CHAPTER - IX

CELL VIABILITY STUDIES OF BIOADHESIVE VAGINAL FORMULATIONS

9.0 CELL VIABILITY STUDY

9.1 Purpose

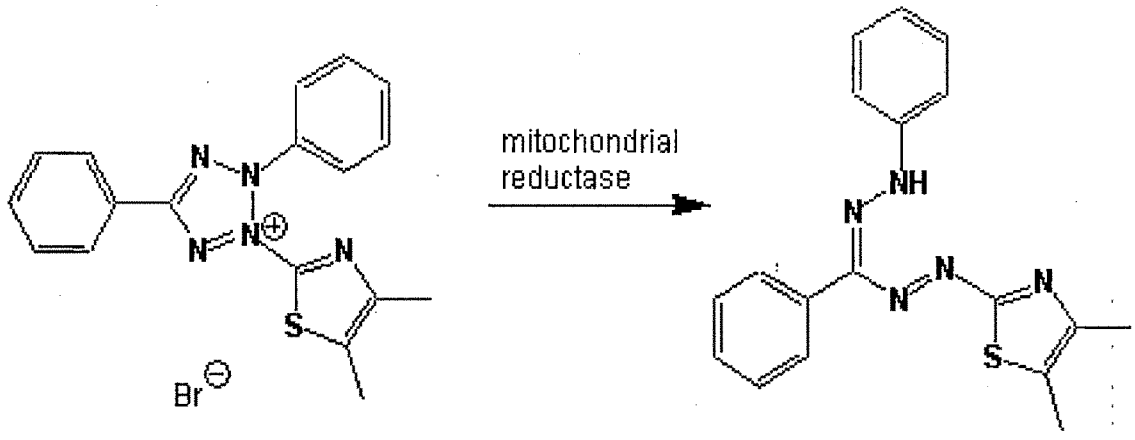
High therapeutic concentrations and prolong retention of the bioadhesive vaginal formulation in vaginal cavity might produce toxicity for the vaginal epithelium cells. Therefore, the optimized bioadhesive vaginal formulations were next tested for cell viability (cytotoxicity).

9.2 Introduction

Cell viability assay was studied to ensure the biocompatibility of the bioadhesive formulations for their intended use (Jung Yun C. *et al.*, 2002). Bioadhesive vaginal formulations are supposed to be administered intravaginally in solid form. In vagina, solid form of the formulation will convert into gel by absorbing large amount of water, providing better retention and absorption. To counteract vaginal clearance mechanism and to achieve desired therapeutic quantity of drugs in vaginal cavity, a very high dosing of formulation is needed. They might produce toxicity for the vaginal epithelium cells in vaginal cavity. Therefore, it becomes important to carry out a cytotoxicity (cell viability study) study for bioadhesive vaginal formulations. Also, cell viability study can provide an idea about the toxic effect of the drug/excipients on vaginal mucosa too.

There are many cytotoxicity assays available; choosing one of them is a very challenging task. The cell viability assay is chosen on the basis of sensitivity of detection, ease of use, reproducibility of data, total cost of running, availability of instrumentation and reagents stability at ambient temperature (while dispensing into several plates). MTT (3-[4-5-dimethylthiazol-2-4]-2, 5-diphenyltetrazolium bromide) assay and trypan blue are the most common assay methods used by researchers to measure the cell viability (Takara K. *et al.*, 2002, Reddy K. V. R. *et al.*, 2004). MTT has the advantage that it is a quantitive method and more sensitive. A linear relationship between cell viability and absorbance can measure the growth rate and death rate. On the other side the trypan blue is qualitative method and indicates only when cell is alive. We used MTT assay in present investigation to measure cell viability.

MTT is a non radioactive cell proliferative assay which measures the reduction of MTT by the mitochondria and/or cytoplasmic succinate dehydrogenase enzyme and to form an insoluble, dark blue insoluble formazan product (Mosmann T. *et al.*, 1983). Only viable cells having dehydrogenase activities are able to reduce significant amounts of MTT dye to formazan. The amount of color produced is directly proportional to the number of viable cells. Most of the mammalian, plant, and yeast cell can be used for the cell viability study during MTT assay.



