7. ANIMAL STUDIES

PAH is a complex, devastating, incompletely understood pathophysiological condition predominantly affecting the small pulmonary arteries. It is characterized by vasoconstriction, right ventricular hypertrophy, intimal lesions, medial hypertrophy, and adventitial thickening of the precapillary pulmonary arteries. The progressive pulmonary hypertension leads to right-sided heart failure and death due to increased afterload on right part of the heart. Increased vasomotor tone and chronic remodeling of the precapillary resistance vessels, including marked vascular smooth muscle cell growth are assumed to be underlying pathogenetic mechanisms. Current therapy is associated with inconvenience of frequent administration; moreover, the parenteral and peroral administration of the drugs to treat PAH is accompanied with non-specific systemic vasodilation leading to many side effects like postural hypotension, headache, flushing and visual disturbances.

A number of animal models have been used to study pulmonary hypertension, most commonly employing hypoxia or monocrotaline. Monocrotaline is a toxin that causes endothelial injury leading to medial hypertrophy in the pulmonary arterioles. Also, pneumonectomized rats injected with monocrotaline have exhibited neointimal overgrowth in addition to medial hypertrophy (1). In our study, model of monocrotaline (MCT) induced chronic pulmonary arterial hypertension was used. MCT causes injury to endothelium of pulmonary arterioles which in turn causes pulmonary artery smooth muscle hypertrophy with persistent severe pulmonary hypertension after one injection in rats (2). We investigated the *in vivo* efficacy of siRNA nanoplexes formulations after administration through intra-tracheal route in treatment of PAH.

All experiments and protocol described in the present study were approved by the Institutional Animal Ethical Committee (IAEC) of Pharmacy Department, The M. S. University of Baroda and 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.

7.1 Methods

7.1.1 Selection of Animals Species

Male wistar rats 7-8 weeks old and weighing between 180-220 g were used to determine efficacy of developed siRNA formulation in treatment of pulmonary hypertension.

For acute toxicity studies female Sprague Dawley rats were used. Female sex was used as females are generally slightly more sensitive to such studies (3). Animals used were healthy, nulliparous and non-pregnant young adults of 8-12 weeks age at the time of study.

7.1.2 Housing and Feeding Conditions

Animal rooms were maintained at 20-25°C and were provided with artificial lighting in the cycles of 12 hr light and 12 hr dark. Individually housed animals were supplied with unlimited supply of conventional rodent diet and drinking water.

7.1.3 Preparation of Animals

Random selection of animals was performed and each animal was marked for identification. Animals were kept in their cages for periods of at least 5 days before dosing sequence was started in order to acclimatize animals with the laboratory conditions.

7.1.4 Monocrotaline (MCT) Administration

Monocrotaline was administered to induce pulmonary arterial hypertension in rats. MCT dissolved in 0.1 N HCl (pH 7.4 adjusted with 0.1N NaOH) was injected subcutaneously at a dose of 60 mg/Kg. Animal grouping was done to have six animals in each group.

7.1.5 Effects of Pulmonary Delivery of FGF2 siRNA Dry Powder Formulations in MCT-induced Pulmonary Hypertensive Rats

Potential effects of FGF2 knockdown was assessed after the development of PH induced by MCT (60 mg/kg s.c.). For treatment, the rats were left untreated for 21 days for induction of PH after MCT injection. Animals then received the treatment with formulations containing FGF2-siRNA (5 nmol/kg). Dry powder formulations containing nanaoplexes equivalent to required dose were rehydrated with nuclease free water and these suspensions were used for Intratracheal administration (**Figure 7.1**). The FGF2-siRNA formulations were administered via intra-tracheal route on day 21 after the MCT injection.

Anaesthesia was given by intraperitoneal injection of xylazine (5 mg/kg) and ketamine (50 mg/kg) and transtracheal instillation of test substances and control substance (Saline) was performed. Trachea was surgically exposed on the ventral side of the neck of the rat and tracheal puncture was performed by a needle just below the larynx. A needle of Hamilton

syringe was inserted into the hole and advanced to the bifurcation of the trachea. Required amount of siRNA nanoplexes formulation were slowly instilled over a 1-min period using a Hamilton syringe. Following instillation, the tubing was withdrawn and a small drop of cyanoacrylate adhesive was placed over the hole to seal the opening. The skin was clothed with 3-0 Dexon sutures. Animals were allowed to recover from anesthesia under a heating lamp. After recovery, animals were housed in individual plastic cages with access to food and water for the remainder of the study.

Group I: Normal control (Healthy rats)

Group II: Positive control (No treatment was given after monocrotaline administration)

Group III: Placebo control (Intratracheal administration of saline)

Group IV: Intratracheal administration of PDPI 1

Group V: Intratracheal administration of PDPI 2

Group VI: Intratracheal administration of PDPI 3

Group VII: Intratracheal administration of LDPI

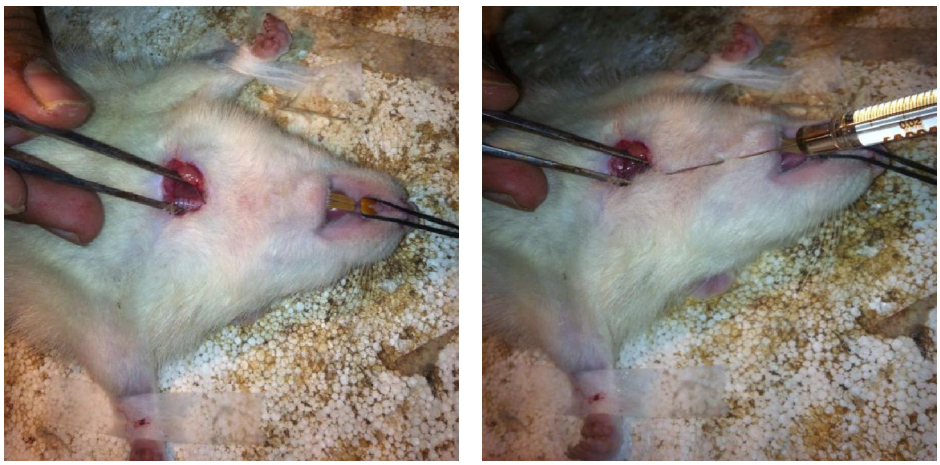


Figure 7.1 Intratracheal administration in rats.

