Chapter 10
In Vivo Studies

Some things, once you do… can never be undone.
-From "Kill Bill Vol 2"

10.1. Acute Toxicity Study

In vivo acute toxicity studies on animals are an essential part of drug development process. Such acute toxicity studies are carried out for various objectives i.e.

1. To determine the Median Lethal Dose (LD_{50}) after a single dose administered through one or more routes, one of which is the intended route of administration in humans.
2. To determine Maximum Tolerated Dose (MTD) and No Observable Effect Level (NOEL).
3. To identify potential target organs for toxicity, determine reversibility of toxicity, and identify parameters for clinical monitoring.
4. To help select doses for repeated-dose toxicity tests.

A number of methods are available to have an insight about the acute toxicity of any chemical or drug product. These include classical Litchfield and Wilcoxon method (Dosing of animals of both sex with increasing amounts of chemical and plotting dose-response curve to determine LD_{50}/MTD). This type of study has a disadvantage that it uses a large number of animals. So two methods are available now as alternatives which reduces the use of animals i.e. Fixed Dose Procedure (FDP) [1] and Up-Down Procedure (UDP) [2]. Both methods produce data consistent with classical LD_{50} methods [3, 4]. Among these methods Up-Down procedure requires the least number of animals (6-10) of single sex and provides results in terms of LD_{50} along with data for the hazard classification system, unlike FDP that does not estimate results in terms of LD_{50} ^{value} [5]. Instead FDP gives better evaluation of the maximum tolerated dose of drug/drug product.

MTD of a drug can be defined as the highest dose of a drug or treatment that does not cause unacceptable side effects. The maximum tolerated dose is determined in clinical trials by testing increasing doses on different groups of people until the highest dose with acceptable side effects is found. Toxicity parameters to be considered include,

1. Mortality
2. Clinical pathology
3. Gross necropsy
4. Weight change
5. Signs of toxicity – convulsions, rashes, akinesia, licking, tremors

Drug doses at or below this level should not induce [6]

- Overt toxicity, for example appreciable death of cells or organ dysfunction,

- Toxic manifestations that are predicted materially to reduce the life span of the animals except as the result of neoplastic development or
- 10% or greater retardation of body weight gain as compared with control animals.

In some studies, toxicity that could interfere with a carcinogenic effect is specifically excluded from consideration.

For determination of MTD of BMP-9 lipoplexes, fixed dose procedure of OECD-Organization for Economic Cooperation and Development was used. Typical protocol includes administration of a drug/drug product in escalating doses through intravenous route and observing animals for any signs of toxicity.

10.1.1 Method

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.

10.1.1.1 Selection of animals species

Female Swiss Albino mice were used for the study as females are generally slightly more sensitive to such studies [4]. Healthy young adult animals (with 8-12 weeks age) which were nulliparous and non-pregnant were used for study.

10.1.1.2. Housing and feeding conditions

The temperature in the animal room was 20-25°C. Artificial lighting with the sequence of 12 hr light and 12 hr dark was kept in animal housing. The animals were housed individually. For feeding, conventional rodent laboratory diets was used with an unlimited supply of drinking water.

10.1.1.3. Preparation of animals

The animals were randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days prior to dosing for acclimatization to the laboratory conditions.

10.1.1.4. Preparation of doses

Test substances (lipoplexes) were administered in a constant dose volume of 20 mL/kg by varying the concentration of the dosing preparation. (The dosing volume was chosen such that the volume did not exceed 2 mL/100g bodyweight). All doses were prepared prior to administration. Above certain dose, only liposomal carrier was tested to ascertain the safety profile of developed liposomal carrier systems.

Lipoplexes were lyophilized and lyophilized lipoplexes were reconstituted with sufficient quantities of normal saline to produce pDNA concentrations desired for administration. All the test substances were sterilized by filtering through 0.2 μ membrane filter prior to administration.

10.1.1.5. Procedures

a) Administration of doses

Prior to dosing, all the animals were fasted by withholding food but not water for 3-4 hr. The fasted body weight of each animal was determined and the dose was calculated according to the body weight.