The prime objective of this experiment is to study the viability of endocervical vaginal epithelial (HeLa-S3) cell lines by MTT assay, following treatment with various concentrations of ITR and CL individually, and their formulations as films and softgels. The experimental results will provide ideas about possible cytotoxic effect of bioadhesive vaginal formulations over vaginal epithelium upon its intravaginal application.

9.3 MATERIALS

Endocervical vaginal epithelial (HeLa-S3) cell lines were procured from National Centre for Cell Science (NCCS, Pune, India). Ham's F12 K medium, fetal bovine serum (10% v/v), L- glutamine, Sodium bicarbonate ($NaHCO_3$), phosphate buffer saline and MTT were purchased from Himedia, (Mumbai, India). Antibiotics (Penicillin/streptomycin) were obtained from sigma Aldrich (Mumbai, India). Trypsine (0.25%) was obtained from Hyclone (Fisher Scientific). 96 well plates (flat bottom, Polystyrene) and cell culture flasks (175 cm^2 polystyrene) were purchased from Tarson (Mumbai, India). All other chemicals used were of research grade.

9.4 EXPERIMENTAL

9.4.1 Media Preparation

The Ham's F12 K medium was mixed with 10% fetal bovine serum, 2.0 mM L- glutamine and 1.5 mg/mL $NaHCO_3$ and 2% antibiotics (Penicillin/streptomycin) in aseptic condition. This combination was used throughout all cell culture experiments.

9.4.2 Cell Culture

HeLa-S3 cell line was grown regularly in cell culture flask. The environmental condition of the flask was maintained at 37°C and 5% CO_2. A sufficient amount of humidity

(approx 90%) was maintained in the incubator. Media was changed in each 48 hours in the culture flask until the cell is confluent and ready to divide. Cells were occasionally observed under the microscope to see the confluency. At 80-90 % confluency cells were divided. Trypsin was used to detach the cells from the bottom of the flask. The detached cells were centrifuged (Remi equipments ltd., Mumbai, India). Supernatant liquid was thrown and the cells were obtained at the bottom of the centrifuge tube. The cells were further seeded in the new 175 cm^2 culture flask and grown. This cycle was repeat until to fulfill the cell demand for MTT experiments.

Take 30µl of cell suspension and mix it with 70 µl of trypan blue. Mix solution well using a pipette. Using a pipette, transfer some of the cell suspension (trypan blue mix) into the hemocytometer overlay with a coverslip. The cell suspension will pass under the coverslip by capillary action. Make sure that the coverslip is not moved once it is place on the grid and the cell solution is added. Place the hemocytometer on the stage of an inverted microscope and Count the live cell as clear form and dead cell as blue cells. Calculate the number of cells per ml using the following formula:

$$\text{Number of cells per ml} = \text{Cell count} \times 2.5 \text{ (chamber factor)} \times 10^3$$

The cells were grown in all the 96 wells of the plate but the only 70 wells were used to study the effect of samples on cell viability. Remaining 14 wells were filled with media only which served as control and 12 wells filled with DMSO served as blank. For one treatment group 7 wells were used.

9.4.3 Sample Preparation for Cell Viability Study

Bioadhesive vaginal formulations are composed of drugs (ITR or CL) and various bioadhesive polymers. We evaluated the cell viability for all the drugs individually and their formulations (film and softgel) and placebo. Stocks of all samples were prepared in PBS with 1mg per ml concentration. Seven different concentrations were prepared by transferring suitable aliquots from a stock to well and maintained upto to 100µl with PBS. At a time in one plate, maximum 8 treatment groups (A-H) were added. The transfers of treatment group were performed in aseptic condition under laminar flow hood.

