Contents

8.1. INTRODUCTION

The success of transdermal drug delivery systems lies in efficiently carrying the drug across skin layers and placing it in systemic circulation at a rate suitable for achieving and maintaining desired therapeutic levels during the course of therapy [1].

It is well-recognized that a drug must successfully cross various skin layers and reach vascularized papillary region to become available for absorption in to systemic circulation [2]. Hence, drug permeation and deposition studies play a crucial role in predicting the in vivo behavior of newly developed formulations via evaluation of their drug carrying potential across skin.

8.2. MATERIALS & METHODS

8.2.1 Materials

Fluorescein-5-isothiocyanate (FITC) was purchased from Sigma-Aldrich, US. Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (4.5 g/L) with L-glutamine and sodium pyruvate and without calcium chloride and sodium bicarbonate; Gamma irradiated Fetal Bovine

Serum; Trypsin-EDTA solution 1X containing 0.25% trypsin, 0.038% EDTA in Hanks' Balanced Salt Solution with Phenol red and without Calcium and Magnesium; Antibiotic Antimycotic solution 100X liquid containing Penicillin (10,000 U/ml), Streptomycin (10 mg/ml) and Amphotericin B (25 µg/ml) in 0.9% normal saline. Dulbecco's Phosphate Buffered Saline (DPBS) without Calcium and Magnesium. Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from Sigma-Aldrich, US.

8.2.2 Reagents

8.2.2.1 Povidone-Iodine solution

4 mL of 7.5% w/v Povidone-Iodine solution was diluted up to 30 mL with distilled water to get 1 %w/v Povidone-Iodine solution.

8.2.2.2 Phosphate buffer saline, pH 7.4 (PBS 7.4)

800 mL water was collected in a 1L glass volumetric flask. 2.38 g of disodium hydrogen phosphate, 0.19 g of potassium dihydrogen phosphate and 8.0 g of sodium chloride were accurately weighed and added to the volumetric flask. The flask was shaken to dissolve the solutes and the volume was made up to the mark with water.

8.2.2.3 Glycerol solution in PBS 7.4 (Gly-PBS 7.4)

15 g glycerol was taken in a 100 mL glass volumetric flask. Around 50 mL PBS 7.4 was added to it and the flask was shaken to uniformly mix the content. The volume was finally made up to the mark with PBS 7.4.

8.2.2.4 Growth media

DMEM supplemented with 10 %v/v FBS and 1 %v/v antibiotic/antimycotic solution was used as growth media. Media was prepared by adding 10 mL FBS and 1 mL antibiotic/antimycotic solution to 89 mL DMEM and mixed well.

8.2.2.5 Treatment media

200 µl of different formulations were diluted to 1 mL with growth media and mixed well.

8.2.2.6 MTT solution

Accurately weighed quantity of MTT was dissolved in Dulbecco's Phosphate Buffered Saline to prepare 5 mg/mL stock. It was filtered

through 0.2 μ syringe filter, collected in to a sterile, amber glass vial and preserved at -20 °C till further utilized.

8.2.3 Skin procurement and preservation

Freshly excised ears of Yorkshire pigs were purchased from Ms. Khoobchand & Sons, New Delhi, India. Isolated pig ears were thoroughly cleaned with povidone-iodine solution, immediately rinsed and soaked in Gly-PBS 7.4 and preserved in dry ice to transport to laboratory. At laboratory, ears were thawed in PBS 7.4 at room temperature and full thickness skin was carefully separated out using scalpel and forceps. The adherent fat was removed and the skin was minutely examined for irregular surface and thickness. The skin was cut in to circular pieces of uniform thickness and a diameter suitable for fastening in franz diffusion cell. These skin pieces were soaked in 15% Gly-PBS 7.4, transferred to zip lock polybags and preserved in deep refrigerator at -70°C for not more than two months [3].

8.2.4 *Ex vivo* Permeation and deposition study

The permeation and deposition profile of both VPN and NPT from their suspension, microneedle patch, optimized nanocarriers and nanocarriers loaded microneedle patch were evaluated across full thickness pig ear skin. The study was carried out using a Franz-type diffusion cell with a receptor chamber volume of 15 ml. Before initiating the permeation experiment, the skin sections was thawed at room temperature and placed on a soft sponge pad impregnated with 30 %v/v ethanol solution in distilled water for equilibration. After 30 minutes' equilibration, skin sections were fastened between donor and receptor chamber of franz diffusion cell keeping stratum corneum towards donor side. Receptor chamber was previously filled with 30 %v/v ethanol solution in distilled water and maintained at 37°C using circulation waterbath. The diffusion media was kept under mild stirring (100 rpm) and dispersions equivalent to 1 mg drug were placed in donor chamber. Microneedle patch containing same amount of drug were applied to skin sections after completion of equilibration step using moderate thumb

