Chapter 7

EX VIVO HISTOPATHOLOGY STUDY

7.1 Introduction

Histopathology, also referred to as cellular pathology, is a complex and a pivotal area of study in modern medicine. It is an extremely detailed branch of science that focuses solely on the anatomical changes that occur in diseased tissue at a microscopic level. This science is so specific that it not only involves performing tests, conducting analysis and gathering data, but interpreting the information gathered and making the final diagnosis to manage the treatment for the specific disease. The histological evaluation of surgical biopsies from affected tissues is a standard way of assessing pathological change and determining treatment in many diseases**.** Histopathologists can use a number of stains and various microscopy techniques to examine and analyze the sample. Each stain has specific properties that help to identify a particular disease, as not every pathogen is visible with every stain. For instance, while haematoxylin-eosin (H/E), is the most common stain used for histopathological studies. [1]

The aim of this study was 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. Histopathological diagnosis can help evaluate any irritancy or necrosis caused to the tissues by the drugs and/or formulations. Formulations when administered are expected to remain non-toxic to the surrounding tissues in contact unless they encounter the site of action. Histopathological studies are hence crucial part of the research work to establish the safety profile of the formulations.

7.2 Materials

Goat vagina was obtained from the local slaughter house. Formalin (10% buffered, Neutral) and Hematoxylin-Eosin stains were procured from Sigma Aldrich, India. Phosphate buffer pH 7.4 and 4.5 were prepared in house using the reagents of analytical grade. Distilled water was prepared using the in house distillation assembly. Isopropyl Alcohol and all other reagents and chemicals used were procured from authentic source and were of analytical reagent grade.

Equipment used:

- Microtome (HM 355S Automatic Microtome, ThermoFisher Scientific, USA)
- Nikon H600L Microscope (Nikon Instruments Inc., NY, USA)

7.3 Histopathology Method [2]

Vaginal tissue was collected from freshly sacrificed goat in a local slaughterhouse. The tissue was cleaned and washed properly with the Phosphate Buffer Saline pH 7.4. Then the vaginal mucosa was separated from the underlying tissue with the help of forceps and scissor. The inner mucosal part, which is expected to remain in contact with the formulations that are intended to be administered intravaginally, was cut into seven small pieces (1 cm X 1cm each) and were put into seven different vials containing 10 ml of following solutions

I. 10 ml of 70% Isopropyl Alcohol (IPA) solution (Positive Control)
II. 10 ml of Phosphate Buffer pH 4.5 solution (Negative Control)
III. 10 ml of Raloxifene Hydrochloride plain drug solution in Phosphate Buffer pH 4.5
IV. 10 ml of Raloxifene loaded Liposomal dispersion
V. 10 ml of Leuprolide Acetate plain drug solution in Phosphate Buffer pH 4.5
VI. 10 ml of Leuprolide Acetate loaded Liposomal dispersion
VII. 10 ml of Raloxifene and Leuprolide (dual drug) loaded Liposomal dispersion

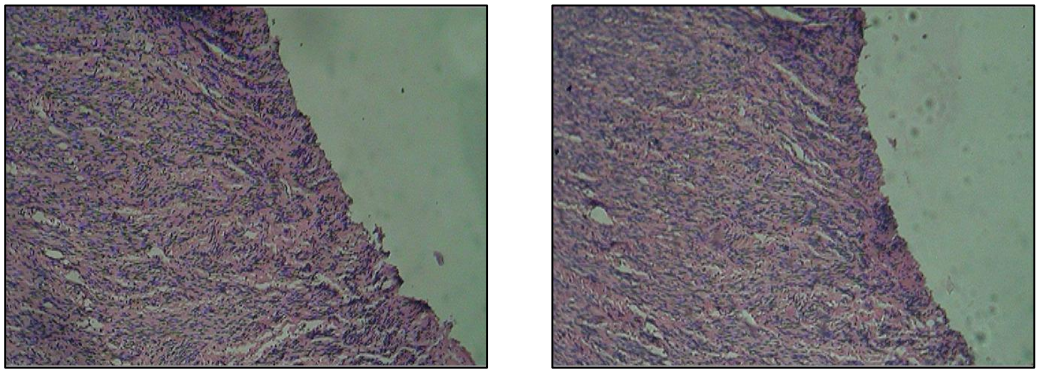
The study was carried out for two different time points 1 week and 2 weeks; i.e. the tissue was kept in contact with the different solutions for 7 days and 14 days as the formulations developed are intended to remain intravaginally for one week. After one and two weeks the tissues were removed from the vials, washed with distilled water and then fixed in 10% buffered formalin individually, dehydrated in graded concentrations of ethanol (70, 90 and 100%), immersed in xylene and embedded in paraffin. The tissues embedded in paraffin blocks were mounted on a Microtome (HM 355S, ThermoFisher Scientific, USA) sectioned at a thickness of 4 microns, mounted on clean glass slides, and stained

