Chapter 8: Animal Studies

8.1 Introduction

The OVA-challenged asthma mice model is the industry gold standard model used to assess pulmonary inflammation. Inhalation of ovalbumin has been having been confirmed to induce an immune response associated with increased airway hyper-responsiveness (AHR). In addition, this model also demonstrates a number of other features characteristic of human asthma covering, cells accumulation into lungs, rise in counts of eosinophils, and high cytokines levels in bronchoalveolar lavage fluid. All the experiments performed and study protocol illustrated in the study were accepted by the Institutional Animal Ethical Committee (IAEC) of Pharmacy Department, Faculty of Technology and Engineering, The Maharaja Sayajirao 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.

8.2 Methods

BALB/c mice of 6-8 weeks old and weighing between 20-25 g were used to determine efficacy of developed siRNA formulations in treatment of airway inflammation.

8.3 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 dark. Individually housed animals were supplied with unlimited supply of conventional rodent diet and drinking water.

8.4 Preparation of Animals

Random selection of mice was performed and each mouse was marked for identification. Animals were kept in cages for periods of at least 7 days before dosing sequence was started in order to acclimatize animals with the laboratory conditions.

8.5 Efficacy study

8.5.1 Ovalbumin (OVA) Challenged inflammatory model

Healthy 6-8 weeks old BALB/c mice (either sex; weighing approx. 20-25 gm) were randomly allocated to eight groups (n=6). Animals of all groups were received water during the experimental period.

Table 8.1: Groups of animals for Efficacy study

Groups	Treatment of Formulations	Numbers of animals/ Group
1	Saline (Normal control)	6
2	Ovalbumin-OVA (Positive control)	6
3	OVA + siRNA-TMC polyplex	6
4	OVA + siRNA-TMC-UAA polyplex	6
5	OVA + siRNA-TMC-PCA polyplex	6
6	OVA + siRNA-TMC-PAA polyplex	6
7	OVA + siRNA-bPEI polyplex	6
8	OVA + siRNA-PEI-UAA polyplex	6

BALB/c mice were sensitised to OVA (10-20 µg per 2 mg alum in PBS) by three intra-peritoneal injections, on day 0 and ovalbumin (50 µg) emulsified with alum on day 14 and day 28. Mice were challenged with an Ovalbumin (100 µg in 40 µl volume total PBS) on day 40-42. Animals were anesthetized by thiopentone sodium (50 mg/kg, i.p) and doses of formulation equivalent to 100 nm siRNA concentration and control as per groups were administered by intratracheal instillation, before the OVA challenge. Mice were euthanized after 72 hr. The lung lobes were isolated, rapidly sliced and divided to make available two samples per mouse which were processed for RNA extraction.

8.6 Gene knockdown efficiency by RT-PCR

BDNF expression was estimated in lungs from mice of normal control, positive control, and from treatment groups. BDNF mRNA level was also measured in mice lungs in all the groups on day 42nd after Ovalbumin administration. Lung tissue samples were

homogenized in cold PBS. Total RNA was extracted by using 100 mg of tissue homogenates. RNA concentrations were measured using spectrophotometric analysis, and integrity of RNA was checked by visual examination of ethidium bromide (EtBr) stained agarose gels. Further mRNA level was quantified by RTPCR.

RT-PCR is a powerful technique for the detection and quantification of mRNA and popular because of high sensitivity, fine reproducibility, and extensive dynamic quantification range(1). RT-PCR permits research scientist to amplify particular pieces of DNA more than a billion-fold (2). In PCR a thermostable polymerase synthesizes a complementary sequence of bases to single strand of DNA containing a double stranded starting point. The starting points can be selected by user corresponding to gene of interest and they are known as primers. During PCR the temperature cycling is used to control the activity of thermostable polymerase and primers binding. At the beginning the temperature is kept at $95^{o}C$ where all double stranded DNA will melt. Then temperature is reduced to ~$60^{o}C$, depending on primer, to allow the primer to bind the target gene. The polymerase consequently binds the double stranded DNA and starts copying. This temperature when repeated several times leads to exponential increase in number of copies of target DNA sequence. The amplified gene can be observed at the end of process by running on agarose gel electrophoresis and staining it wherein the brighter bands will indicate higher copies of DNA. However, in conventional PC the gel-based analysis cannot give time dependent quantity curve.