The test substances were administered via tail vein of animals using sterile single use disposable polystyrene syringes (BD syringes). In the circumstance that a single dose was not possible, the dose was given in smaller fractions over a period not exceeding 24 hr at 1hr time gap between two doses.

b) Main test

The test substance was administered in a single dose by intravenous injection using a polystyrene single-use disposable injection. In the circumstance that a single dose was not possible, the dose was given in smaller fractions over a period not exceeding 24 hr.

Animals should be fasted prior to dosing (e.g. with the mice, food but not water was withheld for 3-4 hr). Following the period of fasting, the animals weighed and the test substance was administered. After the substance was administered, animals were provided with food and water *ad libitum*.

c) Sighting Study

The purpose of the sighting study was to allow selection of the appropriate starting dose for the main study. The test substance was administered to single animals in a sequential manner starting from DOSEfirst to DOSElast. The sighting study was completed when a decision on the starting dose for the main study was made (or if a death is seen at the lowest fixed dose).

The starting dose for the sighting study was selected from the fixed dose levels as described in **Table 10.1.** Starting dose selection was obtained from the available literature showing toxicological data for specific chemicals.

d) Main study - MTD Determination

Single animals were dosed in sequence usually at 48 hr interval. The first animal was dosed at a level selected from the sighting study (**Table 10.3**). A period of at least 24 hr was allowed between the dosing of each animal. All animals were observed for at least 14 days for any signs of toxicity.

If the animal survived, the second animal received a higher dose. If the first animal died or appeared moribund (Moribund status: being in a state of dying or inability to survive, even if treated), the second animal was administered a lower dose.

Animals were to be euthanized by intraperitoneal injection of pentobarbital (50 mg/ml) after study or if moribund status (inability to ambulate, inflammation, anorexia, dehydration, or more than 20% weight loss) was observed. The weight of each animal was recorded immediately before intravenous injection, 1 day after injection, and at the end of study.

e) Numbers of Animals and Dose Levels

1. The action to be taken following testing at the starting dose level is indicated based on the observations. One of three actions will be required; either stop testing and assign the appropriate hazard classification class, test at a higher fixed dose or test at a lower fixed dose. However, to protect animals, a dose level that caused death in the sighting study was not revisited in the main study.

2. A total of five animals of female sex were used for each dose level investigated. The five animals were made up of one animal from the sighting study dosed at the selected dose level together with an additional four animals.

3. The time interval between dosing at each level was determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next dose was delayed until there was confidence of survival of the previously dosed animals. A period of 3 or 4 days between dosing at each dose level is recommended, if needed, to allow for the observation of delayed toxicity. The time interval may be adjusted as appropriate, e.g., in case of inconclusive response.

f) Observations

Animals were observed individually after dosing at least once during the first 30 min, periodically during the first 24 hr, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, except where they needed to be removed from the study and humanely killed for animal welfare reasons or were found dead.

Observations included were changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern. Attention was directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress were humanely killed.

Loss of weight, if more than 20% of initial, or death of animal was considered a positive response at short term outcome (during first 24 hr). For long term outcome death was used as a termination point to stop the test. The duration of observation was determined by the toxic reactions, time of onset and length of recovery period. The times at which signs of toxicity appear and disappear were considered important, especially if there was a tendency for toxic signs to be delayed [7]. All observations were systematically recorded, with individual records being maintained for each animal.

10.1.2 Results and discussion

BMP-9 lipoplexes were administered intravenously to the female Swiss Albino mice as per the dosing protocol given in **Table 10.1** for sighting study. During sighting

study, formulations were administered to a single mice for each dose level and observed for signs of toxicity for 24 hr.

Table 10.1 Sighting Study: Dosing protocol

Group	Dose of lipoplexes equivalent to		
Normal Saline	-		
HSA lipoplexes	50 µg/kg of pDNA	100 µg/kg of pDNA	500 µg/kg of pDNA
CSA lipoplexes			
HDS lipoplexes			
HSA-$(DSS)_6$ lipoplexes			
CSA-$(DSS)_6$ lipoplexes			
HDS-$(DSS)_6$ lipoplexes			

All animals were found healthy and no signs of any toxicity were observed. Results for sighting studies are summarized in **Table 10.2**.