pressure on skin held under a mild tension and immediately fastened in place. 1 mL sample was withdrawn from sampling arm of diffusion cell at each time point up to 24 hours and equal volume of fresh diffusion media was added to maintain total volume. After 24 hours, the skin was withdrawn and its surface was rinsed thrice with 5 mL portions of diffusion media. These washings were collected for estimation of drug retained on skin surface. Rinsed skin was cut into small pieces using scalpel, suspended in methanol and subjected to homogenization under cold conditions for 5 min followed by bath sonication for 15 min. The drug deposited within skin was thus extracted and separated by centrifugation at 5000 rpm and 25°C for 10 min for quantification. Each sample was passed through 0.2 µm membrane filter and drug was quantified in it using HPLC method described earlier in chapter 3A. Cumulative amount permeated per cm2 surface area of skin was calculated and plotted against time. The transdermal steady-state flux (JSS; µg/cm2/h) was calculated from the slope of the terminal linear portion of this graph. Permeation enhancement ratio were also calculated using **Eq. 8-1**.

$$\mathrm{PER} = \frac{J_{SS}^{test}}{J_{SS}^{control}} \qquad \textbf{Eq. 8-1}$$

Where J_{SS}^{test} is steady state flux via test formulation and $J_{SS}^{control}$ is steady state flux via VPN suspension.

8.2.5 *Ex vivo* fluorescence microscopy study

To further demonstrate the permeation behavior of developed formulations, fluorescence microscopy was performed. FITC was selected as a fluorescent dye owing to its close physicochemical resemblance with both VPN and NPT. FITC suspension, its microneedle patch, optimized nanocarriers and nanocarriers loaded microneedle patch were formulated and utilized for the study. FITC loaded UDL, PNP, UDL MNP and PNP MNP were prepared by replacing drug for FITC in optimized compositions as described earlier in respective formulation chapters. Pig

ear skin were thawed at room temperature, equilibrated and fastened on franz diffusion cell in a similar manner as described in earlier section. FITC loaded formulations were applied to stratum corneum side of skin in a similar way as described in earlier section. After completion of 12 hours, skin sectioning was performed in dark environment using cryo-microtome, sections were fixed on glass slide and observed under confocal laser scanning microscope for presence of fluorescence.

8.2.6 *In vitro* cell viability study

8.2.6.1 Cell culture handling and Sub-culturing

Immortalized human keratinocyte (HaCaT) cells were procured from NCCS, Pune and the flask was kept in an anaerobic incubator for 24 h at 37 °C and 5 % CO_2 without removing the media. Later, the culture medium was removed and the adherent cells were rinsed using EDTA-PBS (without calcium and magnesium) solution. Freshly prepared Trypsin-EDTA solution was added to completely cover the cell monolayer and incubated for 10 minutes at 37 °C for detachment of adherent cells. Fresh growth medium was added to stop trypsin activity and the cell suspension was centrifuged at 1200 rpm for 5 min. Supernatant was discarded and the cells were re-suspended in fresh growth medium. Cells were counted using neubauer counting chamber and transferred into new flasks at a plating density of $1x10^4$ cells/cm^2. These flasks were incubated at 37 °C and 5 % CO_2 to facilitate cell growth [4]. The growth media was renewed every third day and passaging was done once the culture attained 80-90 % confluency.

8.2.6.2 MTT assay

The safety of developed formulations was ascertained through viability evaluation of immortalized human keratinocyte (HaCaT) cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [5]. The principle of assay is based on the enzymatic reduction of yellow-colored tetrazolium MTT by mitochondrial dehydrogenase produced in viable (metabolically active) cells. The resulting intracellular purple formazan is solubilized and quantified

spectrophotometrically. Suspension of HaCaT cells in growth media was prepared from its culture using the same method as described above. Cells were then seeded at 5000 cells/well in a 96 well plate and incubated for 24 hours to grow and attach to plate surface. After 24 hours, growth media was removed and 100 µl of fresh treatment media was added to these wells as per the plan summarized in **Table 8-5** and **Table 8-6**.

The plate was incubated for 24 hours after which the treatment media was removed, 50 µl of MTT solution was added to each well and the plate was again incubated for 4 hours. Later, the MTT solution was removed carefully and 200 µl of dimethyl sulfoxide was added to each well to dissolve formazan crystals. The absorbance of resulting solution was measured at 570 nm using 690 XR microplate reader (Bio-Rad, US). Percent cell viability was then calculated considering the cell viability in wells treated with negative control as 100%.