7.1.6 Study Parameters

Increase in right ventricular systolic pressure occurring due to pulmonary arterial vasoconstriction, vascular remodelling and right ventricular hypertrophy along with intimal lesions and medial hypertrophy characterize the PAH. Hence, hemodynamic study, histo-pathological observation, right ventricular hypertrophy measurement and gene expression

study were carried out to mark the progress of the disease and to evaluate the effectiveness of the treatment with siRNA formulations after 42 days of MCT administration.

7.1.6.1 Hemodynamic Measurements

A right heart catheter (PE 50 tubing), attached with heparinized saline filled syringe, was inserted through right jugular vein into the right ventricles for measurement of mean right ventricular systolic pressure. The left carotid artery was cannulated using another catheter for monitoring mean systemic arterial pressure. All pressure measurements were made through reusable BP transducer using chart 5 of Power LAB®. Software setup with PowerLab systems (ML4/30 PowerLab, ADInstruments, Colorado Springs, USA). After exsanguinations, the left lung was fixed in 10% neutral-buffered formalin for histopathological study and the right lung was immediately stored in labelled tubes at -76 ºC for further use in mRNA quantification.

7.1.6.2 Effect on FGF2 mRNA Level in Lung Homogenate

FGF2 expression was examined in lungs from rats of normal control, positive control, Placebo control and from treatment groups. Levels of FGF2 mRNA were also measured in rat lungs in all the groups on day 42nd after MCT administration. Tissue samples from rat lungs were homogenized in cold PBS. Total RNA was extracted as described in chapter 5 by using 100 mg of tissue homogenates. RNA concentrations were determined using standard spectrophotometric techniques, and RNA integrity was assessed by visual inspection of ethidium bromide stained agarose gels. Further process to quantify the mRNA level by RT-PCR was done as given in Chapter 5 (**Table 7.1**).

Table 7.1 Primers for RT-PCR

Primer	Sequence (5'->3')	Template strand	Length	Start	Stop	T_m
Rat FGF2 primers						
Forward primer	TCCATCAAGGGAGTGTGTGC	Plus	20	746	765	59.96
everse primer	TCCGTGACCGGTAAGTGTTG	Minus	20	884	865	59.97
Product length	139					
GAPDH primers						
Forward primer	TGTGAACGGATTTGGCCGTA	Plus	20	91	110	59.96
Reverse primer	GATGGTGATGGGTTTCCCGT	Minus	20	298	279	60.03
Product length	208					

7.1.6.3 Right Ventricular Hypertrophy Measurement

Atria were removed from the isolated hearts and dissection was done to separate right ventricle (RV) from left ventricle (LV) and septum (S). Ventricles and septum were dried well by blotting and were weighed accurately. Weight of RV (g) and total combined weight of LV and S (g) was determined and ratio of weights of RV and LV+S was taken and expressed as a percentage as an index of right ventricular hypertrophy.

7.1.6.4 Histopathology

Fixed left lobes of rat lungs were cut from anterior, posterior and the hilus part and embedded in paraffin. Section of 5 μm thickness were cut using microtom (MICROM) and mounted on slides. Hematoxylin-Eosin staining was performed on mount sections using regular histopathological technique. Stained sections were microscopically evaluated and photographs were taken on Nikon Eclipse E600 microscope mounted with camera using NIS-Elements software. Sections were evaluated for the presence of inflammatory reactions, musculinization of arteries and medical thickening of peribronchial arteries as it leads to development of the disease and increment of pressure on arterial walls.

7.1.7 Acute toxicity study

7.1.7.1 Preparation of Doses

Synthesized polymers and placebo liposomes were administered in a constant dose volume of 2 mL/kg by varying the concentration of the dosing preparation. Volume of dose was chosen based on the literature that showed that lower volumes (<1 mL) show uneven distribution of the test preparations in the lungs while higher volumes (>2 mL) can show potential vehicle effects as well as coughing effects in some species and subsequent loss of administered dose of preparation (4). All doses were prepared and sterilized by passing through a 0.2 μ membrane filter prior to administration.

7.1.7.2 Administration of Doses

Rats were weighed prior to anaesthesia. Anaesthesia was given by intraperitoneal injection of xylazine (5 mg/kg) and ketamine (50 mg/kg) and transtracheal instillation of test substances and control substance (Saline) was performed. Trachea was surgically exposed on the ventral side of the neck of the rat and tracheal puncture was performed by a needle just below the larynx (5). Rats administered with polymer solution and placebo liposomes were assigned as test animals and rats administered with vehicle only were assigned as placebo control animals. Penetration into lower airways and lungs was ensured by placing rats in dorsal recumbency during recovery form anaesthesia.

7.1.7.3 Numbers of Animals and Dose Levels

1. For each carrier evaluated (i.e. AA, AH, AL and placebo liposomes), 6 female rats were used. Two dose levels were chosen and for each dose level used, three female rats were used.