Table 10.2 Results of Sighting Study

Group/Formulation	Dose	Observation	
		Toxicological Signs/symptoms#	Mortality
Normal saline	-	None	None
HSA lipoplexes	50 µg/kg of pDNA	None	None
	100 µg/kg of pDNA	None	None
	500 µg/kg of pDNA	None	None
CSA lipoplexes	50 µg/kg of pDNA	None	None
	100 µg/kg of pDNA	None	None
	500 µg/kg of pDNA	None	None
HDS lipoplexes	50 µg/kg of pDNA	None	None
	100 µg/kg of pDNA	None	None
	500 µg/kg of pDNA	None	None
HSA-$(DSS)_6$ lipoplexes	50 µg/kg of pDNA	None	None
	100 µg/kg of pDNA	None	None
	500 µg/kg of pDNA	None	None
CSA-$(DSS)_6$ lipoplexes	50 µg/kg of pDNA	None	None
	100 µg/kg of pDNA	None	None
	500 µg/kg of pDNA	None	None
HDS-$(DSS)_6$ lipoplexes	50 µg/kg of pDNA	None	None
	100 µg/kg of pDNA	None	None
	500 µg/kg of pDNA	None	None

#Observations included were changes in skin and fur, eyes and mucous membranes, respiratory distress, symptoms related to autonomic and central nervous systems such as tremors, convulsions etc., lethargy, and coma.

After performing the sighting study, 100 µg/kg of pDNA and 500 µg/kg of pDNA were considered for the main study. Main test was performed using the dosing protocol shown in **Table 10.3**. Four animals were administered with the selected doses of pDNA making up total of 5 animals per group including one animal from sighting study.

Table 10.3 MTD Study: Dosing Protocol

Group	Dose of lipoplexes equivalent to	
Normal Saline	-	-
HSA lipoplexes	100 µg/kg of pDNA	500 µg/kg of pDNA
CSA lipoplexes	100 µg/kg of pDNA	500 µg/kg of pDNA
HDS lipoplexes	100 µg/kg of pDNA	500 µg/kg of pDNA
HSA-$(DSS)_6$ lipoplexes	100 µg/kg of pDNA	500 µg/kg of pDNA
CSA-$(DSS)_6$ lipoplexes	100 µg/kg of pDNA	500 µg/kg of pDNA
HDS-$(DSS)_6$ lipoplexes	100 µg/kg of pDNA	500 µg/kg of pDNA

Results for the MTD study are summarized in **Table 10.4**. All groups showed no signs of toxicity after administration of test substance. In all groups, formulation was considered safe at the maximum dose of 500 µg/kg of pDNA administered as the lipoplexes.

As study was not performed on the higher concentrations of pDNA, actual MTD was not reached in the study. The MTD might be considered higher than the evaluated dose. However, the total dose evaluated is considerably higher that would provide the *in vivo* therapeutic efficacy. Based on the calculations of the cationic lipid doses required for the delivery of the evaluated amounts of pDNA (500 µg/kg), the doses of the cationic lipids that can be considered to be safe are 0.61 mg/kg for His-SA, 0.72 mg/kg for Car-SA and 1.85 mg/kg of His-DSPE administered as lipoplexes.

Table 10.4 Results for Main test

Group	Observations		
	Change in weight of animals	**Toxicological Signs/symptoms#**	**Mortality**
Normal Saline	-	None	None
HSA lipoplexes	**	None	None
CSA lipoplexes	**	None	None
HDS lipoplexes	**	None	None
HSA-$(DSS)_6$ lipoplexes	**	None	None
CSA-$(DSS)_6$ lipoplexes	**	None	None
HDS-$(DSS)_6$ lipoplexes	**	None	None

#Observations included were changes in skin and fur, eyes and mucous membranes, respiratory distress, symptoms related to autonomic and central nervous systems such as tremors, convulsions etc., lethargy, and coma.

*** indicates statistically insignificant change in weight after 14 days with $p<0.005$.*

Considering the fact that the formulations is targeted to the cells inhabiting the bone marrow, the locally expressed quantity of the BMP-9 protein will be sufficiently

high to provide therapeutic activity high enough to induce density of bone forming cells within the bone marrow. This provide robust bone formation for longer period of time reducing the frequencies of administration and at doses lower enough than used in this study. Based on these acute toxicity studies, the developed formulations and synthesized lipids can be considered safe for *in vivo* administrations.