8.3. RESULTS & DISCUSSION

Results obtained for various permeability and safety indicating studies are presented and discussed below

8.3.1 Ex vivo permeation and deposition of VPN

The data obtained for VPN permeation across full thickness pig ear skin are summarized in **Table 8-1**. The formulations can be arranged in following order of increasing permeability: VPN Suspension<VPN MNP<VPN PNP<VPN UDL<VPN PNP MNP<VPN UDL MNP. A slight improvement in VPN permeation was observed with VPN PNP (J_{ss} - 1.648 µg/cm^2/h) as compared to VPN suspension. However, after skin microporation (VPN PNP MNP), an 8-fold increase in permeation (J_{ss} – 9.753 µg/cm^2/h) was observed. The results reflected inability of PLGA nanoparticles to overcome skin barrier layer and necessity of physical breaching to achieve significant permeability enhancement. In case of VPN UDL, a 5-fold increase in permeation was observed through intact skin (J_{ss} – 6.019 µg/cm^2/h) owing to the ultradeformable nature of these carriers that enables them to permeate through pores much smaller than their size. A further improvement in VPN permeability was evident (PER

- 9.1) through VPN UDL MNP (J_{ss} – 11.091 µg/cm²/h) indicating the synergistic effect of microporation of UDL permeability [6].

Table 8-1. Ex vivo drug permeation of VPN across full thickness pig ear skin

Time (h)	Amount permeated per unit area (µg / cm²)					
	VPN Suspension	VPN MNP	VPN UDL	VPN UDL MNP	VPN PNP	VPN PNP MNP
0.25	0.09±0.01	0.25±0.01	0.51±0.03	0.7±0.06	0.16±0.01	0.99±0.08
0.5	0.25±0.02	0.66±0.06	1.21±0.08	1.88±0.15	0.32±0.02	1.62±0.15
1	0.60±0.04	1.24±0.09	3.47±0.32	5.22±0.52	0.99±0.06	3.69±0.28
2	1.14±0.10	1.97±0.19	6.02±0.55	8.63±0.85	1.69±0.15	7.36±0.58
3	1.56±0.10	2.64±0.16	9.33±0.70	13.12±1.29	2.8±0.18	10.32±0.99
4	4.07±0.28	5.03±0.43	20.54±1.63	28.12±2.72	6.24±0.46	23.82±1.37
6	6.56±0.65	7.54±0.51	30.64±2.05	45.16±3.9	8.89±0.74	36.40±2.60
8	8.66±0.63	9.58±0.90	43.85±2.72	61.24±4.08	12.29±0.77	52.77±4.80
20	21.81±1.84	26.56±2.42	107.26±9.03	170.13±14.29	30.29±2.65	143.92±11.49
24	26.68±2.13	34.26±2.20	131.34±10.86	214.49±15.25	36.88±2.55	182.93±11.85
J_{ss}	1.218	1.927	6.019	11.091	1.648	9.753
PER	1.0	1.6	4.9	9.1	1.4	8.0

Table 8-2. VPN distribution profile after 24 h of permeation experiment

Formulation	Drug permeated across skin (%)	Drug deposited within skin (%)	Drug retained on skin surface (%)
VPN Suspension	8.38 ± 0.46	14.84 ± 1.14	75.42 ± 3.94
VPN MNP	11.58 ± 0.70	39.43 ± 3.86	47.61 ± 3.72
VPN UDL	41.24 ± 3.72	25.47 ± 2.19	29.58 ± 2.02
VPN UDL MNP	67.35 ± 3.56	21.19 ± 1.88	9.61 ± 0.51
VPN PNP	10.76 ± 0.59	19.26 ± 1.26	68.54 ± 4.65
VPN PNP MNP	57.44 ± 5.65	33.46 ± 2.37	7.42 ± 0.49