Table 9.1 Sample planning of test samples in 96 tissue culture well plates

Transwells No. I											
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A_1 10µl	A_3 30µl	A_5 50µl	A_7 70µl	A_8 80µl	A_9 90µl	A_{10} 100µl	I_1 10µl	J_1 10µl	Control 100µL	Control 100µL	Blank 100µl
B_1 10µl	B_3 30µl	B_5 50µl	B_7 70µl	B_8 80µl	B_9 90µl	B_{10} 100µl	I_3 30µl	J_3 30µl	Control 100µl	Control 100µl	Blank 100µl
C_1 10µl	C_3 30µl	C_5 50µl	C_7 70µl	C_8 80µl	C_9 90µl	C_{10} 100µl	I_5 50µl	J_5 50µl	Control 100µl	Control 100µl	Blank 100µl
D_1 10µl	D_3 30µl	D_5 50µl	D_7 70µl	D_8 80µl	D_9 90µl	D_{10} 100µl	I_7 70µl	J_7 70µl	Control 100µl	Control 100µl	Blank 100µl
E_1 10µl	E_3 30µl	E_5 50µl	E_7 70µl	E_8 80µl	E_9 90µl	E_{10} 100µl	I_8 80µl	J_8 80µl	Control 100µl	Control 100µl	Blank 100µl
F_1 10µl	F_3 30µl	F_5 50µl	F_7 70µl	F_8 80µl	F_9 90µl	F_{10} 100µl	I_9 90µl	J_9 90µl	Control 100µl	Control 100µl	Blank 100µl
G_1 10µl	G_3 30µl	G_5 50µl	G_7 70µl	G_8 80µl	G_9 90µl	G_{10} 100µl	I_{10} 100µl	J_{10} 100µl	Control 100µl	Control 100µl	Blank 100µl
H_1 10µl	H_3 30µl	H_5 50µl	H_7 70µl	H_8 80µl	H_9 90µl	H_{10} 100µl	Blank 100µl	Blank 100µl	Blank 100µl	Blank 100µl	Blank 100µl

Sample to be analysed.....

A. **$ITRF_{65}$ film** — **$ITRF_{65}$** film weight eq. to 100mg of ITR dissolved in 10ml PBS

B. **F_{86} film** — **F_{86}** film weight eq. to 100mg of CL dissolved in 10ml PBS

C. **CL** — 100mg CL dissolved in 10ml PBS

D. **ITR** — 100mg ITR dissolved in 10ml PBS

E. **ISF_{59}** — **softgel** content weight eq. to 100mg of ITR dissolved in 10ml PBS

F. **SF_{79}** — **softgel** content weight eq. to 100mg of CL dissolved in 10ml PBS

G. **Placebo 1** — **$ITRF_{65}$** placebo dissolved in 10ml PBS buffer

H. **Placebo 2** — **F_{86}** placebo dissolved in 10ml PBS buffer

I. **Placebo 3** — **ISF_{59}** placebo dissolved in 10ml PBS buffer

J. **Placebo 4** — **SF_{79}** placebo dissolved in 10ml PBS buffer

9.4.4 Cell Viability Study using MTT Assay

MTT assay is designed for spectrophotometric quantification of cell growth and viability (Denizot F *et al.,* 1986) by measuring change in color. In this assay, the yellow color water soluble MTT compound is reduced by mitochondria and/or cytoplasmic succinate dehydrogenase enzyme present in the living cells and to form purple color water insoluble formazan crystals (Niks M *et al.,* 1990, Maria-Elisa P., 2003). But, formazan crystals can be solubilized by using solubilizing agents. DMSO and acidified 10% sodium dodecyl sulfate in 0.1 MHCl are most commonly used solubilizing agents. The optical density (OD) of this colored solution is measured at 570 nm with a 96-well multiscanner ELISA reader with DMSO serving as blank. No formazan crystals are formed in dead cells. The intensity of color produced by formazan crystals is directly proportional to number of living cells present in the sample. All MTT experiments were performed under aseptic condition and performed in triplicate.

Day 1

The HeLa-S3 cell line was grown in cell tissue culture flask using Ham's F12 K medium supplemented with 10% fetal bovine serum, 1.5 mg/mL $NaHCO_3$ and 2.0 mM L- glutamine. Media of the flask was changed at regular intervals (48 hours) until flask becomes 80-90% confluent. Cells were trypsinized, detached and centrifuged. Exponentially growing HeLa-S3 epithelial cells are seeded into 96-well plate containing Ham's F12 K media at a density of 10^6cells/well. The volume for each well was maintained to 100 µl with media. The cells are allowed to grow for 24h at 37 °C and 5% CO_2 prior exposure to vaginal formulation.