10.2 *In vivo* performance study

In vitro study of the therapeutic activity of a gene delivery system demonstrates the ability of the system to act on the target cells. However, the delivery system when administered in vivo, is going to face several hurdles before it reaches the therapeutic site. *In vitro* simulation studies such as serum stability studies, effect of electrolytes, etc. may give preliminary idea on the potential of the delivery system. However, performing in vivo studies in animals more close idea to the actual performance of the gene delivery system.

In vivo therapeutic activity can be confirmed through use of osteoporosis models in animals. A few examples are, ovariectomy induced osteoporosis, glucocorticoid induced osteoporosis, alcohol abused osteoporosis, fracture repair model, post-menopausal osteoporosis, genetic models, orthotopic bone formation model, immobilization model etc. [8, 9]. Selection of a model and choice of animal species depend on several factors such as disease condition simulation; differences between animal and human physiology; animal size, gender and age; ease and time for osteoporosis induction etc.

Among these models, ovariectomy induced osteoporosis has been widely accepted as a suitable model for gene delivery systems [10-13]. Specifically, ovariectomized rats show extensive cancellous bone as well as cortical bone loss [8]. Cancellous bone loss occurring after ovariectomy in rapid growing rats is primarily due to altered bone growth [8, 14]. On the contrary, ovariectomay in mature rats (rats with mature skeletal development) leads to cancellous and cortical bone loss due to a different mechanism i.e. abnormal bone remodelling [8].

In the present investigation, ovariectomized female Sprague Dawley rat model has been used for investigation of therapeutic potential of the BMP-9 gene delivery system. Osteoporosis induced rats were given the intravenous dose of BMP-9 lipoplexes and x-ray imaging was performed to track the changes in cancellous and cortical bone changes.

10.2.1 Method

Overiectomized (OVX) rats were used as an animal model for evaluating the therapeutic activity of the developed gene delivery system. Study protocols were approved by IAEC (Institutional Animal Ethical Committee, Pharmacy Department, The Maharaja Sayajirao University of Baroda, Vadodara, India). Sprague Dawley Rats of 25-26 weeks age were selected. Animal care was observed according to the guidelines of CPCSEA

(Committee for the Purpose of Control and Supervision of Experiments on Animals, India). Rats were be anaesthetized using xylazine (15 mg/kg) and ketamine (100 mg/kg) injection [15]. Dorsal mid-lumbar area was shaved and swabbed with surgical scrub with ethyl alcohol. Single incision was made on the skin and in muscle wall on both sides below approximately 1/3 of the distance between the spinal cord and the ventral midline. The ovary and the oviduct were exteriorised from peritoneal cavity through the incision and vasculature was ligated using surgical absorbable sutures. The ovary along with a part of the oviduct was removed and remaining tissues were returned to the peritoneal cavity. The incision was closed in two layers i.e. muscle layer followed by skin layer using sterile absorbable sutures. Wound was wiped with alcohol swab and povidone iodine cream was applied to the wound. The ovary on the other side was removed similarly. High degree of aseptic procedure will be maintained throughout the operation. Animals were observed intermittently for the healing of the wound. Animals were housed for induction of osteoporosis for 8 weeks.

Sham operated rats were used to see the induction of osteoporosis. After 8 weeks of induction period, rats were given intravenous dose of BMP-9 lipoplexes (equivalent to 30 μg pDNA per rat). After treatment, rats were housed again for recovery period of 8 weeks and X-ray radiography was performed. X-ray radiographs were analysed for osteopenic trabecular (cancellous bone) sites and quantitative measurements were performed on the thickness of the cortical bone. For cortical bone thickness, the 5 sites in the region just below the greater trochanter and 5 sites in the region of calcar of each femoral bone (right and left) were selected while for endosteal diameter measurement, 5 sites in each (left and right) femoral shaft region were analyzed. Image analysis was carried out using image analysis software ImageJ ver. 1.50c (National Institute of Health, USA).

Table 10.5 Animal groups for osteoporosis induction and treatment

Group	Surgery	Treatment
Sham Operated group	Sham operated	None
Control group	OVX	None
Treatment I	OVX	HSA lipoplexes
Treatment II	OVX	HSA-$(DSS)_6$ lipoplexes
Treatment III	OVX	HDS lipoplexes
Treatment IV	OVX	HDS-$(DSS)_6$ lipoplexes

Statistical analyses of the results was carried out using Prism 6.0 software using ANOVA with Tukey's multiple comparison test considering the $p < 0.05$ as a significant difference.