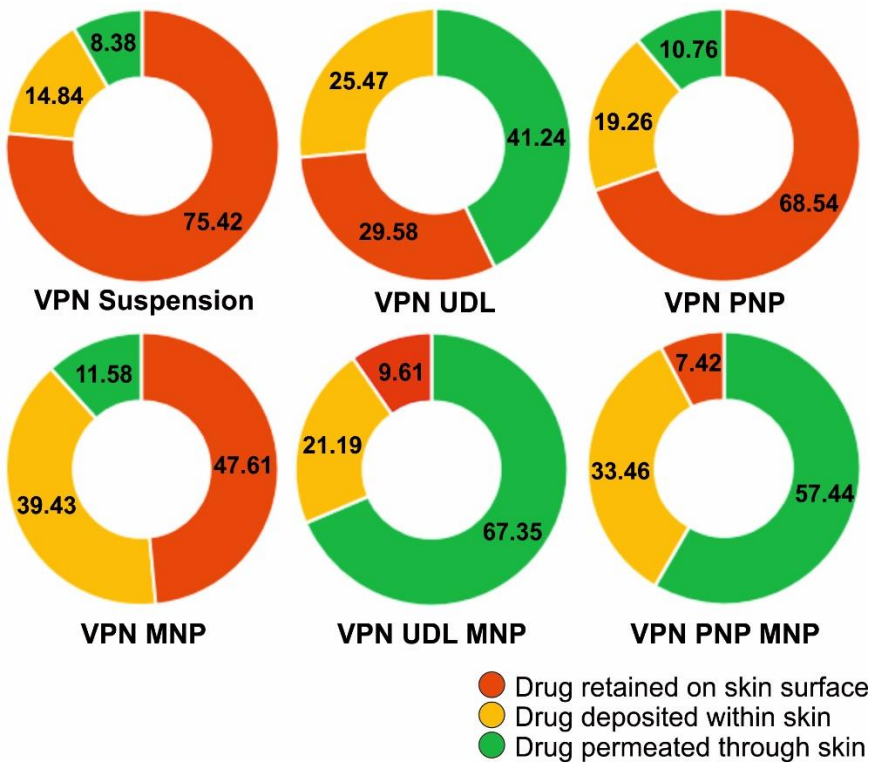

Fig. 8-1. VPN distribution profile from its formulations after 24 h of permeation experiment on full thickness pig ear skin

The data of VPN distribution through these formulations at 24 hours (after completion of permeability experiment) are presented in **Table 8-2** and illustrated in **Fig. 8-1**. Maximum deposition of VPN was observed in VPN MNP owing to the lipophilic nature of drug that might have made it difficult to permeate through relatively hydrophilic dermis region. The VPN deposition through UDL was found lesser as compared to PNP indicating a better permeation potential of ultradeformable liposomes over PLGA nanoparticles.

Similarly, the data obtained for NPT permeation across full thickness pig ear skin are summarized in **Table 8-3**. The formulations can be arranged in following order of increasing permeability: NPT Suspension<NPT PNP<NPT MNP<NPT UDL<NPT PNP MNP<NPT UDL MNP. A very slight improvement in NPT permeation (PER – 1.2) was observed with NPT PNP (J_{ss} - 1.944 µg/cm²/h) as compared to NPT suspension. However, after skin microporation (NPT PNP MNP), a 5.6-fold increase in permeation (J_{ss} – 9.285 µg/cm²/h) was observed. The results reflected inability of PLGA nanoparticles to overcome skin barrier

layer and necessity of physical breaching to achieve significant permeability enhancement [7]. In case of NPT UDL, a 4.3-fold increase in permeation was observed through intact skin (J_{ss} – 7.197 µg/cm²/h) owing to the ultradeformable nature of these carriers that enables them to permeate through pores much smaller than their size. A further improvement in NPT permeability was evident (PER – 6.5) through NPT UDL MNP (J_{ss} – 10.858 µg/cm²/h) indicating the synergistic effect of microporation of UDL permeability.

Table 8-3. Ex vivo drug permeation of NPT across full thickness pig ear skin

Time (h)	Amount permeated per unit area (µg / cm²)					
	NPT Suspension	NPT MNP	NPT UDL	NPT UDL MNP	NPT PNP	NPT PNP MNP
0.25	0.10±0.01	0.16±0.01	0.61±0.05	0.87±0.07	0.13±0.01	0.93±0.07
0.5	0.29±0.01	0.39±0.03	1.31±0.11	2.27±0.19	0.36±0.02	2.42±0.19
1	0.80±0.06	1.15±0.10	4.04±0.34	5.19±0.33	0.99±0.08	5.79±0.53
2	1.21±0.06	1.82±0.09	5.98±0.59	8.40±0.57	1.59±0.11	8.53±0.51
3	1.88±0.12	2.99±0.18	9.68±0.87	13.28±0.82	2.13±0.18	12.89±1.12
4	4.84±0.31	6.59±0.45	21.75±1.92	29.36±1.71	4.55±0.42	28.37±1.53
6	7.54±0.62	10.16±0.58	32.64±2.25	46.52±2.50	7.83±0.68	43.06±3.56
8	10.22±0.65	14.90±1.23	46.24±3.68	65.82±3.92	11.37±1.01	59.84±3.28
20	29.23±1.53	38.40±2.68	119.36±8.69	180.93±17.12	32.06±2.13	153.43±8.00
24	35.89±2.71	47.16±4.23	148.15±9.23	224.36±12.97	39.84±2.48	190.57±15.28
J_{SS}	1.665	2.189	7.197	10.858	1.944	9.285
PER	1.0	1.3	4.3	6.5	1.2	5.6