2. The dosing level of each carrier was chosen based on the amount of the polymer or lipid amount required for delivery of therapeutic concentration of siRNA i.e. 5 nmoles/kg. Dosing levels of carriers are given in **Table 7.2.**

Table 7.2 Dosing levels of carriers

Sr No	Formulation	Doses (mg/kg)	
1.	AA	150 µg/kg	750 µg/kg
2.	AH	150 µg/kg	750 µg/kg
3.	AL	150 µg/kg	750 µg/kg
4.	Liposomes (on lipid basis)	4 mg/kg	20 mg/kg
5.	Saline (Normal Control)	-	-

7.1.7.4 Clinical Observations

Animals were observed closely and frequently following instillation and all observations were recorded systematically for each animal. Animals found in moribund status or showing severe pain or distress was to humanely kill as described earlier. Poor appearance of animals immediately after instillation of dose was not considered a treatment related clinical sign. Observations to record included any changes in skin or fur, behaviour patterns, tremors, convulsions, diarrhoea, sleep, salivation, coma etc. Respiratory distress signs like dyspnea, bradypnea, apnea or hyperpnoea, etc. were checked if present. Animals were weighed individually after dosing (day 0), twice weekly and at the time of death or euthanasia. Animals were observed for a period of 14 days after administration of doses.

7.1.7.5 Bronchoalveolar Fluid Examination

At the end of the study, animals were euthanized by an overdose of intraperitoneal injection of pentobartital (75 mg/mL). The trachea was exposed and cannulated with a 20-gauge catheter. After instillation of 1.5 mL of cold sterile PBS three times through the trachea into the lung, BALF was recovered at 50% to 60% of the original volume. The BALF was centrifuged for 10 min at 1500 rpm. Cell pellets were resuspended in cold sterile PBS having 0.2% EDTA solution.

For total WBC count, erythrocytes present in the BALF preparation were lysed by adding 1% (v/v) glacial acetic acid (6) to eliminate the possibility for falsely elevated WBC count of the BALF. Geimsa staining was performed for better visualization of cells. 10 µL of sample was loaded on the haemocytometer. And cells were counted on a microscope.

Differential count of WBC was performed to determine the number of different leucocytes i.e. monocyte, eosinophils, polymorphonuclear cells, lymphocyte etc. in BALF. Total cell were counted per cubic milimeter and percentage of each cell type was calculated. Calculations were performed for each dose level of carrier administered.

7.1.7.6 Histopathological Examination of Lung

Histopathological examination of left lung was performed after the end of the acute toxicity study. Same protocol as used in the Section 7.1.6.4 was used for histopathological evaluation of lung.

7.2 Results and Discussion

7.2.1 Treatment in MCT Administered Rats

Efficacy of the formulations was evaluated after treatment with siRNA formulations in disease induced rats in the respective groups. Only-MCT treated animals showed strikingly increased mean RVSP (56.80 mmHg ±2.06 mmHg) (**Figure 7.2**) and %RVH (53.63%±2.79%) (**Figure 7.3**). However, there was no significant difference in mean systemic arterial pressure (114.50-123.66 mmHg) amongst controls and all formulation treated groups (**Figure 7.4**) which means monocrotaline selectively induced PAH. Almost 30% of the animals died in positive control group by this time, which might be due to right heart failure caused by extensive vasoconstriction and hypertrophy in untreated animals. Treatment with siRNA dry powder formulation through pulmonary delivery showed significant reduction in mean RVSP and mean %RVH in all the formulation treated groups ($p<0.05$) on day 42 after MCT administration and day 21 after siRNA treatment. After 21 days, animals treated with intratracheal instillation of siRNA formulations showed significant difference in mean RVSP and %RVH when compared with positive control animals.

The decrease in FGF2 mRNA levels induces relaxation and anti-proliferative effects on vascular smooth muscle. Thus the above seen trend in hemodynamics and RVH was further supported by decrease in level of FGF2 mRNA levels found in the lung homogenates of the rats in different groups. All the formulations were able to decrease FGF2 mRNA levels in rat lungs (**Figure 7.5** and **Figure 7.6**) as compared to the placebo and positive control.

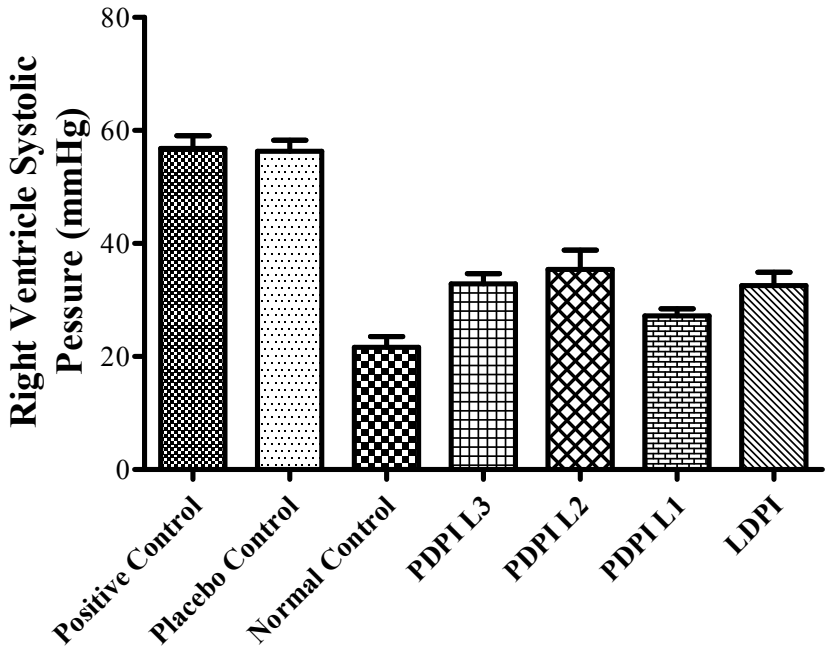


Figure 7.2 Right ventricular systolic pressure in MCT induced pulmonary hypertensive rats after treatment with various formulations.