In Real Time PCR this process is monitored in real time using fluorescent probes of double stranded DNA and detecting them with a camera. The RT-PCR offers several benefits such as: a direct look into the reaction, the precise calculation of reaction efficiency eliminating the necessite to run gels and performing a really quantitative analysis of gene expression rather than semi-quantitative as in normal PCR(3). The RT-PCR uses cyanine dyes e.g. SYBR green I and BEBO, which do not interfere with polymerase chain reaction. The primer-dimer formation can be simply identified from melt curve. The primer design is also a critical aspect of RT-PCR. It depends on choice of amplicon as well. The amplicon is generally kept to < 300 base pairs SYBR green based detection while 50-150 base pairs for probe-based detection. The primers are generally 15

– 20 base pair and contain 20-80% CG units. Care should be taken to avoid formation of dimers in SYBR green based detection or should be verified from melt curve.

Protocol:

In vivo mRNA knockdown efficiency of siRNA polyplex formulations was assessed in order to quantify the gene silencing potential of the BDNF silencing RNA. RT-PCR quantify the mRNA expressed in lung tissue homogenate containing different siRNA polyplex formulations. Total RNA was isolated using TRIzol and reverse transcription into cDNA was carried out by RNA to cDNA conversion kit. BDNF mRNA was quantified using RT PCR (Step one) using SYBR Green Mastermix, forward and reverse primers and 2 ng of cDNA in 20 μL volume. Housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the mRNA expression of BDNF gene. The reaction protocol and specification followed are described below:

8.6.1 Primers selection

Primer design tool of NCBI (National Center for Biotechnology Information)was referred for primer selection. The primers for human BDNF siRNA were 5′-CATAAGGACGCGGACTTGTACA-3′for   forward   and   5′-AGACATGTTTGCGGCATCCA -3′ for reverse which will form a PCR product 200 bp. Primers for GAPDH were 5′-AATGTGTCCGTCGTGGATCTG-3′ for forward and 5′-CAACCTGGTCCTCAGTGTAGC -3′ for reverse which will form a PCR product of 130 bp.

8.6.2 Total RNA isolation

Analysis of gene expression also depends on integrity of isolated RNA; therefore isolation of intact total RNA is primary requisite for gene quantification. The absolute quantification that normalize specific mRNA expression against total RNA (g/g of total RNA). The long mRNA is prone to degradation by RNase enzyme during tissue sampling, RNA purification and storage. In addition to the cellular RNase there are several other RNases that are present in environment. The RNA samples may get contaminated by DNA and even minor quantities can get amplified in PCR. Therefore, a properly optimized laboratory protocol was used for RNA extraction:

1. 1 mL TRIzol reagent (1 mL/10cm^2) was added to each well in the plate and kept incubated for 5 min at room temp.

2. The sample was transferred to 2ml of autoclaved eppendrof tube (DEPC treated, RNase free). Then 200µl of chloroform was added and mixed vigorously for 15-20 sec and incubated for 2-3 min at room temperature conditions.

3. The instrument was pre-maintained and samples were centrifuged at 12,000g for fifteen minutes at 2-8^{0}C.

4. Then half of aqueous phase, above the fairly visible interphase, was transferred to fresh eppendrof tubes. The aqueous phase contains both RNA and DNA, however, RNA, being of smaller fragments, resides in the top of aqueous phase.

5. To the aqueous phase 500µlof isopropyl alcohol (IPA) was added and incubated at for 10 min at room temperature. Further, sample was incubated at -20°C to precipitate the RNA.