Table 8-4. NPT distribution profile after 24 h of permeation experiment

Formulation	Drug permeated across skin (%)	Drug deposited within skin (%)	Drug retained on skin surface (%)
NPT suspension	11.27 ± 0.91	15.69 ± 1.17	71.95 ± 6.61
NPT MNP	14.81 ± 1.05	40.67 ± 3.40	43.48 ± 4.11
NPT UDL	46.52 ± 2.89	23.81 ± 1.26	27.96 ± 1.48
NPT UDL MNP	70.45 ± 6.26	19.76 ± 1.22	8.44 ± 0.75
NPT PNP	12.51 ± 1.13	20.87 ± 2.07	64.38 ± 3.65
NPT PNP MNP	59.84 ± 3.88	31.57 ± 1.80	7.28 ± 0.46

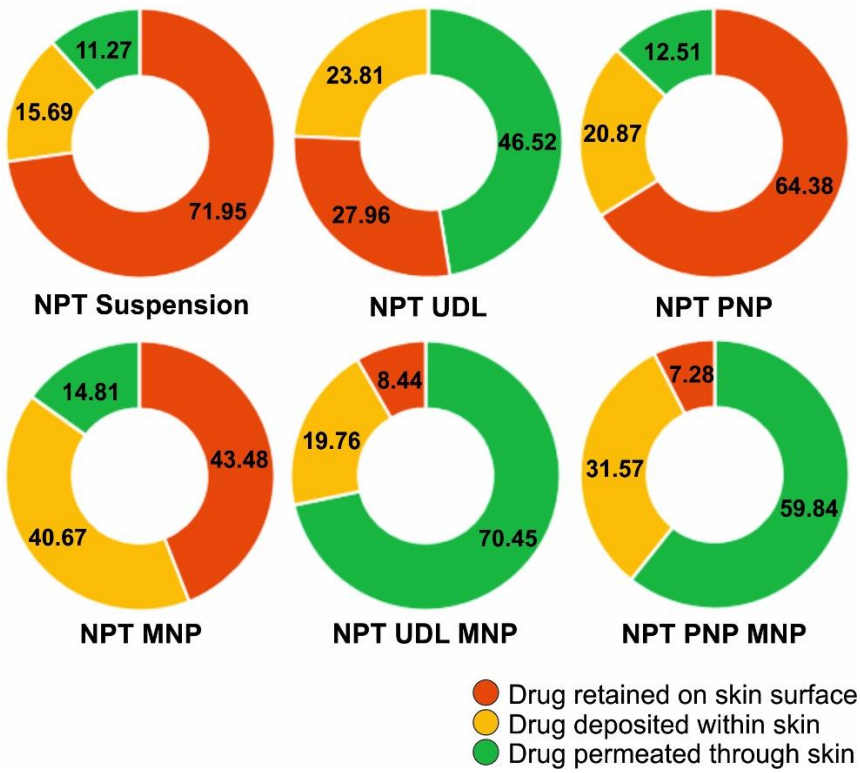

Fig. 8-2. NPT distribution profile from its formulations after 24 h of permeation experiment on full thickness pig ear skin

The data of NPT distribution through these formulations at 24 hours (after completion of permeability experiment) are presented in **Table 8-4** and illustrated in **Fig. 8-2**. Maximum deposition of NPT was observed in NPT MNP owing to the lipophilic nature of drug that might have made it difficult to permeate through relatively hydrophilic dermis region [8]. The NPT deposition through UDL was found lesser as compared to PNP indicating a better permeation potential of ultradeformable liposomes over PLGA nanoparticles.

8.3.2 *Ex vivo* fluorescence microscopy study

Fluorescence microscopic images of pig ear skin sections, after 12h of treatment with FITC loaded formulations, are presented in **Fig. 8-3** and **Fig. 8-4**. Negligible fluorescence was observed in sections of skin treated with FITC suspension. The formulations can be arranged in following order of increasing fluorescence: FITC Suspension<FITC PNP<FITC MNP<FITC UDL<FITC PNP MNP<FITC UDL MNP.