10.2.2 Results and discussion:

Ovariectomy was performed in mature rats (rats with mature skeletal development) and hence, leads to cancellous and cortical bone loss due to a mechanism representative of abnormal bone remodelling [8]. This indicates a situation of increased bone resorption and decreased bone formation. The X-ray radiograph of the ovariectomized rat show significantly reduced radio-opacity (or increased radiolucency) specifically in the femoral bones and lumbar vertebral region as compared to the sham operated rats (**Figure 10.1**). Additionally, significantly reduced cortical bone thickness and increased endosteal diameter ($p < 0.05$) indicates the induction of osteoporosis. This indicates the development of osteoporosis after overiectomy. The overiectomized rats were then divided in different treatment groups. Vehicle control group (OVX rats with normal saline administration) was kept for comparison of bone morphology change with and without treatment with the developed gene delivery system.

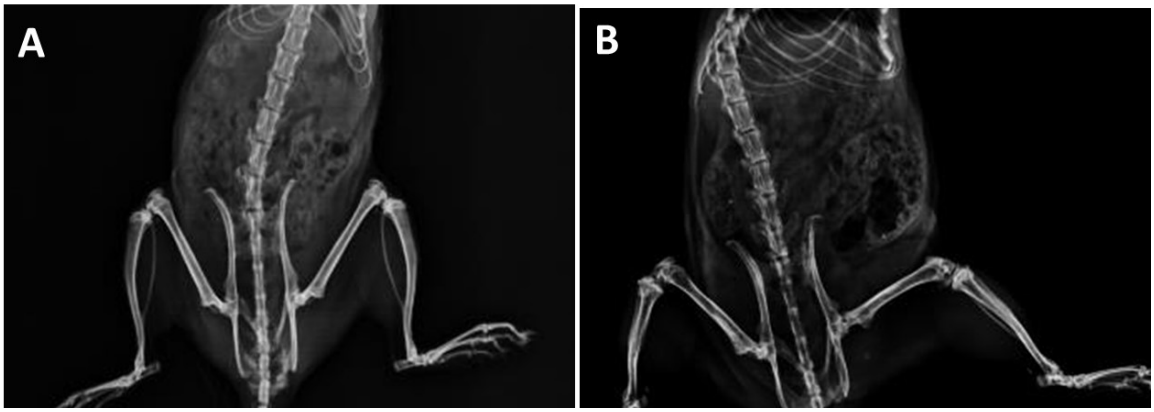

Figure 10.1 X-ray radiographs of the Sham operated and ovariectomized rats after 8 weeks osteoporosis induction period.

Rats were administered with 30μg of pDNA intravenously. The effect of the selected peptide conjugated and non-conjugated lipoplexes were evaluated. X-ray radiographs of the animals of different treatment groups are shown in the **Figure 10.2**. Cortical bone thickness and endosteal diameter were determined for each group of animals

are shown in **Table 10.6**, **Figure 10.3** and **Figure 10.4**. The induction of the osteoporosis was confirmed from X-ray radiograph and cortical bone thickness of the sham operated group and ovariectomized group after 8 weeks induction period. Vehicle control had no effect on the cortical bone thickness as well as endosteal diameter ($p > 0.05$). However, all the lipoplex formulations led to significant increase in the cortical bone thickness ($p<0.005$) and reduction in endosteal diameter ($p<0.05$). As it can be seen from the figure, there is an apparent increase in the radiopacity of the femoral and lumbar vertebral bones as compared to the vehicle control group. Additionally, in the hip bone region (in epiphysis and metaphysis regions) there is an increased radiopacity indicative of the trabecular bone growth.

The results indicate that the formulation is able to reach the target site and induce production of the BMP-9 gene. Vasculature of bone comprises extremely fenestrated capillaries of 80-100 nm diameter and sinusoids (single layer of endothelial cells devoid of supporting cells) [16]. This absence of ordered structure of vessels reflects a high degree of permeability [17] and leads to heavy accumulation of colloidal particles in the bone apart from liver and spleen[18]. The size of the developed lipoplexes in the region of the ≤125 nm supports the passive accumulation of the formulation in the bone. This supports the extravasion and bone marrow interstitial accumulation of lipoplexes.