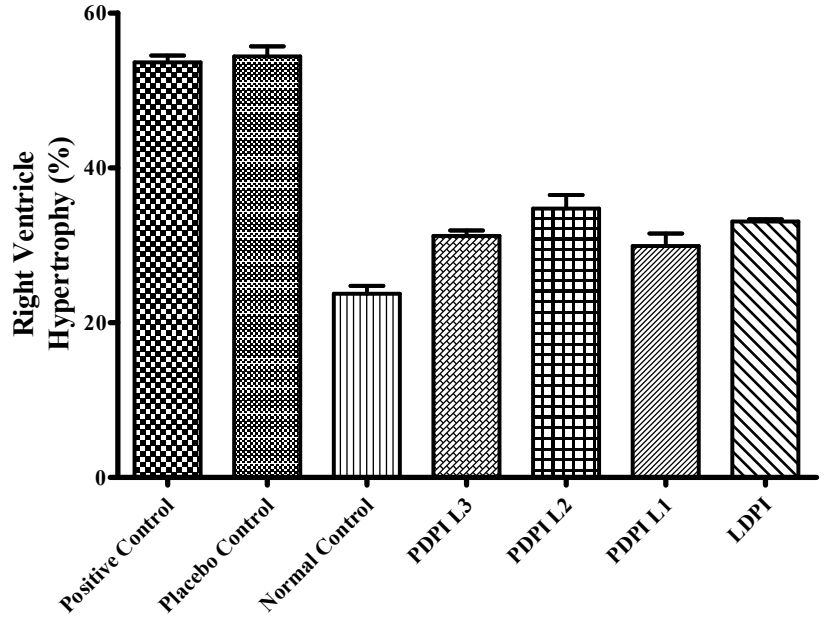


Figure 7.3 Right ventricular hypertrophy (%) in MCT induced pulmonary hypertensive rats after treatment with various formulations.

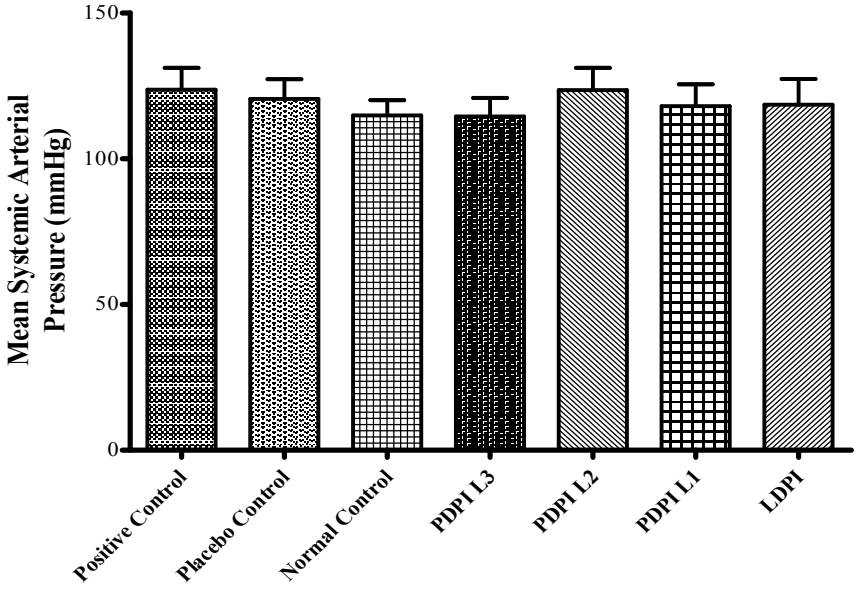


Figure 7.4 Mean systemic arterial pressure in MCT induced pulmonary hypertensive rats after treatment with various formulations.

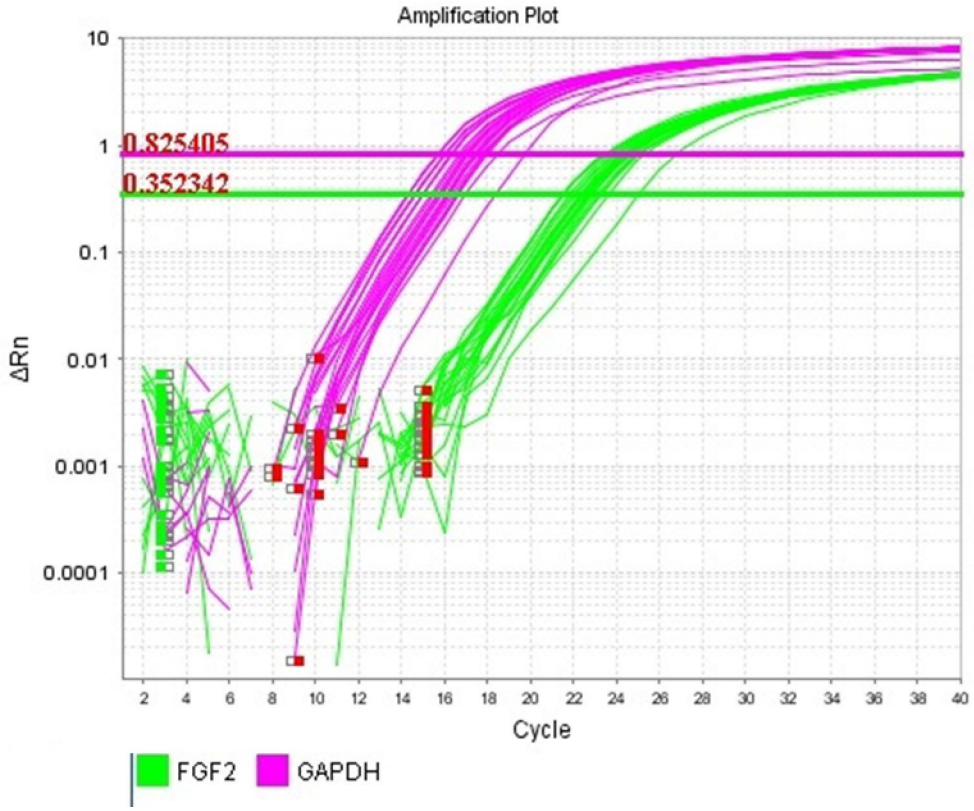


Figure 7.5 Amplification plot of gene expression study by RT-PCR.