with haematoxylin-eosin. The slides of controls and treated mucosal tissues were examined using a fluorescence microscope (Nikon H600L Microscope, Nikon Instruments Inc., NY, USA) for any lesions or damage to the epithelium. Photomicrographs of vaginal mucosal tissues were also taken.

7.4 Results and Discussion

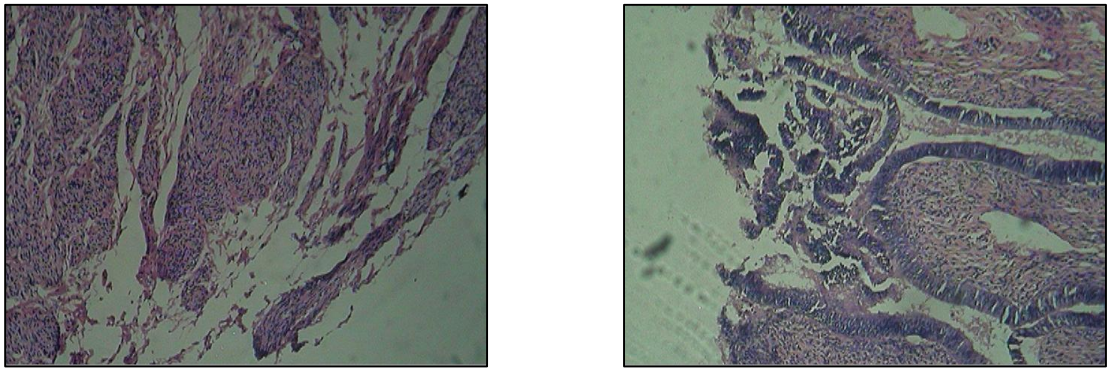
Histologically normal vaginal tissue comprises of (i) the inner mucosal epithelial stratum, (ii) a lamina propria containing thin-walled veins, (iii) the intermediate muscularis stratum, and (iv) the external adventitial layer. The inner mucosal epithelial stratum adheres strictly to the muscular stratum. Its nonkeratinized squamous epithelium is raised by two median, anterior and posterior, longitudinal ridges, between which are the rugae divided by sulci of variable depth, the vaginal columns. [3] Any abnormality in these structures caused by the formulations in contact can be evaluated by Histopathological study. Ideally, formulation should be non-toxic and non-irritant to the tissues with which it stays in contact for longer period of time so as to be a safe drug delivery.

As seen in the results of histopathological studies, image of Phosphate Buffer pH 4.5 treated vaginal mucosa (Figure 7.1; Negative control) showed intact tissue and squamous epithelium of the vaginal mucosa at both the time points 1 and 2 weeks. While the section That was IPA treated (Figure 7.2; Positive control) shows disruption and thinning of mucosal membrane, as there was damage to the epidermal cells. The tight intact squamous epithelium is seen broken and disrupted at intervals.



A) **B)**

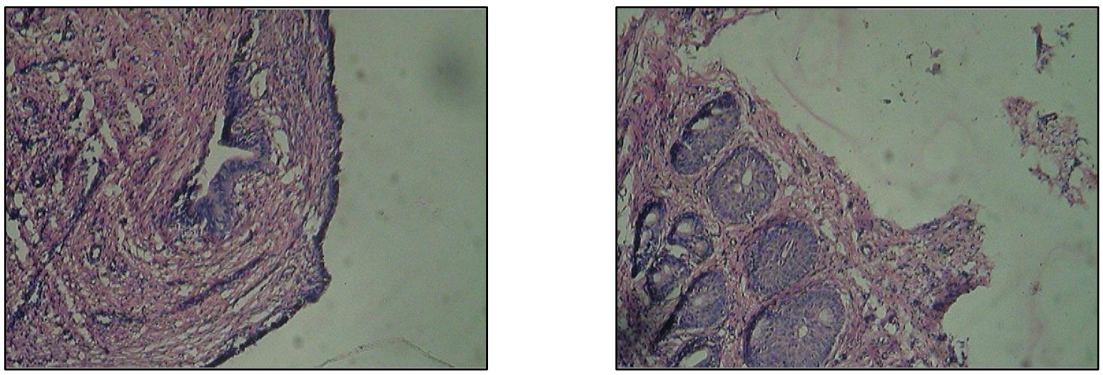
Figure 7.1 Vaginal tissue treated with Phosphate Buffer pH 4.5 (negative control) for A) One week B) Two weeks



