Chapter 12

SUMMARY AND

CONCLUSION

12.1 Summary

Uterine leiomyomas, commonly known as fibroids, are well-circumscribed, non-cancerous tumors arising from the myometrium (smooth muscle layer) of the uterus. Leiomyomas are the most common solid pelvic tumor in women, causing symptoms in approximately 25% of reproductive age women. However, with careful pathologic inspection of the uterus, the overall prevalence of leiomyomas increases to over 70%, because leiomyomas can be present but not symptomatic in many women. Various reproductive dysfunctions, which it causes, include pelvic pressure, uterine bleeding, recurrent miscarriage, premature labor, fetal malpresentations and infertility.

Endometriosis, characterized histologically by the presence and growth of endometrial glands and stroma outside the uterine cavity, is a chronic recurring disease commonly encountered in women during their reproductive age. It is thought to affect up to an estimated 10% of women, with a higher prevalence in women who present with infertility (15–35%) and pain. It causes chronic pelvic pain, infertility, dysmenorrhoea, deep dyspareunia, erratic pelvic pain, dyschezia (pain with defecation), and haematuria.

Ovarian steroids, estrogen and progesterone, are important factors for tumor growth and endometriosis. Estradiol stimulates the proliferation of leiomyoma Smooth Muscles Cells, whereas progesterone appears to delay or inhibit programmed cell death (apoptosis) in uterine Smooth Muscles Cells. Ovarian steroids also influence the secretion of growth factor peptides and the expression of their receptors, with both estrogens and progestogens inducing expression of vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF). All these growth factors promote the angiogenesis leading to proliferation, migration and differentiation of tumor cells.

Current treatment options include medical, surgical and ablative therapies. Surgical and ablative therapies leads to severe morbidities and are not preferred therapies for women who wish to preserve her fertility. Among the medical therapy, non-hormonal therapy includes NSAIDS that are generally prescribed for symptomatic relief from pain. While for hormonal therapy it mainly consists of four classes of medications: Combined

estrogens and progestogens, Progestogens, Gonadotrophin releasing Hormone Analogue (GnRH analogue) and Selective Estrogen Receptor Modulator (SERM). Combined estrogens and progestogens leads to severe side effects like weight gain, mood changes, breast tenderness, reduced libido, acne etc which makes them non patient acceptable. GnRH analogue and SERM promises better efficacy for the management and treatment of fibroids and endometriosis. Among the GnRH analogues, Leuprolide acetate is the preferred drug due to lesser side effects and cost effectiveness than other agents of the class. On the other hand, Raloxifene Hydrochloride a SERM, shows anti estrogenic effect on uterine myoma cells and hence a suitable candidate for the management of fibroids and endometriosis.

Currently Leuprolide acetate is administered by parenteral route making the delivery very painful and the systemic side effects make it non-patient acceptable. Similarly in the case of Raloxifene, the drug has very less oral bioavailability (2%) and so very lesser amounts may reach its site of action i.e. uterus. Hence, for both the type of agents vaginal delivery can prove to be a promising approach targeting the drug to the site of action. Studies have reported that drugs on intravaginal administration are targeted to the uterus where their tissue concentration is amplified and systemic absorption is minimized which limits the circulating level and side effects. This phenomenon is known as first uterine pass effect. Despite the variety of formulations for intravaginal therapy like creams, gels, tablets, suppositories, their efficacy is often limited by a poor retention at the site of action, leakage and messiness. Liposomes have been chosen as the formulation due to its advantages like biocompatible and biodegradable nature, non toxic, and ability to entrap both lipophilic as well as hydrophilic drug. It also provides a targeted drug delivery. A prolonged vaginal residence time can be achieved by the use of Intravaginal Rings (IVR).

Hence the aim of the present investigation was to target the drugs available for fibroid and endometriosis treatment i.e. GnRH analogue and SERM to uterus by vaginal route to achieve maximum therapeutic effect by lowest possible dose, reduce the side effects of drugs by avoiding the systemic absorption and provide a safe and economic therapy for fibroids and endometriosis.