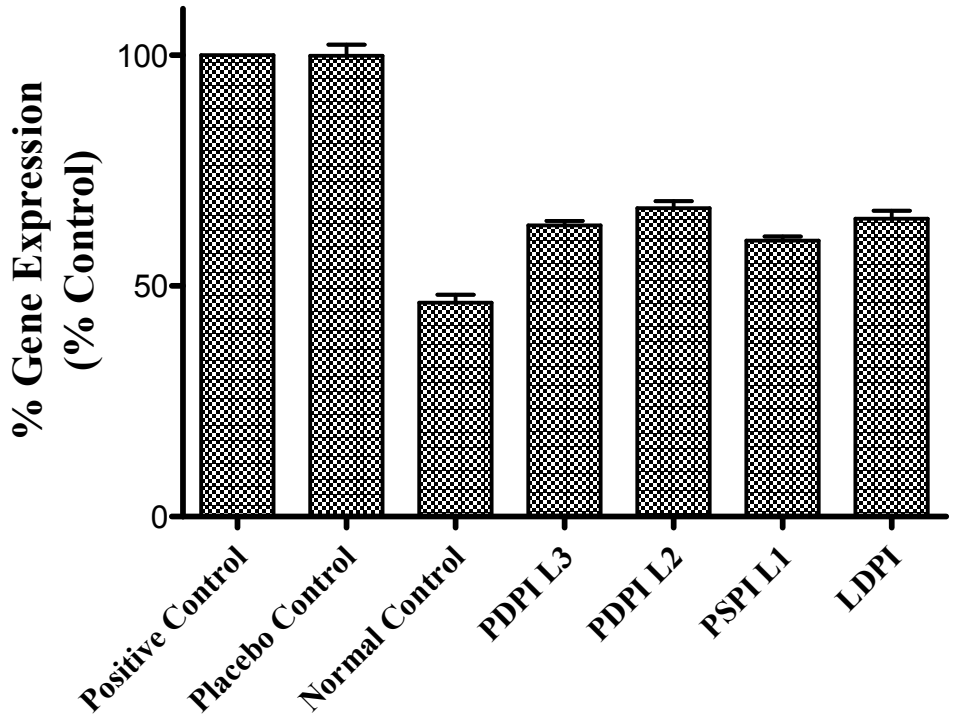
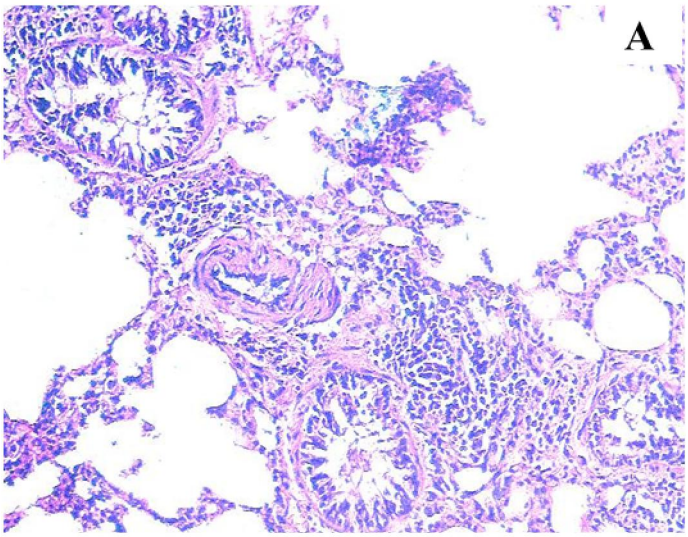


Figure 7.6 % Gene expression of FGF2 mRNA in MCT induced pulmonary hypertensive rats after treatment with various formulations.