6. The sample was centrifuged at 12, 000g for 10min at 2-8^{0}C to obtain the RNA pellet. The supernatant was removed and 75% ethanol was added to wash the pellet by mixing with vortex again centrifuged at 7500g for 5min at 2-8^{0}C

7. The supernatant was removed and pellet was allowed to semi air dry. The washed pellet was dissolved in 50µl DEPC treated water by incubation at 55-60^{0}C for 10min.

8. The RNA was checked on agarose gel by loading 2 µl of sample with loading dye. The RNA concentration was estimated by optical density (1 O.D = 33 µg/ml) using nanodrop spectrophotometer and the purity was checked from A260/A280 ratio which was between 1.8-2.1

Table 8.2 Details of primers

Primer	Sequence (5'->3')	Template strand	Length	Tm	GC%
BDNF					
Forward primer	CATAAGGACGCGGACTTGTACA	Plus	22	58.2	51
Reverse primer	AGACATGTTTGCGGCATCCA	Minus	20	59.3	50
GAPDH primers					

Forward primer	AATGTGTCCGTCGTGGATCTG	Plus	21	59.6	52.5
Reverse primer	CAACCTGGTCCTCAGTGTAGC	Minus	21	60.7	54.8

8.6.3 RNA to cDNA conversion

RNA is converted to cDNA to store the information in RNA in a stable form as RNAs are highly unstable and sensitive and are prone to degrade by the RNases enzymes. On the other hand, DNA is fairly stable. The RNA to DNA conversion is brought about by RNA-dependent DNA polymerase, recognized as reverse transcriptase. Using RNA as template it can produce cDNA. It also requirements for a primer with a free 3′-OH group. During conversion reaction the primers annealed to the 3′-end of the mRNA. 3′-end of the primer is extended by the reverse transcriptase producing a RNA-DNA hybrid molecule. At last using RNase H or alkaline hydrolysis, the RNA strand of RNA-DNA hybrid molecule is digested. The following step wise protocol was used for cDNA synthesis:

1. Conversion Kit was used to convert RNA to cDNA the high capacity RNA-to-cDNA.
2. Kit components were removed from their storage conditions and allowed to thaw on ice.
3. 1.5 microgram of RNA /20 μL of reaction was used for conversion.
4. The Reaction set up used is given in table 8.3.

Table 8.3 Parameters for RNA to cDNA conversion

Component	Volume/ Reaction (μL)
Sample	9
2✕ RT Buffer	10
20✕ RT Enzyme Mix	1

5. To the each well of 48 well plate, 20 µL of RT (reverse transcription) reaction mix was added for real time PCR.

6. Plate was sealed with sealer and centrifuged to spin down the contents and to remove air bubbles. Plate was kept in the holder of PCR system and following cycle given in Table 8.4 was run:

Table 8.4 Steps of PCR cycle

Parameters	Step 1	Step 2	Step 3
Temperature	45 °C	95°C	4
Time	30 min	10 min	Storage

8.6.4 Real Time PCR Reaction

Once the cDNA was obtained from mRNA, gene expression quantification was studied on RT-PCR using SYBR green based detection and gene knock-down was accessed with respect to the control analysis. The reaction was adjusted as per below composition Table 8.5.

Table 8.5 : Parameters for quantification of mRNA

Component	Volume/ Reaction (µL)
Primer- Forward	0.7
Primer- Reverse	0.7
cDNA	1.5
Master Mix	7.5
NFW	q.s. to 15

15 µL of RT reaction mix was added to the each well of 48 well plate for real time PCR. Plate was sealed with sealer film and centrifuged to settle down the contents and to remove air bubble, if any. Then, Plate was placed in the holder of RT-PCR and cycle was adjusted as per following details:

Table 8.6: Details for RT-PCR cycle steps

Parameters	1 Step	Step 2	Cycles No
Temperature	95°C	60°C	45
Time	15 sec	60 sec	

8.7 Bronchoalveolar lavage fluid (BALF) examination

The airway and lungs were washed three times with 5ml of PBS to total volume 15 ml. The recovered BALF was centrifuged. Determination of total cell counts, differential cell count which are indicator of the inflammatory cells influx of cells in to the lung, assessed from bronchoalveolar fluid (BALF) by hematoxylin and eosin (H & E) staining. In brief, Lungs were surgically removed, cleaned, and weighed to check edema formation if present. The trachea of the animal was exposed and cannulated with a 20-gauge catheter. Lungs were then lavaged with 5 ml PBS instilled through the trachea and collected after 30 sec. BAL fluid collected was centrifuged for 10 min at 1500 rpm and then the supernatant was stored at -20°C. Differential cell counts were studied on smears comprising at least 400 cells in numbers, which were stained with H&E(4, 5).

8.8 Histopathological examination of lung

After BAL fluid recovery, the lungs were inflated with 2 ml phosphate buffer saline. Lung tissue was excised from the whole lung, fixed with 10% formaldehyde, and embedded in paraffin. Various paraffin sections (1-3 μm thick) were cut using microtome (MICROM) and mounted on slide and stained with (H&E). Then sections of tissue were observed under phase contrast microscope (Nikon Corporation, Japan) and images were taken using NIS-Elements software. Sections were assessed for the presence of inflammatory reactions, arteries masculinization etc.

8.9 Statistical analysis

Experiments were performed in triplicate. Unless stated, data are represented as the mean ± SD. The statistical significance of the findings was determined using a Student's t-test where $p < 0.05$ denotes significant difference.

8.10 Result and discussion

Gene knockdown efficiency

Asthma and chronic obstructive conditions are mainly characterized by airway inflammation and airway obstruction. It is reported that pro-inflammatory mediators like cytokines and growth factors like neurotropins releases in the inflammatory conditions of the airways. BDNF, is one of the chief neurotropins upregulated in the pulmonary inflammatory conditions. Hence, upregulation of the BDNF can be reduced through RNA interference mechanism by silencing RNA.BDNF mRNA knock down was studied in mice by Ovalbumin induced inflammation model. Ovalbumin, known agent or allergen to induce inflammatory conditions. Results of the in vivo mRNA knock down efficiency of the siRNA demonstrated that BDNF siRNA efficiently down regulates the BDNF gene. Table 6.6 demonstrates the gene expression in different treatment group in comparison to control group. Polyplexes formulations administered animal groups exhibited gene silencing up to 64 % *in vivo*. Modified TMC and Modified PEI based polyplexes showed higher gene silencing compared to the polyplexes prepared from the native polymers. Figure 7.2 and figure 7.3 showed Amplification plot, Melt Curve of BDNF and GAPDH in the RT PCR Study. Results of the BDNF mRNA showed that modified TMC based polyplexes showed 51 % to 57 % gene knockdown efficiency. While, Only TMC based polyplexes demonstrated about 46 % gene silencing ($p < 0.05$). On the other hand, PEI based and PEI-UAA polyplexes showed 60% and 64% gene silencing efficiency. From the results, it can be said that developed novel cationic polymers based non viral vectors based siRNA systems efficient gene knock down activity which show very promising potential as a vectors for siRNA therapeutics for the treatment of pulmonary inflammatory conditions.

Table 8.7: Gene expression (%) of BDNF mRNA in OVA induced inflammatory mice

Sr. No.	Formulations/Treatment	Gene Expression* (%)
1	Saline Control	98.90 ± 1.09
2	Positive Control	99.10 ± 0.84
3	siRNA-TMC Polyplex	54.28 ± 1.79
4	siRNA-TMC-UAA Polyplex	43.14 ± 1.62
5	siRNA-TMC-PCA Polyplex	47.74 ± 1.57
6	siRNA-TMC-PAA Polyplex	49.63 ± 0.92
7	siRNA-bPEI Polyplex	40.78 ± 0.81
8	siRNA-bPEI-UAA Polyplex	36.26 ± 1.02

