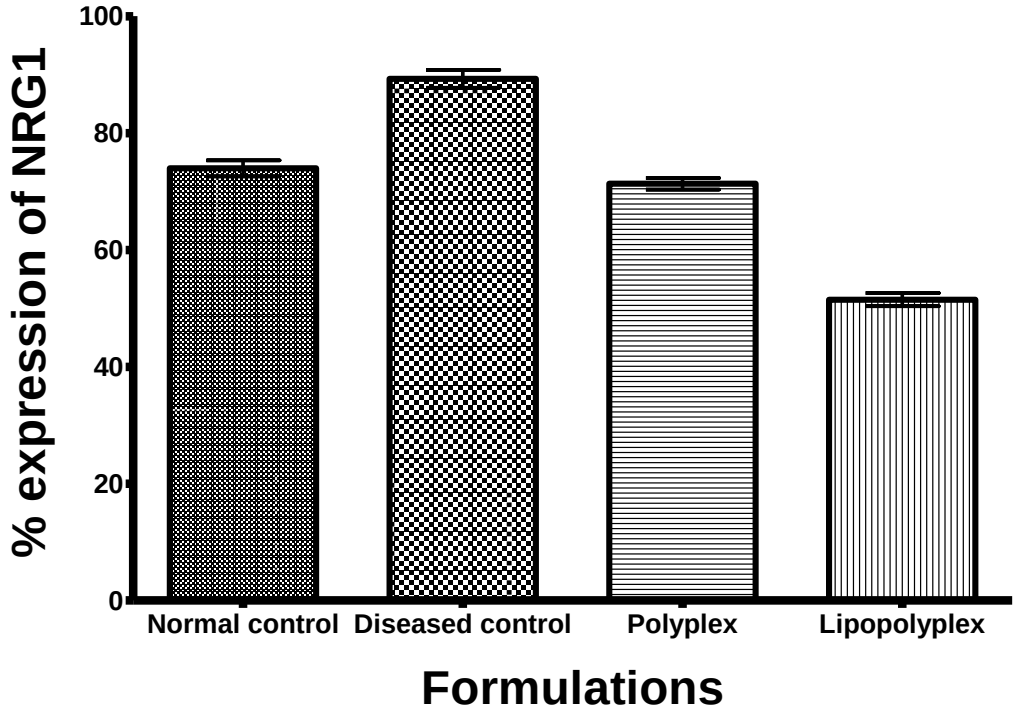

Figure 8. 5: Graphical representation of % NRG1 expression using western blot

Table 8. 7: % NRG1 expression of different treatment groups using western blotting

Treatment	**% NRG1 expression Mean ± SD**
Normal control	73.96 ± 1.36
Diseased control	89.29 ± 1.52
Biodegradable bPEI-Chi polyplex	71.33 ± 0.99
Lipopolyplex	51.49 ± 1.14

8.9.4.2 RT-PCR

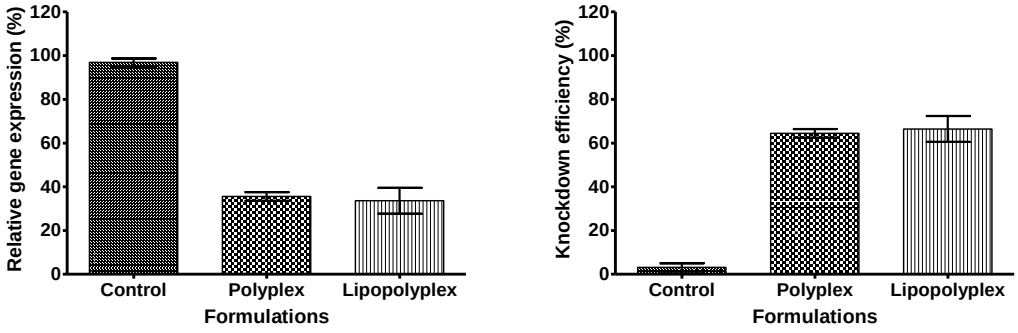

Figure 8. 6: Gene expression and knockdown efficiency of NRG1 siRNA estimated by RT-PCR

Table 8. 8: % NRG1 expression and knockdown efficiency of different treatment groups estimated by RT-PCR

Treatment	**% NRG1 expression Mean ± SD**	**Knockdown efficiency Mean ± SD**
Control	96.83 ± 1.86	3.17 ± 1.86
Biodegradable bPEI-Chi polyplex	35.58 ± 2.01	64.42 ± 2.01
Lipopolyplex	33.60 ± 5.95	66.40 ± 5.95

Figure 8. 6 represent the results of gene expression study performed by RT-PCR and expressed as % knockdown efficiency and relative gene expression (w.r.t. control) and summarized in Table 8. 8.

The results of NRG1 protein levels in diseased as well as treated groups estimated by western blotting (Table 8. 7) proved that there was considerable reduction of protein expression from 89.29 ± 1.52% to 71.33 ± 0.99% (Polyplex) and 89.29 ± 1.52% to 51.49 ± 1.14% (Lipopolyplex) when normal protein level in control group was about 73.96 ± 1.36% which depicted considerable reduction in protein expression on siRNA administration. Furthermore, gene expression levels in disease model and treatment groups, performed by RT-PCR, showed approximately 60% gene knockdown in treated groups as compared to disease control group as shown in Figure 8. 6. This improved efficacy in polyplex and lipopolyplex formulation may be attributed to increased uptake and release of siRNA from the conjugated vector and thereby results in higher accumulation of siRNA in cytosol. This along with reduced degradation of siRNA causes higher therapeutic efficiency at same concentration as compared to naked siRNA. Thus, vector conjugated siRNA can effectively result in higher gene knockdown, in turn downregulate overexpressed NRG1 gene during schizophrenic condition and thereby maintaining optimum gene expression.

8.10 References

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