Analytical methods for the estimation of drugs

UV-Spectrophotometric methods were developed for both Leuprolide acetate and Raloxifene Hydrochloride separately. Leuprolide acetate showed linearity in the range of 40-100 µg/ml with R^2 value of 0.999 both in distilled water and simulated vaginal fluid pH 4.2 (SVF). While for Raloxifene Hydrochloride linearity was observed in the range of 2-10 µg/ml with R^2 value of 0.998 in Methanol: Chloroform (1:9) and R^2 value of 0.999 in SVF. The developed methods were accurate and precise. LOD and LOQ values for Leuprolide acetate were 0.652 µg/ml and 2.17 µg/ml respectively, in distilled water. Where as in SVF, LOD and LOQ values were 1.27 µg/ml and 4.25 µg/ml. Similarly for Raloxifene Hydrochloride, LOD and LOQ values were 0.044 µg/ml and 0.147 µg/ml respectively, in Methanol: Chloroform (1:9) and in SVF the values were 0.045 µg/ml and 0.150 µg/ml.

Simultaneous estimation method for Raloxifene Hydrochloride and Leuprolide acetate was also developed in SVF pH 4.2. The developed method was accurate and precise. The developed method could detect (LOD) and quantify (LOQ) 1.30 µg/ml and 4.34 µg/ml of LA respectively while 0.140 µg/ml and 0.476 µg/ml of RLX respectively.

The reported LCMS methods with some modifications were used to analyze the concentration of Leuprolide acetate and Raloxifene Hydrochloride separately in rabbit plasma. The retention time obtained was 3.72 ± 0.01 min for Leuprolide acetate and 1.72 ± 0.07 min for Raloxifene Hydrochloride.

Preformulation and Preliminary Optimization

Identification of both the drugs as well as drug-excipient compatibility was assured using FTIR and DSC analysis. Drug-excipient compatibility is necessary for safe and stable formulation development. Preliminary trials were carried out to formulate liposomes. Dehydration-Rehydration method was chosen for entrapment of hydrophilic drug Leuprolide acetate while thin film hydration method for lipophilic drug Raloxifene Hydrochloride based upon maximum entrapment efficiency obtained. Sonication cycle and speed of rotation of RBF were optimized as process parameters while, type of lipid

and volume of hydration media were optimized as formulation parameters. Speed of rotation of RBF was kept constant at 120 rpm for the development of liposomal formulations and sonication cycle was kept constant at 60 % amplitude 0.6 s, for 3 cycles of one minute for size reduction. DSPC was chosen as lipid and 2 ml distilled water as hydration medium to hydrate the dry lipid film.

Formulation and Optimization of Liposomal formulations

Liposomal formulation for Raloxifene Hydrochloride (RLX-Liposomes) prepared using thin film hydration was optimized using 3^2 full factorial design keeping independent variables as Lipid: Cholesterol ratio and Hydration time at three levels (low, medium and high). The responses measured were vesicle size and % entrapment efficiency (% EE). The result of optimization as suggested by software Design Expert (version 9.0.0.7) was that the optimized formulation had vesicle size of 122.1±1.2 nm with 90.96±1.4 % EE with over all desirability of 0.910 at 2:1 Lipid: Cholesterol ratio with 1.5 hours hydration time. While, liposomal formulation for Leuprolide acetate (LA-Liposomes) prepared using dehydration-rehydration method, optimized using 3^2 full factorial design gave the optimum vesicle size of 432.5±2.1 nm with 74.63±1.1 % EE with overall desirability of 0.937 at 3:1 Lipid: Cholesterol ratio with 0.5 hours hydration time. Liposomal formulation with dual drug entrapment (RLX-LA Liposomes) prepared using dehydration-rehydration method, optimized using 3^2 full factorial design gave the optimum vesicle size of 354.4±1.0 nm with 90.91±1.1 and 74.3±1.0 % EE for Raloxifene Hydrochloride and Leuprolide acetate respectively, with overall desirability of 0.673 at 3:1 Lipid: Cholesterol ratio with 0.5 hours hydration time. Based upon results it was observed that independent variables had no significant effect on the vesicle size of RLX liposomal formulation but significantly affected the entrapment efficiency. As the Lipid: Cholesterol and Hydration time was increased, % EE increased. In the case of LA-Liposomes, similar effect was observed; independent variables had no significant effect on the vesicle size but significantly increased % EE with increasing Lipid: Cholesterol and Hydration time. While for dual drug entrapped (RLX-LA Liposomes), vesicle size increased with increasing Lipid: Cholesterol and Hydration time. % EE was also found to increase significantly with increasing Lipid: Cholesterol and Hydration time. Each

optimized formulation was freeze dried as liposomal gel in medical grade silicone tubing to form intravaginal rod insert.