Day 2

On the day of treatment (after 24 hours of incubation period) Ham's F12 K media of each well was replaced with fresh medium. Test samples (**A-J**) were placed on top of the cells in well as describe in Table 1. Seven wells were treated with one treatment group. The culture plate is further incubated for 24 hours at 37 °C.

Day 3

After 24 hours incubation, cells were washed with PBS to remove the formulation and 100 µl of fresh medium with 10 µl of MTT solution (5 mg/ml in 0.1 M PBS, pH 7.2) was added to each well. Wells containing only medium and MTT were considered as negative controls (without formulation treated). Plates are then incubated for 4 h at 37 °C and 5% CO_2 in incubator. After incubation, MTT reaction medium was discarded and cells were washed

with PBS. Then, add 100 µl DMSO in each well to dissolve blue formazan crystals and measured optical density (OD) at 570 nm with a 96-well multiscanner ELISA reader. Absorbance obtained with DMSO serve as blank. The percent viability was calculated by the following formula:

$$\text{Cell viability} = \frac{\text{OD of the test sample}}{\text{OD of the control sample}} \times 100$$

4.5 RESULTS AND DISCUSSION

Cell lines procured from NCCS, Pune were free from any kind of bacterial and fungal contamination. Percentage viability of cell lines was studied by using Trypan blue dye exclusion technique. The % viability of *HeLa* cell line was found between 72-78% which is most suitable to perform cytoxicity studies.

Table 9.2 Percentage cell viability and characterization of cell line

Cell line	Live Cell count	Total cell count	% Cell viability
HeLa- S3	16.6×10^4	23.2×10^4	72%
HeLa- S3	120×10^4	154×10^4	78%
HeLa- S3	240×10^5	320×10^5	75%

In order to determination of safety of bioadhesive vaginal formulations against vaginal epithelial cell, MTT assay was performed using HeLa-S3 cell lines. Cell viability study was performed with all the drugs individually and their formulations (film and softgel) and placebo. Absorbance obtained with blank was substrated from absorbance values obtained with test and control sample. Result of cell viability with respect to ITR and their formulations is enumerated in **Table** 9.3.

It is assumed that media supports cell growth fully and hence the cell viability with media treatment group (control) is considered 100%. ITR and their formulations equivalent to concentration range from 100µg to 1000µg was used to evaluate the cell viability. **Fig.**9.1 shows the cellular viability of HeLa-S3 cells which was investigated over various concentrations of ITR and their optimized vaginal formulations and/or placebo. As can be seen from Table **9.3,** $ITRF_{65}$ film and its placebo did not show any cytotoxic effect in HeLa-S3 cells at concentration up to 500µg. Nevertheless, in presence of high concentration (1000 µg) of $ITRF_{65}$ film and placebo, cell viability was decreased by 6% and 2%

respectively. While softgel formulation of ITR was decreased viability of HeLa-S3 cells upto 10-11%. Placebo softgel was also decreased cell viability by 9-10%. This may be due to higher amount of PEG 400 used in softgel preparation.

Table 9.3 Percentage cell viability against **ITR** and their optimized vaginal formulations and/or placebo.

Samples	% Cell viability at different concentration						
	100µg	300µg	500µg	700µg	800µg	900µg	1000µg
ITR	99.20	99.31	98.42	97.04	95.87	94.60	93.74
$ITRF_{65}$ film	99.56	99.12	98.86	97.63	95.22	93.84	93.08
ISF_{59}	96.52	95.82	93.76	92.29	90.85	89.14	89.57
*Placebo 1	99.39	99.22	98.67	97.70	97.81	97.43	97.54
*Placebo 3	96.21	95.88	94.48	93.14	91.46	92.72	91.87

*Placebo 1 – Film palcebo, *Placebo 3- softgel placebo

Table 9.4 Percentage viability of HeLa-S3 cells against CL and their optimized vaginal formulations and/or placebo.