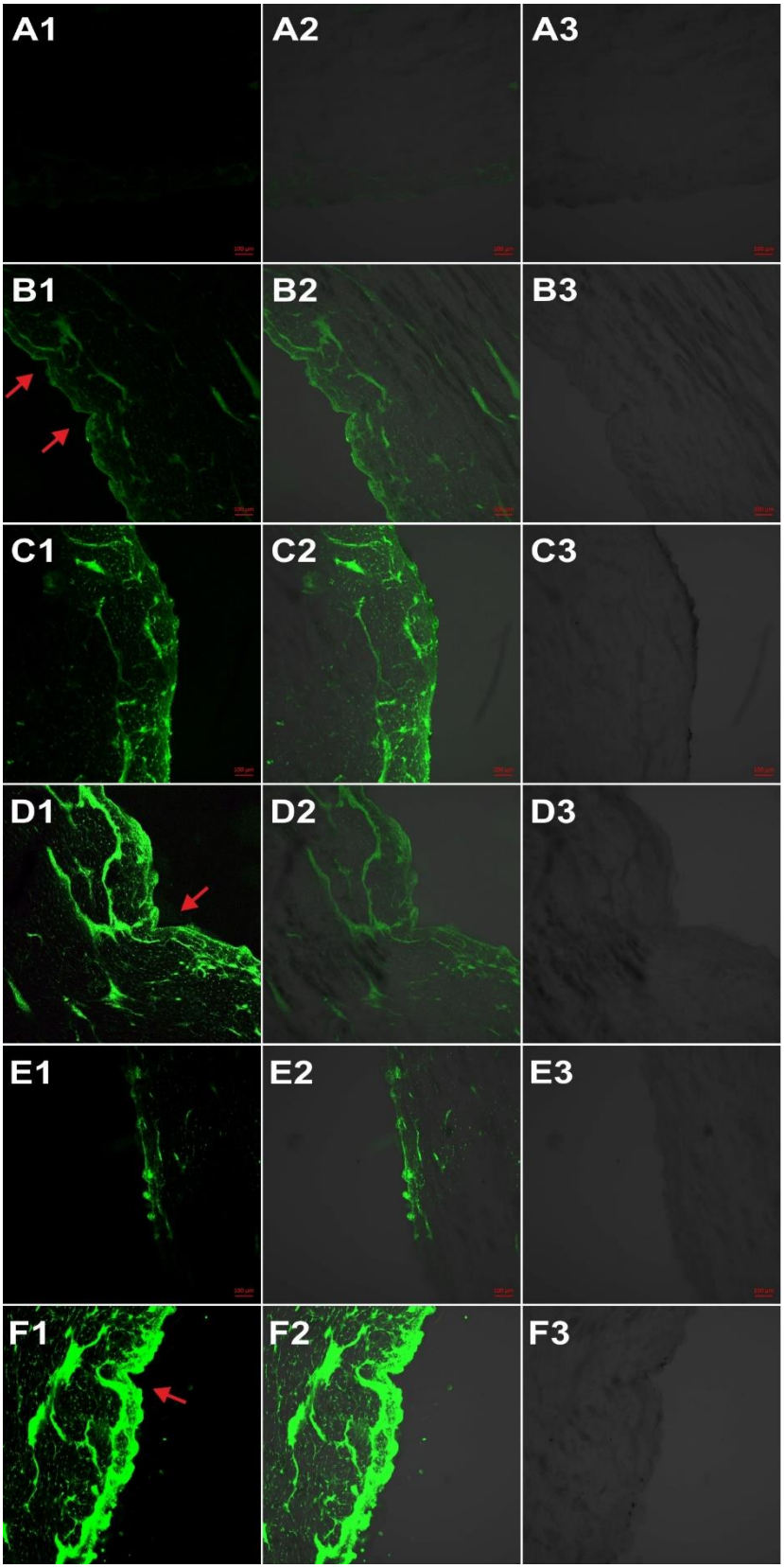

Fig. 8-3. Fluorescence microscopic images of pig ear skin sections after 12h of treatment with A) FITC suspension, B) FITC MNP, C) FITC UDL_{VPN}, D) FITC UDL MNP_{VPN}, E) FITC PNP_{VPN}, F) FITC PNP MNP_{VPN}