Characterization of liposomal formulations

All the three optimized formulations were characterized for zeta potential, drug loading (%w/w), morphological analysis by TEM and SEM and in vitro drug release. Optimized batch of RLX-Liposomes had zeta potential of +2.13±1.0 mV. Optimized batch of LA-Liposomes had zeta potential of -23.3±1.1 mV while for dual drug entrapped RLX-LA Liposomes it was -22.9±1.2 mV indicating the physical stability of the liposomes. Drug loading for RLX-Liposomes was found to be 25.29±1.1 %w/w, for LA-Liposomes it was 9.29±1.2 %w/w. In the case of dual drug entrapped RLX-LA Liposomes loading of RLX was 18.79±1.5 % w/w and for LA it was 7.65±1.1 %w/w within the vesicles. TEM and SEM analysis revealed that all the liposomal formulations contained discrete, spherical and uniform vesicles with size in agreement with that obtained by dynamic light scattering (Malvern Zetasizer).

RLX loaded liposomes was able to sustain the drug release for up to 132 hours (approx. 6 days). Where as, the entire pure RLX drug was released within 8 hours. In the case of LA loaded liposomes, drug release could be achieved for 108 hours (approx. 5 days). Pure LA was entirely released into the diffusion medium within 4 hours. Similar results were obtained for dual drug entrapped liposomes. There was not a significant difference between the drug release rate from the individual formulations and dual drug entrapped formulation. Drug release kinetics when studied by applying various mathematical models to the drug release data, zero order was found to follow for all the liposomal formulations. While IVRs was able to sustain the drug release up to 144 hours (6 days) for both RLX as well as LA.

Histopathology study

Histopathology study using goat vaginal mucosa was performed to observe the pathological changes in the vaginal mucosa in contact with the drugs and drug loaded

formulations, administered intra vaginally to target the uterus. Results revealed no signs of irritancy or damage to the mucosal tissue upon contact with the liposomal formulations.

In vitro cell cytotoxicity study

In vitro cytotoxicity studies (MTT Assay) were performed on MCF-7 cell line at different concentrations (0.001, 0.01, 0.1, 1, 10 µg/ml) of drugs and drug loaded liposomal formulations (std. RLX, std. LA, RLX-Liposomes, LA-Liposomes and RLX-LA Liposomes) at three different incubation times (12, 24 and 48 hours). Results demonstrated that RLX-Liposomes (86.41 % cell inhibition after 48 hours for 10 µg/ml concentration) was more effective in causing cell cytoxicity than the standard Raloxifene drug (73.44 % cell inhibition after 48 hours for 10 µg/ml concentration). Similarly, LA-Liposomes caused more cell cytotoxicity (88.24 % cell inhibition after 48 hours for 10 µg/ml concentration) than standard Leuprolide drug (74.19 % cell inhibition after 48 hours for 10 µg/ml concentration). For dual drug entrapped (RLX-LA Liposomes) formulation, the cytotoxicity was significantly more (90.47 % cell inhibition after 48 hours for 10 µg/ml concentration) than the both the individual formulations. Liposomal formulations showed lower values of IC50 at all the time points than the standard drugs which can be interpreted in a way that lower dose of liposomal formulation is required to inhibit cell growth than the standard drug. IC50 value after 48 hours of incubation showed that LA liposomes (0.56) had 28 fold higher value of IC50 and RLX liposomes (0.83) had 41 fold higher value of IC50 than dual drug entrapped liposomes indicating the highest efficacy of the dual drug entrapped liposomal formulation (RLX-LA Liposomes).

Results of apoptosis study showed that liposomal formulations had more counts of cells in apoptotic phase (66.4 for both RLX-Liposomes and LA-Liposomes) than as compared to the standard drugs (57.4 for RLX and 33.4 for LA). Highest apoptotic count could be registered by RLX-LA dual drug entrapped liposomes (75.22). The augmented apoptotic activity of combined drug formulation in comparison to individual liposomal formulations can be due to the synergism of both the drugs. Standard RLX and LA shows

stronger arrest at G0/G1 phase compared to their respective liposomal formulations. Where LA-Liposomes shows more cell arrest in S-Phase preventing its progression to M phase. RLX-LA liposomes showed more cell accumulation in G2/M phase than as compared to individual liposomal formulations.