Samples	% Cell viability at different concentration						
	100µg	300µg	500µg	700µg	800µg	900µg	1000µg
CL	98.47	98.76	97.88	97.13	95.89	93.84	92.49
F_{86} film	98.67	98.17	97.59	96.35	95.76	93.6	92.61
SF_{79}	95.85	95.52	94.77	92.28	90.12	88.95	88.21
*Placebo 2	98.75	99.00	98.84	98.2	99.06	98.53	97.84
*Placebo 4	96.6	96.35	95.85	94.85	93.65	92.68	91.35

*Placebo 2 – Film palcebo, *Placebo 4- softgel placebo

The cellular viability of HeLa-S3 cells which was investigated over various concentrations range from 100µg to 1000µg of CL. Results of all the samples are given in **Table** 9.4. There is no cytotoxicity observed with CL, F_{86} film and their placebo against HeLa-S3 cells at concentration up to 500µg. At higher concentration (1000 µg), F_{86} film and their placebo was decreased cell viability by 8%, and 3% respectively.

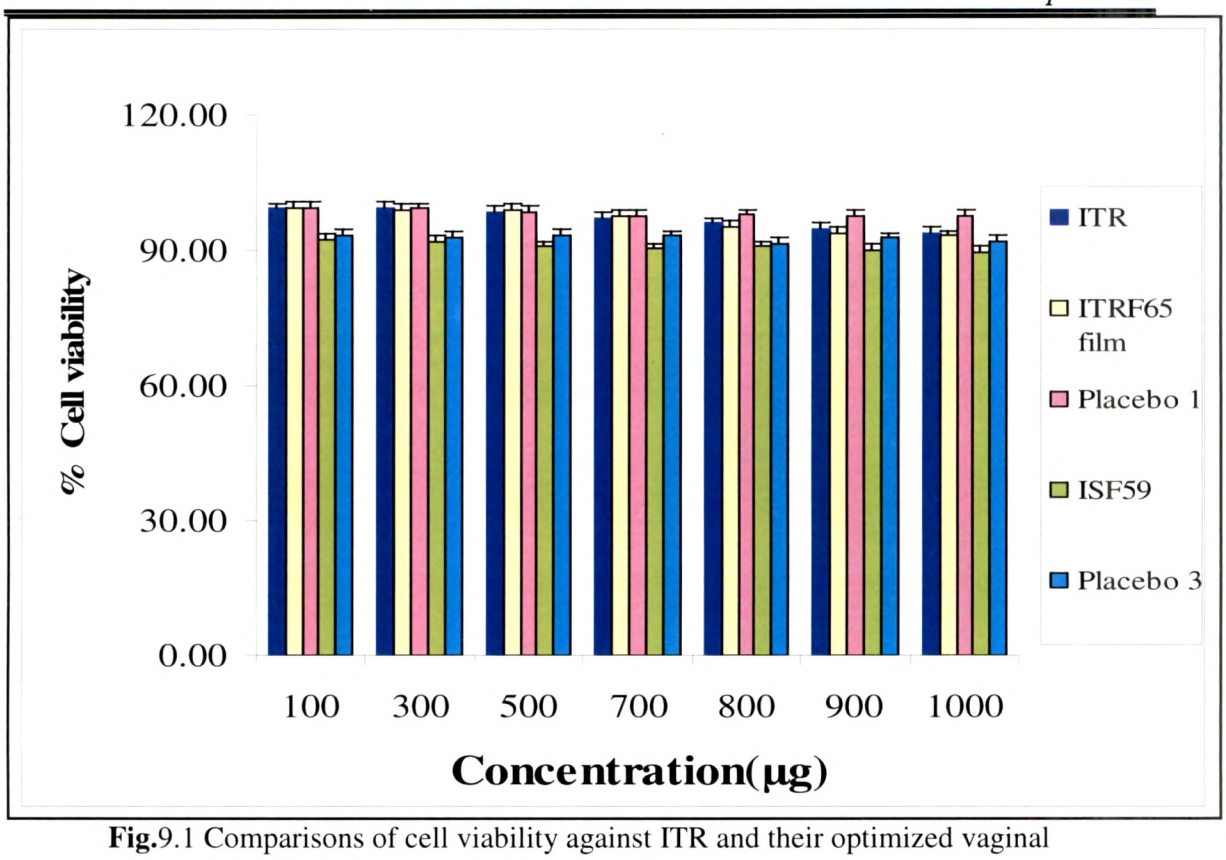


Fig.9.1 Comparisons of cell viability against ITR and their optimized vaginal formulations and/or placebo.