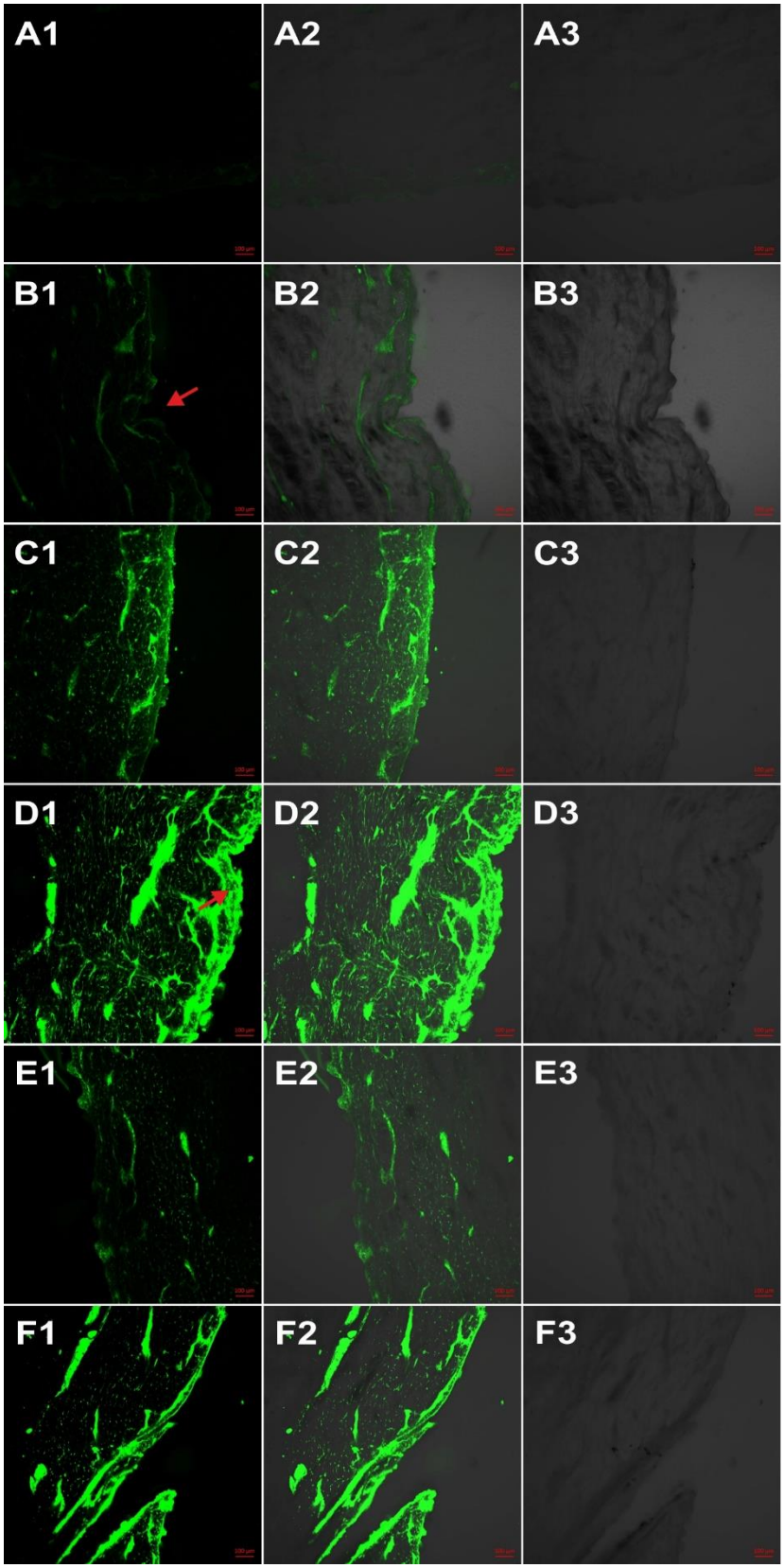

Fig. 8-4. Fluorescence microscopic images of pig ear skin sections after 12h of treatment with A) FITC suspension, B) FITC MNP, C) FITC UDL_{NPT}, D) FITC UDL MNP_{NPT}, E) FITC PNP_{NPT}, F) FITC PNP MNP_{NPT}

The fluorescence microscopic data were found in-line with the *ex vivo* permeation and deposition data where maximum fluorescence was observed in section of skin treated with FITC PNP MNP and FITC UDL MNP. Thus, results confirmed the enhancement permeation through developed nanocarriers loaded fast dissolving microneedle patches.

8.3.3 *In vitro* cell viability evaluation

The cell viability data for VPN formulations are summarized in **Table 8-5** and graphically illustrated in **Fig. 8-5**.

Table 8-5. *In vitro* cell viability data for VPN formulations in HaCaT cell line

Treatments	Absorbance		% cell viability	SD
	Mean	SD		
Negative Control- PBS 6.8	0.527	0.036	100.00	6.75
VPN suspension	0.445	0.036	84.50	6.80
Placebo UDL MNP	0.496	0.018	94.27	3.47
VPN UDL MNP	0.474	0.027	90.03	5.13
Placebo PNP MNP	0.494	0.023	93.78	4.35
VPN PNP MNP	0.454	0.039	86.23	7.41
Positive Control- Triton X 100	0.140	0.041	26.61	7.78