*Values are represented as Mean ± SD,n=3
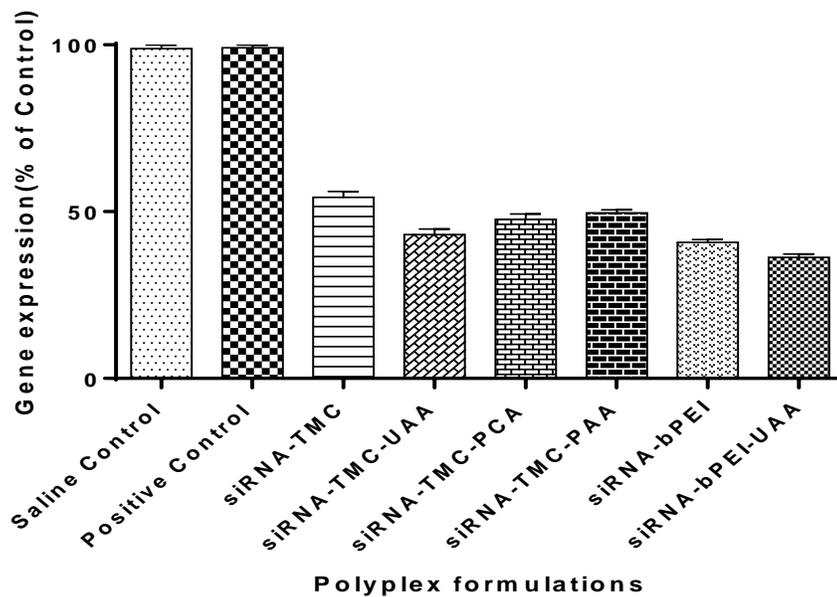
Figure 8.1: Gene expression (%) of BDNF mRNA in OVA induced inflammatory mice
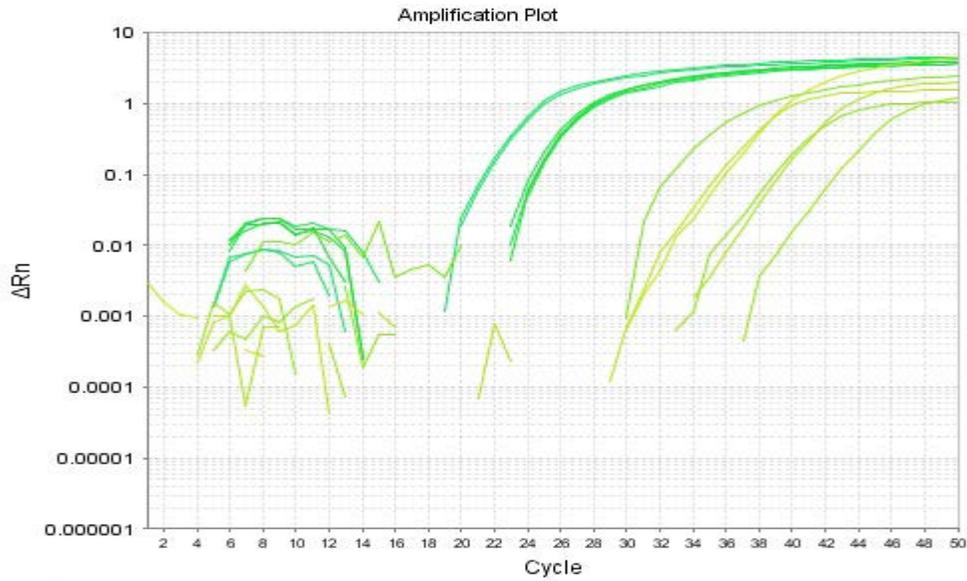
Figure 8.2: Amplification plot of BDNG mRNA(Green) and GAPDH(Yellow)