MCT-induced PH in rats is related to PA-SMC proliferation, collagen accumulation, and inflammation (7, 8). Therefore, we evaluated the effect of FGF2 knockdown on the basis of histopathology. Arterial medial thickening in the small pulmonary arteries and inflammatory changes were observed after 42 days of MCT administration as compared to control animals (**Figure 7.7**). Arterial medial thickening in the pulmonary microvasculature were curbed by the pulmonary delivery of all nanoplexal formulations. Histopathology studies revealed suppression of the arterial medial thickening, reduced muscularization of pulmonary arteries and moderated inflammatory reaction (9) with siRNA nanoplexes formulation treated groups (**Figure 7.7**). Izikki et al. demonstrated proliferating cell nuclear antigen (PCNA) immunohistochemistry which indicated that FGF2-siRNA markedly reduced SMC proliferation within the arterial wall. Masson trichrome stain suggested reduced accumulation of collagen fibres stained blue (10). Moreover, in case of siRNA nanoplexes treated groups, significantly negligible inflammatory response, medial thickening and muscularization was observed which might be due to their cellular delivery of siRNA formulations in the lungs.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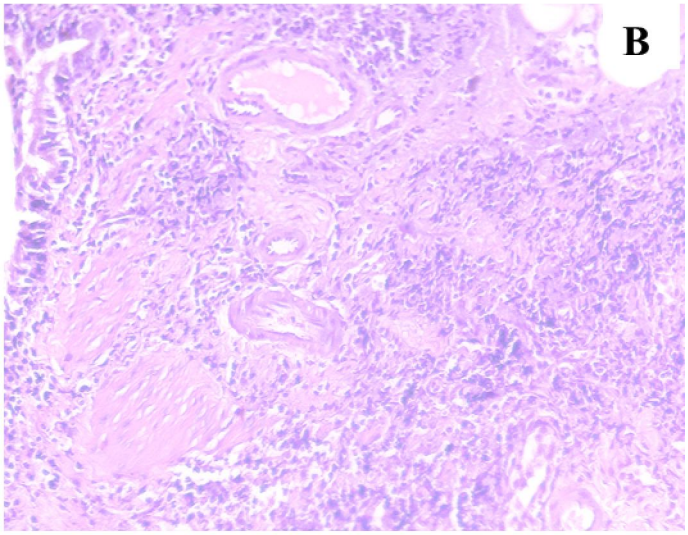
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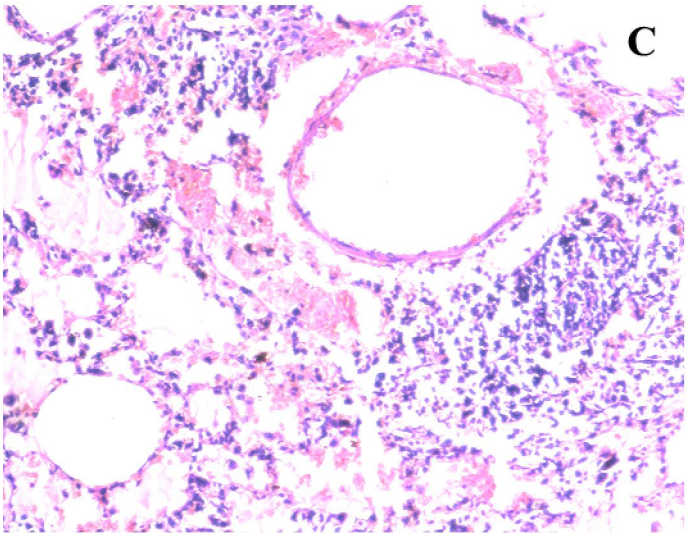
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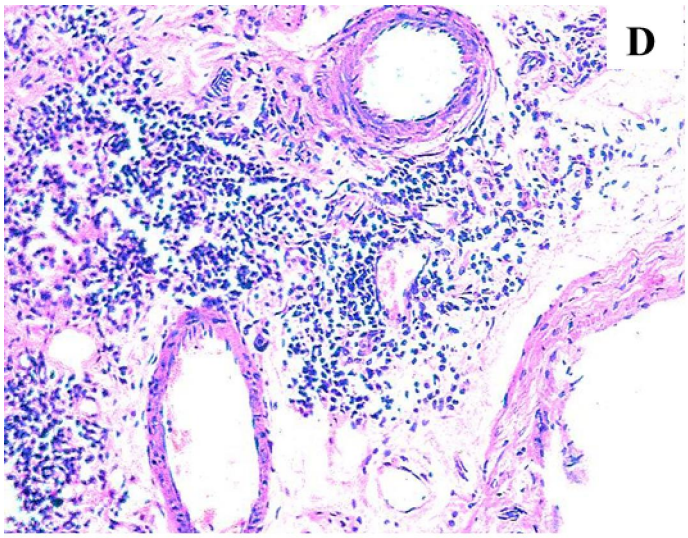
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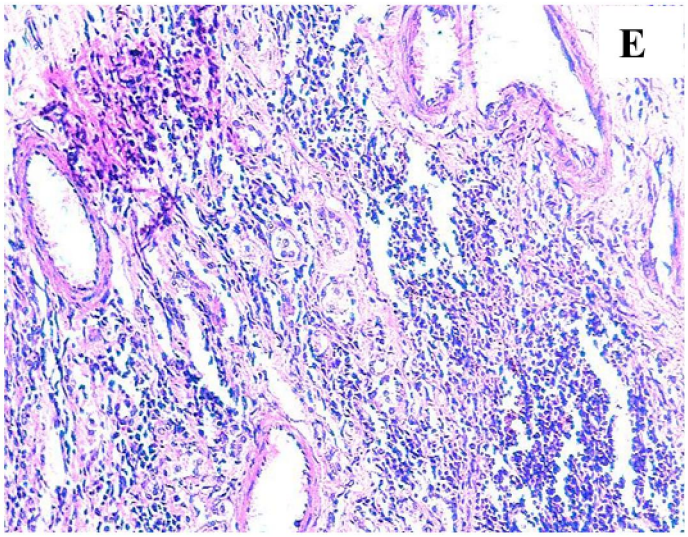
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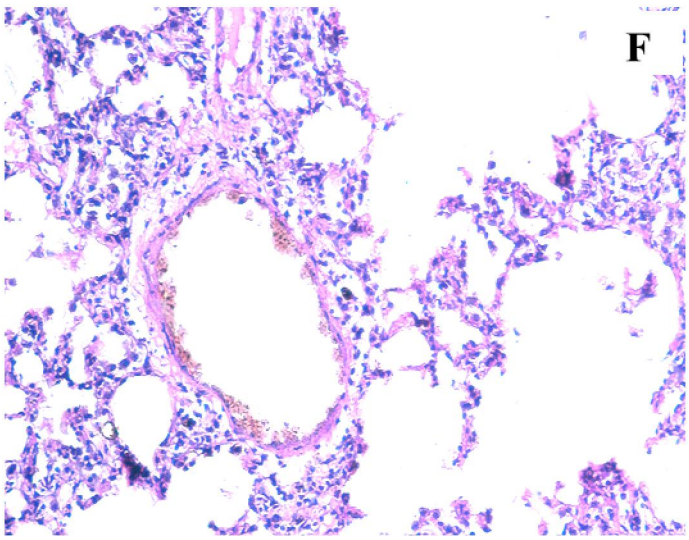
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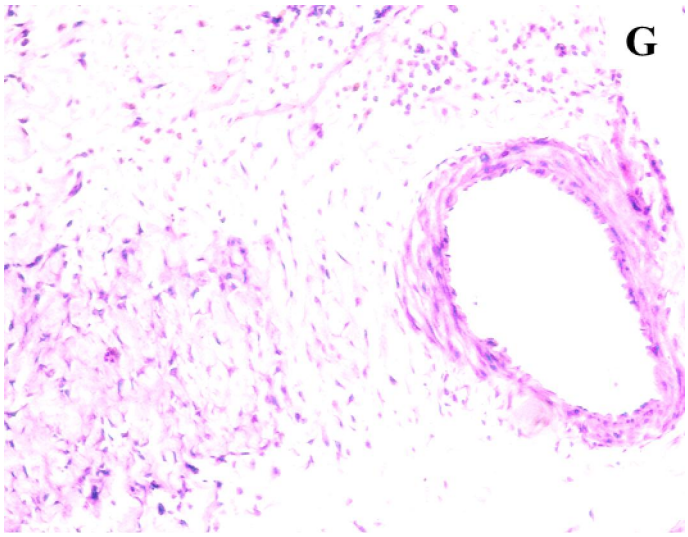