Accumulated lipoplexes would be easily taken up by the mesenchymal stem cells leading to expression of BMP-9 in bone marrow and subsequent induction of differentiation of the osteoblastic precursor cells to osteoblasts. This build-up of the bone formation cells leads to increased bone formation as indicated by the trabecular (cancellous) bone formation which can be seen as increased radiopaque regions in the bone epiphysis, metaphysis and bone shaft region. Secondly, the effect of the targeted lipoplexes was significantly higher as compared to nontargetd lipoplexes. This is due to increased retention of the targeted lipoplexes in the bone trabecular space due to binding of the aspartate residues to the bone formation surfaces [19].

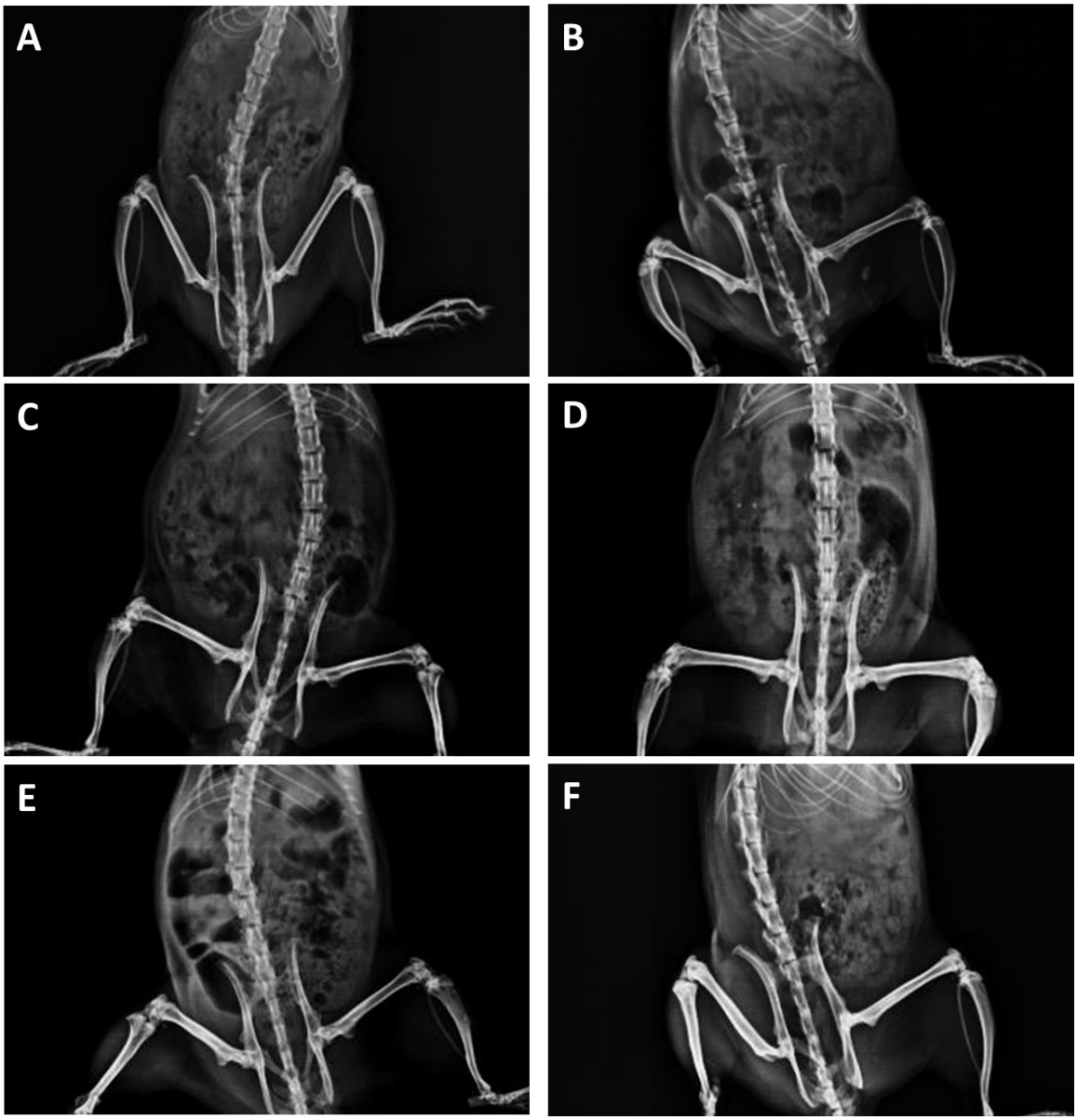

Figure 10.2 X-ray radiographs of the representative animals of each group: A: Sham operated, B: Vehicle control, C: Treatment I (HSA lipoplexes), D: Treatment II (HSA-$(DSS)_6$ lipoplexes, E: Treatment III (HDS lipoplexes), F: Treatment IV (HDS-$(DSS)_6$ lipoplexes

Table 10.6 Effect of different treatments on the Cortical thickness and endosteal diameter of the femoral bones after induction of osteoporosis.