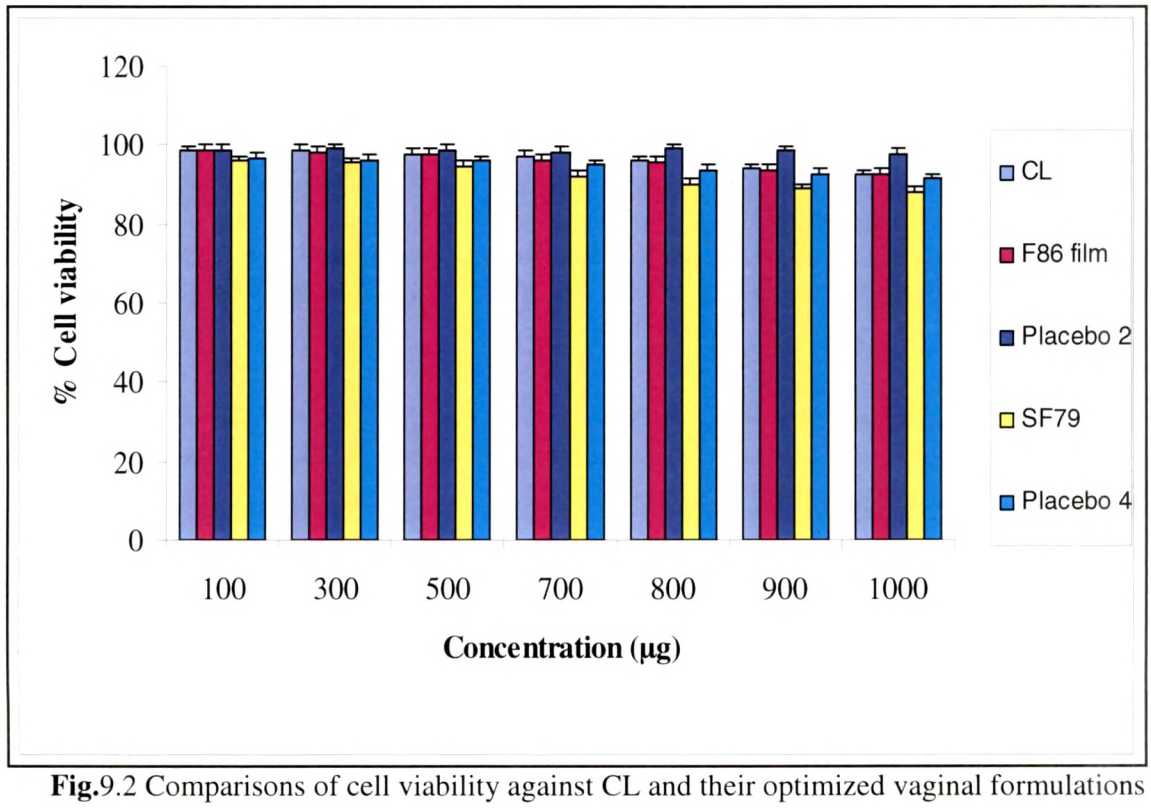


Fig.9.2 Comparisons of cell viability against CL and their optimized vaginal formulations and/or placebo.

Fig. 9.2 shows comparisons of cell viability against CL and their optimized vaginal formulations and/or placebo. Softgel preparation of CL (SF_{79}) was reduced cell viability upto 12%. This may be due to higher concentration of PEG 400 because placebo of softgel was also reduced cell viability upto 9%. The results were in agreement with previous cytotoxicity studies of PEG-based intravaginal formulations which showed greater cytotoxicity of formulations. (Jung Yun C. *et al.*, 2002).

4.6 CONCLUSION

ITR and CL did not exhibit significant toxicity across the given concentration range. Concentration dependent toxicity was noted with respect to each drug and their formulations. All the optimized vaginal formulations were found to be non-toxic because of they showed cell viability more than 85%. However, the PEG-based softgel formulation of CL and ITR was greater reduced cell viability of HeLa-S3 cells than the film formulation. The overall results obtained during this investigation suggest that all the developed bioadhesive vaginal formulations were non toxic in nature. Therefore, they can be safely used intravaginally without affecting cell viability of vaginal mucosa. They will offer substantial benefits for improving women's health.

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