Figure 7.7 Histopathology of Positive control (A), Placebo Control (B), Normal Control (C) and treatment with PDPI L3 (D), PDPI L2 (E), PDPI L1 (F) and LDPI (G).

7.2.2 Acute Toxicity Studies

The dosing level of each carrier was chosen based on the amount of the polymer or lipid amount required for delivery of therapeutic concentration of siRNA i.e. 5 nmol/kg. In case of modified PEIs required amount of polymer was 132 µg/kg. Similarly, the corresponding amount of liposomal carrier on lipid basis was 3.67 mg/kg. Hence, first dosing levels that were used to evaluate the acute toxicity of carriers were 150 µg/kg in case of polymers and 4 mg/kg in case of liposomal carrier. Toxicity of carrier was also determined at a dose 5 times higher than initial dose i.e. 750 µg/kg and 20 mg/kg respectively for polymeric carriers and for liposomal carriers.

All animals were found healthy and no mortality was observed during the period of 14 days neither any sign of any clinical toxicity was observed. Weights of animals were not changed after dosing and change in animal weights was within the limits of 5% of initial weights.

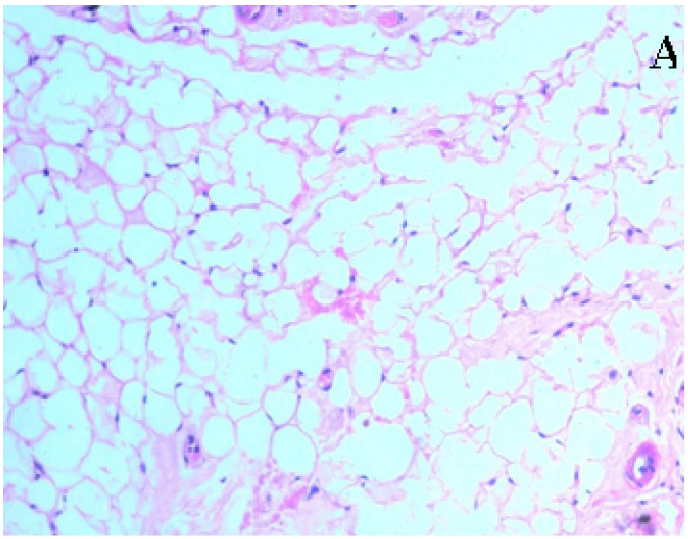
Cationic carriers are shown to be toxic to cells. This is due to charge based toxicity that cause aggregation on cell surfaces and impair cell surface functions (11). Also, intracellular processes critical to cells are impaired due to cationic polymers. Report can be found on the nectrotizing and membrane permeabilizing toxicity of cationic polymers (12). Cationic lipids also cause cytotoxicity by pore formation and leakage of cellular ingredients

from cell membrane as well as aggregation of cell surfaces. Further DOPE enhances DOTAP mediated pore formation effect which can augment cytotoxic activity of cationic liposomes.

Despite of reported toxicity of cationic carriers, modification of carrier systems proposed in this thesis, i.e. modification of PEI with BOC-amino acid and modification of DOTAP/DOPE based system by incorporation of DPPC and cholesterol may be accounted for low *in vivo* toxicity of developed carriers. From the results, it was observed that optimized carriers did not show any toxicity even at 5 times higher dose than that required at therapeutic level. Moreover, these polymers and liposomal carriers are ultimately to be used as polyplex and lipoplex formulations which are the complexes of cationic polymers and cationic liposomes with negatively charged siRNA, toxicity profile of carriers can be extrapolated to the case of polyplexes and lipoplexes as well. This can be ascribed to significant charge neutralization of cationic polymers and liposomes by this interaction. This rule out the possibility of any toxicity of formulated nanoplex formulations.