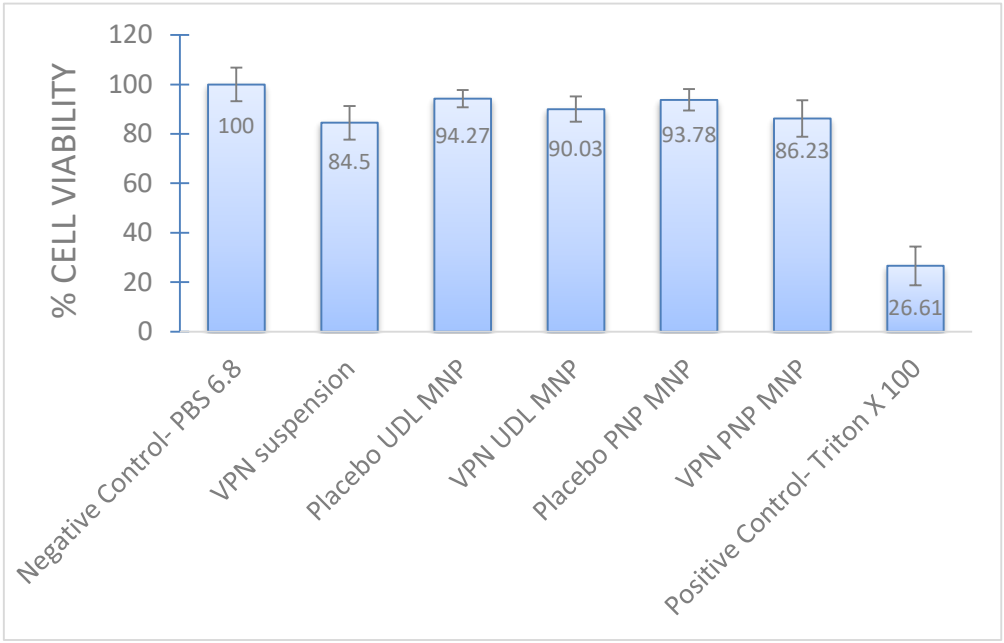

Fig. 8-5. Percent viability of HaCaT cells after exposure to VPN formulations or appropriate controls

A significantly lower viability of cells treated with Triton X 100 (26.61 %) indicated validity of the positive control. The viability of cells treated with VPN loaded nanocarriers were found significantly higher

than positive control and near to negative control. This indicated a less toxic nature of developed formulations.

The cell viability data for NPT formulations are summarized in **Table 8-6** and graphically illustrated in **Fig. 8-6**.

Table 8-6. *In vitro* cell viability data for NPT formulations in HaCaT cell line

Treatments	Absorbance		% cell viability	SD
	Mean	SD		
Negative Control- PBS 6.8	0.527	0.036	100.00	6.75
NPT suspension	0.435	0.020	82.61	3.70
Placebo UDL MNP	0.508	0.021	96.47	3.96
NPT UDL MNP	0.473	0.036	89.74	6.84
Placebo PNP MNP	0.498	0.019	94.60	3.61
NPT PNP MNP	0.450	0.037	85.51	7.09
Positive Control- Triton X 100	0.140	0.041	26.61	7.78

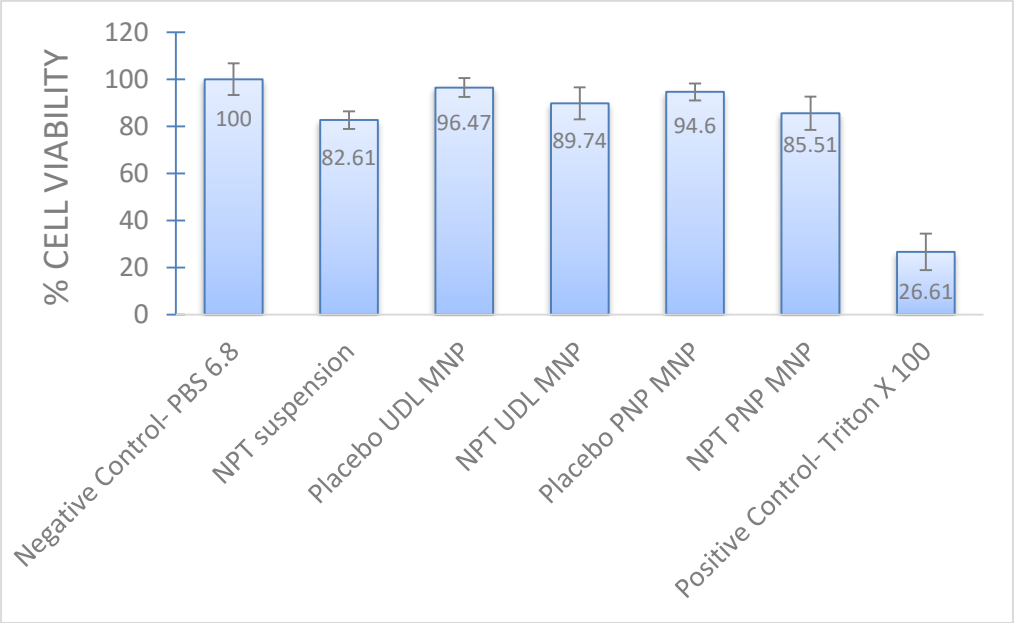

Fig. 8-6. Percent viability of HaCaT cells after exposure to NPT formulations or appropriate controls

The viability of cells treated with NPT loaded nanocarriers were found significantly higher than positive control and near to negative control. This indicated a less toxic nature of developed formulations.

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