(a) (b)
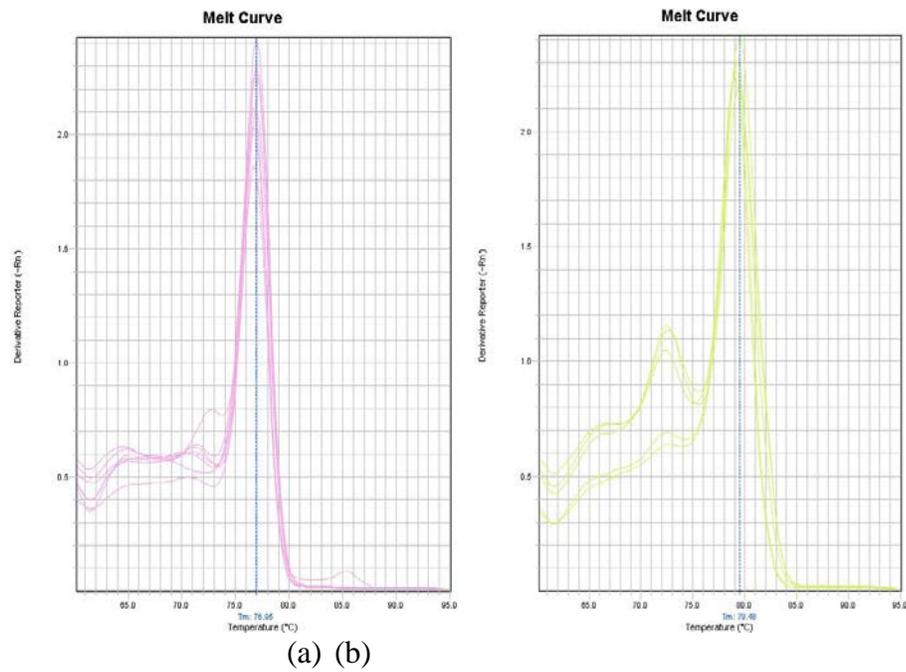
Figure 8.3: Melt Curve of (a) BDNF and (b) GAPDH

Bronchoalveolar lavage fluid (BALF) examination

Bronchoalveolar fluid examination shows picture of the airway inflammation as in the inflammatory conditions increased in the cells, cellular infiltrates in the lung, release of the pro-inflammatory mediators like cytokines and interleukins. Here, we have examined the BAL fluid for the increase in total cell counts and differential cell counts like lymphocytes, eosinophils, neutrophils, macrophages, etc. eosinophils recruitments, macrophages and lymphocytes to the airways is a known feature of asthma and pulmonary obstructive conditions, and the degree of eosinophil cells infiltration is associated with the significance of patients' conditions. These cells often play a significant role in airway inflammation and hyper-responsiveness of airways.

Analysis of the inflammatory cells in the BAL fluid samples showed that total cell counts were significantly enhanced by ovalbumin sensitization in the mice model. However, total cell counts were decreased by siRNA polyplexes formulations treatment. Particularly, the eosinophils counts were elevated in the Ovalbumin challenged mice than in the control animal group and lower in the polyplex formulations treated animals than in the Ovalbumin challenged group of animals.
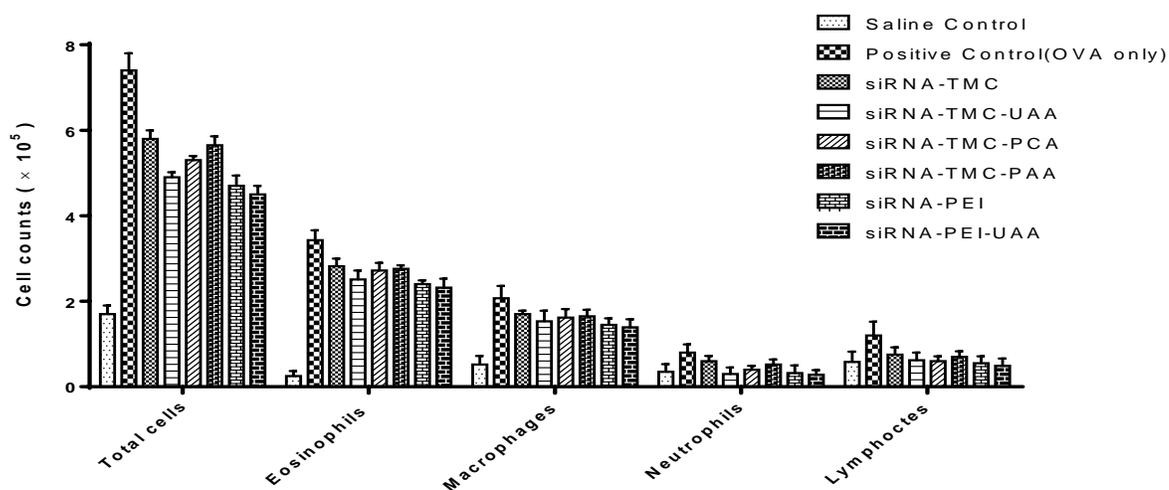