Table 7.3 Cell counts in bronchoalveolar lavage fluids

Group	Dose	Total Cell Count	Polymorphs (%)	Lymphocyte (%)	Eosinophil (%)	Monocytes (%)
Normal Control	--	4575±318	53.35±5.16	40.15±5.86	3.30±1.41	3.20±0.46
AHP	150 µg/kg	5100±424	55.30±5.23	38.40±4.80	3.80±1.13	2.50±1.17
	750 µg/kg	5575±459	59.00±2.82	35.15±2.61	3.00±1.1	2.85±0.23
ALP	150 µg/kg	5000±353	55.65±2.89	37.20±3.11	4.00±0.98	3.15±0.23
	750 µg/kg	5400±282	60.15±3.04	35.50±3.53	2.00±0.98	2.35±1.00
AAP	150 µg/kg	5150±212	55.30±3.81	39.20±1.69	3.65±2.33	1.85±1.64
	750 µg/kg	5425±318	56.45±3.46	37.25±3.88	3.45±0.63	2.85±0.01
Liposome	4 mg/kg	5000±424	57.30±2.82	37.05±2.33	3.40±0.56	2.25±0.10
	20 mg/kg	5625±742	62.25±3.88	31.15±4.45	3.85±1.2	2.75±0.47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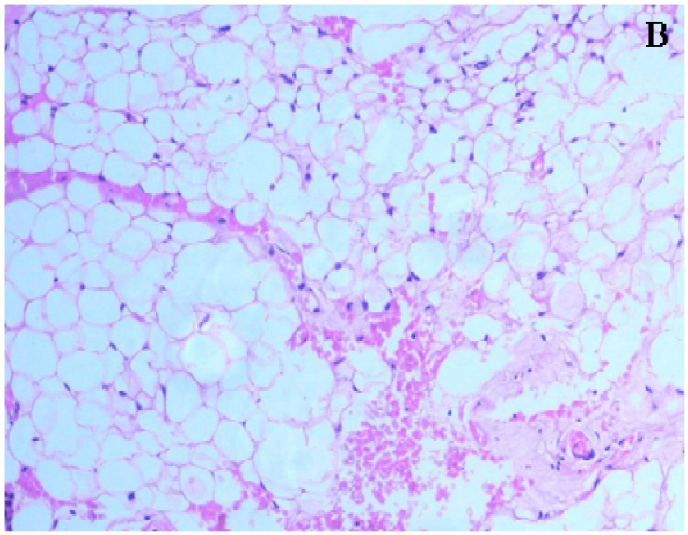
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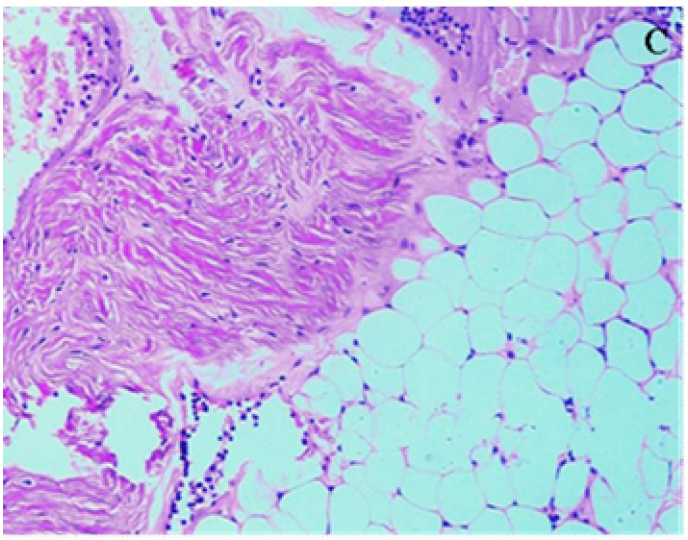
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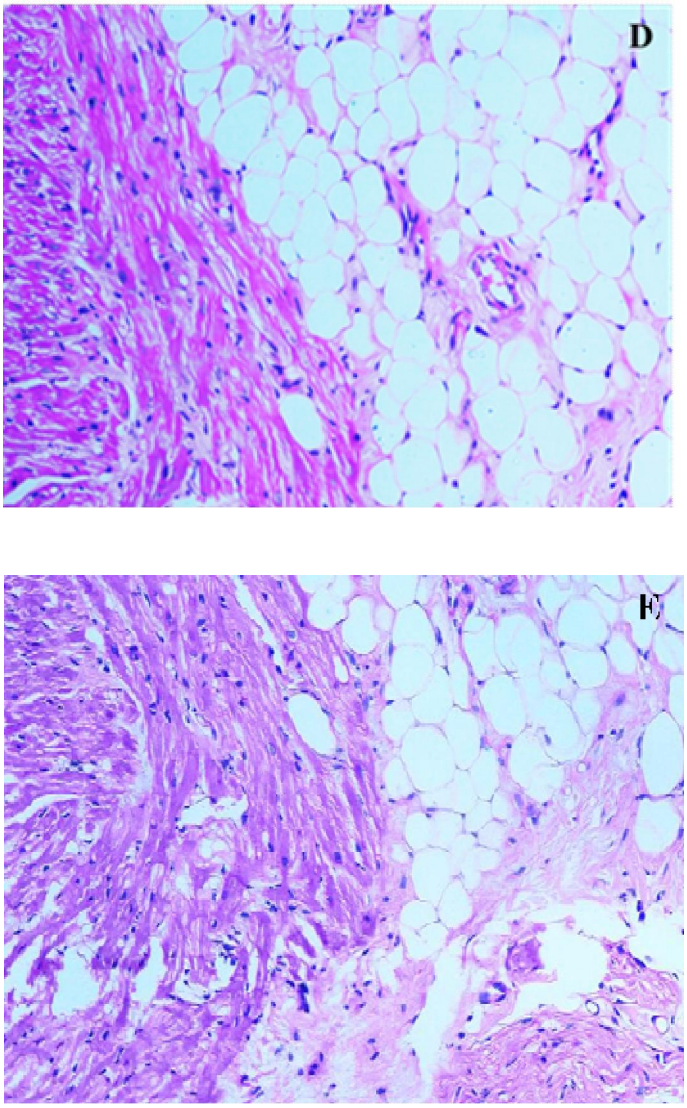

Figure 7.8 Histopathology of lung tissue sample at 5 times higher dose of Control (A), ALP (B), AAP (C), AHP (D) and liposomes (E).

Examination of total cell count and differential cell count in broncholaveolar lavage fluid showed that total count of cells was comparable to that of control. Histopathological examination of lung sections however did not show sign of toxicity (**Table 7.3** and **Figure 7.8**). The tissue endothelium was found to be intact. Hence, from acute toxicity studies, it can be concluded that polyplex and lipoplex formulation developed from modified PEI based and liposomal carrier would be non-toxic at therapeutic levels to be used for *in vivo* treatment of PAH.

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