Group	Surgery	Treatment	Cortical Bone thickness (mm)	Endosteal circumference (mm)
Sham Operated group	Sham operated	None	0.82±0.13	1.18±0.08
Control group (Vehicle control)	OVX	None	0.53±0.08	1.56±0.10
Treatment I	OVX	HSA lipoplexes	0.74±0.08	1.31±.11
Treatment II	OVX	HSA-$(DSS)_6$ lipoplexes	1.07±0.10	1.17±.07
Treatment III	OVX	HDS lipoplexes	0.73±0.09	1.34±.13
Treatment IV	OVX	HDS-$(DSS)_6$ lipoplexes	0.86±0.08	1.28±.010

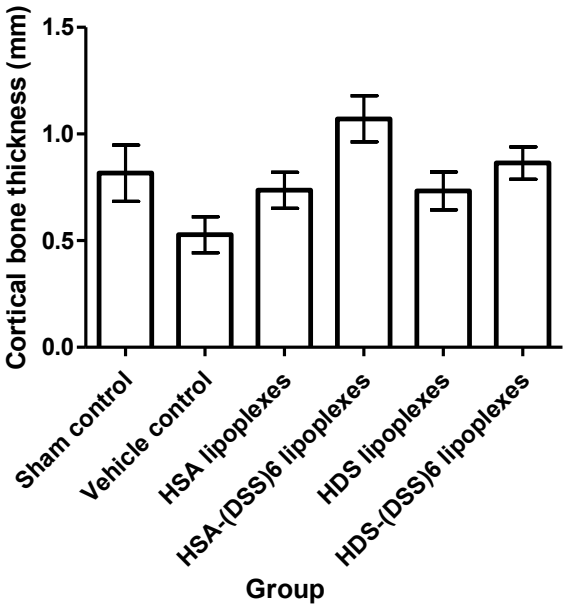

Figure 10.3 Effect of different formulations on the cortical bone thickness of femoral bones of the osteoporosis induced rats

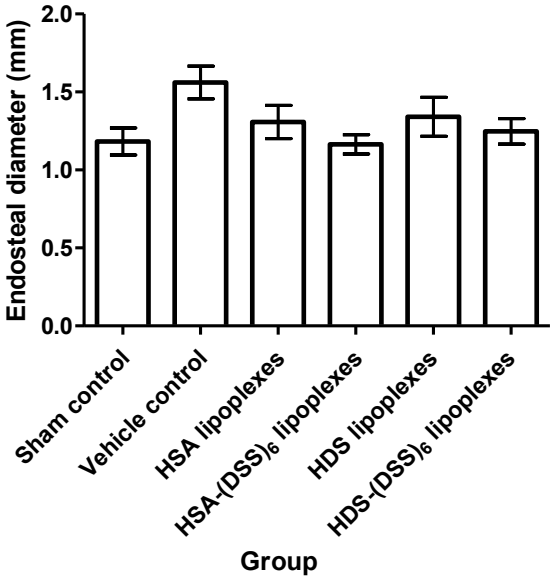

Figure 10.4 Effect of different formulations on the Endosteal diameter of femoral bones of the osteoporosis induced rats

Results indicate that selected formulations were able to induce bone formation in osteoporotic rats. However, targeted lipoplexes were more effective in the recovery of the bone than the non-targeted lipoplexes. The results conclude the effectiveness of the developed formulation for osteoporosis treatment through intravenous administration.

10.2 References

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