Figure 8.4 : Total cell counts and differential cell counts in BALF

**Histopathological examination of lung**

To assess the anti-inflammatory activity by silencing BDNF gene, we assessed lung specimens of mice with a microscope. The findings of the examination showed that less or no inflammation was present in the lungs of the mice in the saline control group. In opposition, the lungs of the mice in the Ovalbumin challenged animals (Positive control) showed inflammation of epithelium remarkably as well as extensive cellular infiltration. Nevertheless, the lungs of the mice in the formulations treated groups of animals displayed considerably better lung pathology in comparison with those of the mice in the Ovalbumin challenged animals demonstrating potential of gene silencing RNA therapeutics for the treatment of obstructive airway conditions. Figure 8.5 showed histopathology of lung with saline control, positive control and after treatment with different polyplex formulations.
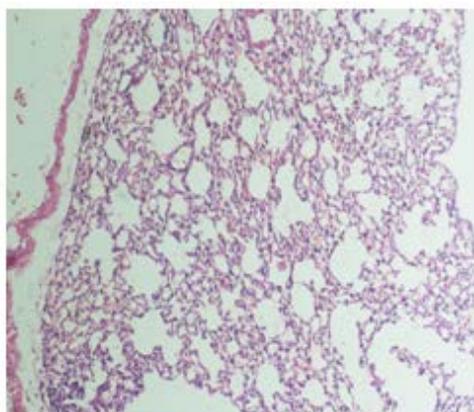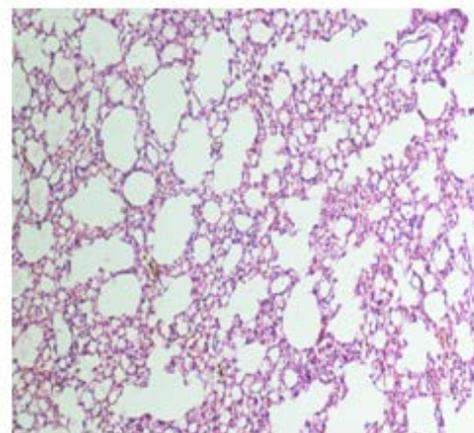