A) **B)**

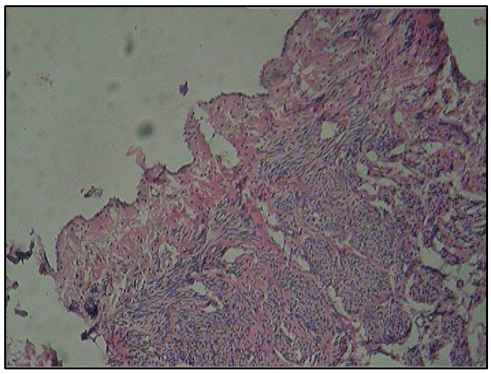
Figure 7.2 Vaginal tissue treated with Isopropyl Alcohol (positive control) for A) One week B) Two weeks

The sections treated with plain standard RLX and plain standard LA shows little damage but not so significant like that caused by IPA to the tissue, as seen in the Figures 7.3 (std. RLX) and 7.4 (std. LA). As the exposure time increased from one week to two week, the damage caused was also increased but not causing major disruption. This suggests that plain drugs cannot be administered intravaginally to target the uterus, as they are liable to cause tissue damage and irritancy to the vaginal mucosa. They need to be formulated and entrapped within a carrier that can protect the surrounding tissues and cells.



A) **B)**

Figure 7.3 Vaginal tissue treated with standard RLX for A) One week B) Two weeks



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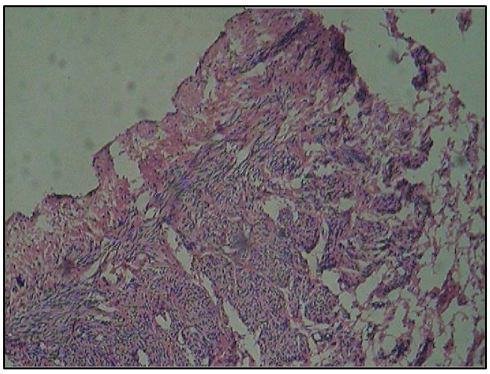
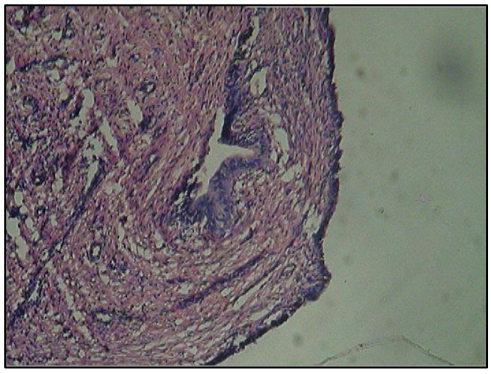


B)

Figure 7.4 Vaginal tissue treated with standard LA for A) One week B) Two weeks

RLX-Liposomes, LA-Liposomes and RLX-LA Liposomes when compared to the negative and positive controls as well as standard drugs, showed no abnormality. There was no change in the epithelium lining with no signs of irritancy or damage to the mucosal tissue as seen in the Figures 7.5, 7.6 and 7.7. The formulations seemed to be tolerated well even after two-week exposure. The results suggest good intravaginal acceptance of the drug when formulated as liposomal formulations. The formulations thus proved to be non-toxic and safe for the intravaginal administration.