In vivo biodistribution study

In order to examine the uterine drug targeting efficiency, in vivo biodistribution studies were carried out using ^{99m}Tc labeling of drugs. The mean labeling efficiency of RLX was >98 % at pH 4.5, while for LA it was >98 % at pH 7.0. Incubation of both ^{99m}Tc-RLX and ^{99m}Tc-LA in human serum and 0.9 % saline at 37^{o}C revealed that the labeling of both the drugs was extremely stable. DTPA challenge study demonstrated that the labeling efficiency of the radio labeled complex did not alter significantly in the presence of DTPA indicating the higher binding affinity of technetium with RLX and LA. When ^{99m}Tc-RLX loaded liposomes were administered via vaginal route, maximum amount of drug was found in uterus even after 24 hours (74.94%) with no distribution in any other organs of the body. In the case of ^{99m}Tc-RLX plain drug administration only 28.24 % was able to retain in uterus after 4 hours, while ^{99m}Tc-RLX loaded liposomes at same time point showed 2.7 fold higher concentration. After 4 hours, further imaging, showed no radioactive counts in uterus in case of plain drug administration. ^{99m}Tc-LA loaded liposomes when administered via vaginal route, 90.21 % of drug was found in uterus even after 24 hours, while in case of ^{99m}Tc-LA plain drug administration, 20.78 % was found after 4 hours. This showed that liposomes showed 4.5 fold higher concentration in uterus than plain drug. After 4 hours further imaging showed no radioactive counts in case of plain drug administration.

Pharmacodynamic and Pharmacokinetic study

Pharmacodynamic study was performed to observe the effect of liposomal formulations on the uterine fibroids induced in rabbits and to compare the effect of three different formulations viz. LA-Liposomes, RLX-Liposomes and RLX-LA-Liposomes. Three different rabbits, induced with uterine fibroids were administered intravaginally with

Liposomal Leuprolide loaded rod insert (IVR), Liposomal Raloxifene Hydrochloride loaded rod insert (IVR) and Liposomal dual drugs (LA-RLX) entrapped rod insert (IVR). The results showed that uterine lumen in the case of RLX-liposomal formulation treated rabbit after 30 days though showed the regression effect but not as significantly as LA-liposomal formulation treatment. While, rabbit administered with RLX-LA (dual drug loaded) liposomal formulation, showed highest fibroid regression effect than the individual formulations.

Pharmacokinetic study revealed the presence of very negligible % of administered dose of both the drugs in rabbit serum. After 24 hours of drug loaded intravaginal rod insertion in rabbits, the drug concentration in serum was found to be only 0.02 % for LA-Liposomes while, for RLX-Liposomes it was 0.53 %, as quantified by LCMS-MS technique. The results of pharmacokinetic studies could be very well correlated with the in vivo biodistribution study showing maximum amount of the drug retained in uterus even after 24 hours.

Stability study

Short-term accelerated stability study for three months was performed according to the ICH Q1A (R2) guidelines for all the three liposomal formulations as well as the Intra Vaginal Rod inserts. The vesicle size and entrapment efficiency were determined at 0, 1, 2, 3 month to study the stability of liposomal dispersions. While the Intra Vaginal Rod inserts were examined for physical appearance and In Vitro drug release. Results showed that there was no significant difference between the measured parameters at initial time and after three months indicating the stability of developed formulations.

12.2 Conclusion

RLX-Liposomes, LA-Liposomes and dual drug loaded (RLX-LA Liposomes) were successfully prepared and optimized using 3^2 full factorial design. All the three liposomal formulations had desired size range with high drug entrapment efficiency and loading. Liposomes appeared spherical and discrete as confirmed by TEM and SEM with

no signs of aggregation. Drug release was sustained up to 132 hours for RLX-Liposomes and 108 hours for LA-Liposomes. IVR of both the drugs could give release up to 6 days. Histopathology study revealed the non-toxicity of all the liposomal formulations upon contact with vaginal tissue. Biodistribution study by Gamma Scintigraphy revealed the preferential uptake of liposomes by the uterus when the formulation was administered by vaginal route. In comparison to Plain drug, liposomes were able to concentrate in higher amount and were retained within the uterus for longer period of time. In vitro cell cytotoxicity study revealed highest % cell inhibition caused by dual drug entrapped liposomes with lowest IC50 value and highest apoptotic count. Pharmacodynamic study showed maximum fibroid regression effect of dual drug loaded (RLX-LA) liposomes. Negligible concentration of drugs could be detected in rabbit serum after intravaginal delivery of liposomes indicating the potential of the route to target the uterus. All the developed formulations were found to be stable upon storage under refrigerated conditions. Hence, uterine targeting by vaginal route seems to be a promising approach for the treatment of disorders related to uterus where the tissue concentration of the drug exceeds the systemic absorption anticipating a dose reduction needed to elicit the therapeutic action and avoidance of the side effects. Extended research involving preclinical and clinical trials may further prove the potential of the formulation in drug targeting to the uterus via vaginal route.