(a) Saline Control
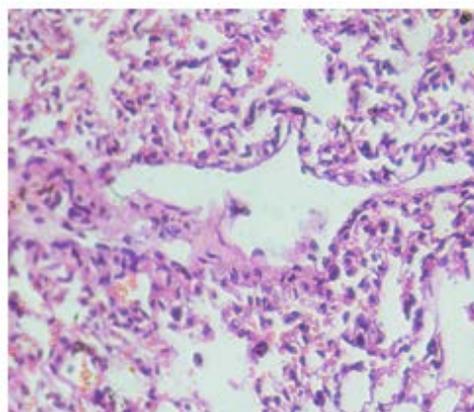
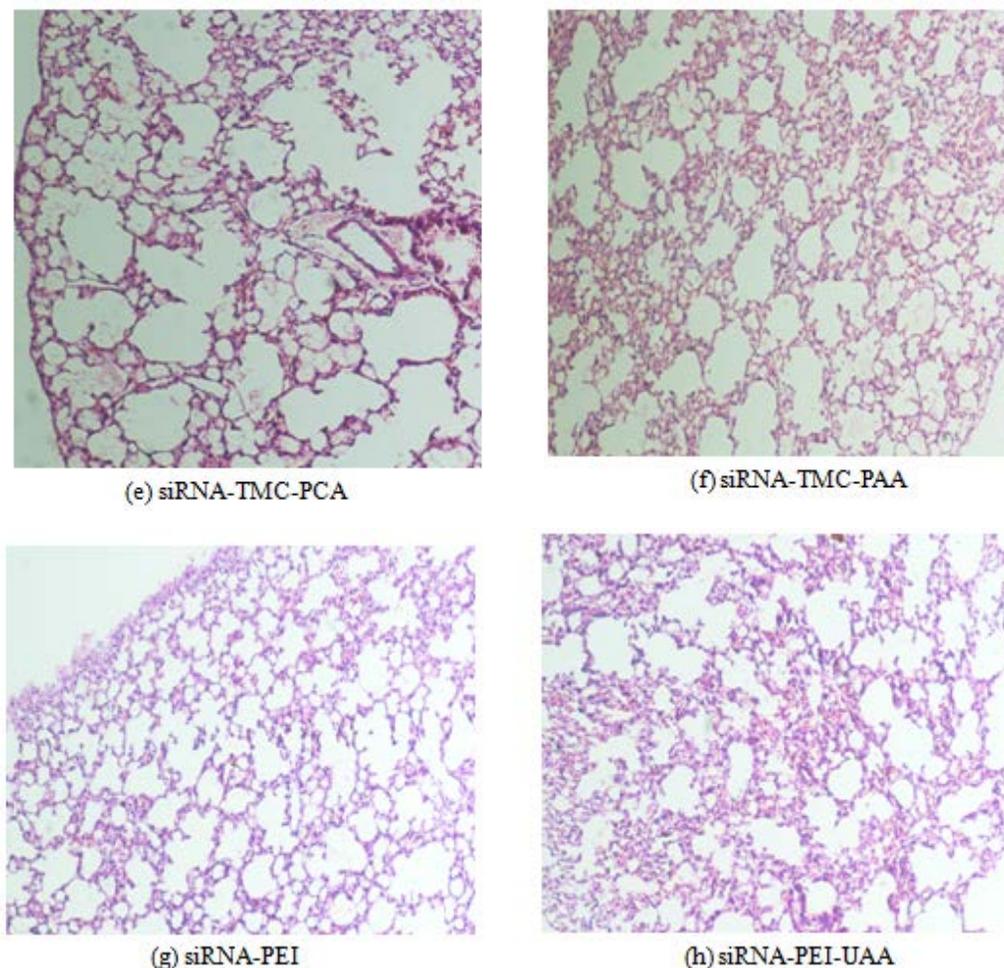(b) Positive Control
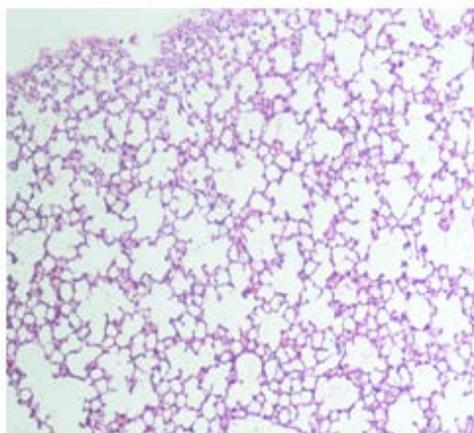
(c) siRNA-TMC

(d) siRNA-TMC-UAA

Figure 8.5: Histopathology of lung of control and after treatment with formulations

8.11 References:

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