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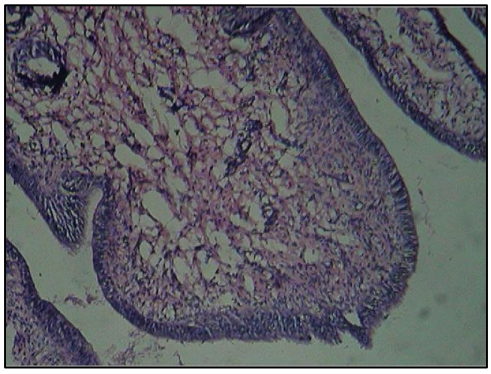
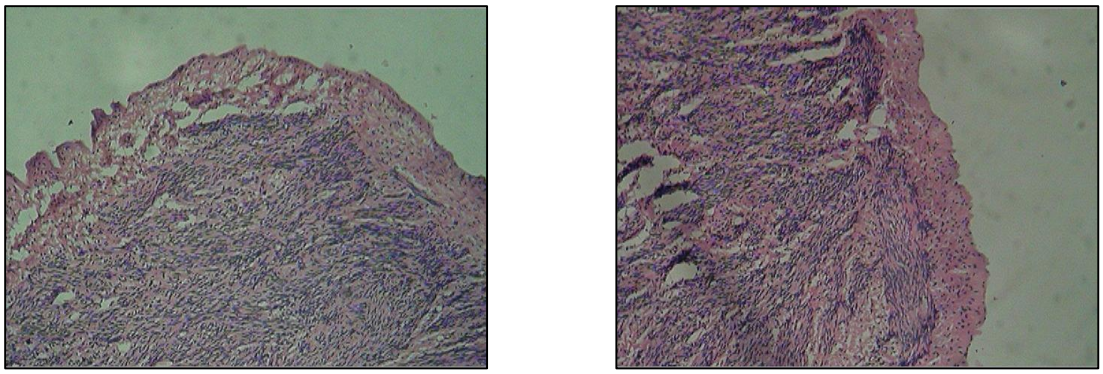


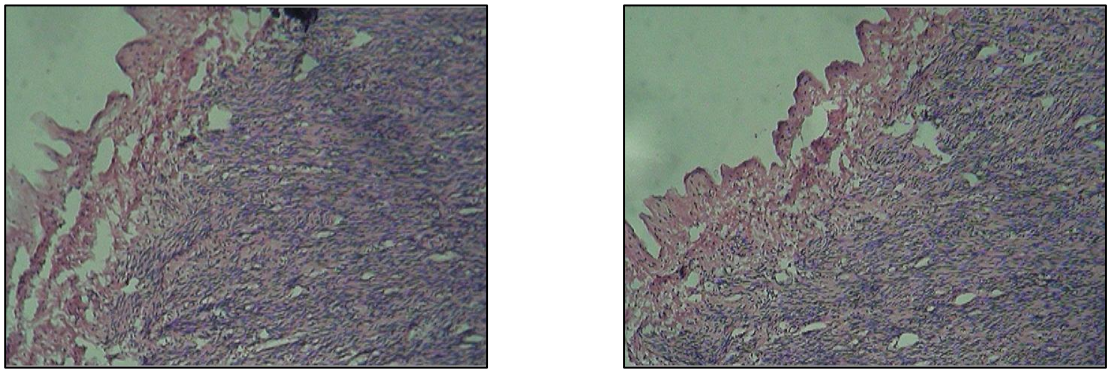
B)

Figure 7.5 Vaginal tissue treated with RLX-Liposomes for A) One week B) Two weeks



A) **B)**

Figure 7.6 Vaginal tissue treated with LA-Liposomes for A) One week B) Two weeks



A) **B)**

Figure 7.7 Vaginal tissue treated with RLX-LA-Liposomes for A) One week B) Two weeks

Liposomes being made up of inert, biodegradable, and biocompatible lipids do not cause any damage to the tissues. Moreover, they entrap the drugs within their bilayers and aqueous core, as a result of which there is no direct exposure of drugs to the tissues. This will prevent the necrosis and toxicity caused to the tissues.

7.5 References

1) http://www.microscopemaster.com/histopathology.html as accessed on 14th July 2016.

2) J. D'Cruz, Barbara Waurzyniak and Fatih M. Uckun 2004. Mucosal Toxicity Studies of a Gel Formulation of Native Pokeweed Antiviral Protein Toxicol Pathol 32: 212

3) Jannini, E.A., d'Amati, G. and Lenzi, A., 2006. Histology and immunohistochemical studies of female genital tissue. In Women's sexual function and dysfunction: Study, diagnosis and treatment (pp. 125-133). Taylor and Francis.