

https://dspace.org/

Design and mechanistic evaluation of novel pore-regulated polymer matrices for transmucosal drug delivery

Adeleke, Oluwatoyin Ayotomilola

2012-02-14
http://hdl.handle.net/10539/11308

Downloaded from a DSpace repository. https://dspace.org/

CHAPTER FIVE

Investigating The Potential Toxicity And Biocompatibility Of The Optimized, Drug Loaded Pore-Regulated Polymer Matrices Employing Histomorphological And Cytological Assays

5.1. INTRODUCTION

In Chapter four, the performance of the optimized, drug loaded P-RPM formulations under *in vivo* physiological conditions employing the large white pigs as an animal model was discussed. The optimized P-RPM formulations, loaded with either PS or CBZ, displayed the potential to initiate and maintain the permeation of drug molecules through the buccal mucosal site in the pig model and this further substantiated the *in vitro* drug release and *ex vivo* permeation evaluations described in Chapters two and three. These assessments were carried out quantitatively by measuring changes in drug plasma levels with time and qualitatively utilizing Fourier Transform Infrared Spectrophotometry. The generated quantitative data were subjected to PK-PD modeling which produced relevant pharmacokinetic and pharmacodynamic information and an *In vitro-In vivo* correlation model. Thus far, remarkable results have been obtained from assessing the overall *in vitro* and *in vivo* performances of the novel P-RPM formulation being investigated in this entire research. Consequently, it is essential to also evaluate its potential toxicity and biocompatibility at the cellular and tissue levels employing mammalian cell cultures and biopsies isolated from the site of application of the P-RPM in the pig model.

The increase in interest of developing safe, efficient and reliable transmucosal drug delivery systems has prompted a necessity to ascertain that an acceptable level of biocompatibility exists between drug formulation and the site of application (Illum, *et* al., 1994). Histological examinations have been utilized to evaluate the potential toxicity and biocompatibility of transmucosal drug delivery systems (Illum *et al*., 1994; Şenel *et al*., 1998; Xiang *et al*., 2002; De Caro *et al*., 2008). The evaluation of the biocompatibility/toxicity of a drug delivery system involves an understanding of inflammatory processes, healing and foreign body responses which are generally considered as tissue or cellular host response to injury which is a measure of toxic impact towards the application of such formulations. Though it can be anticipated that majority of therapeutic formulations exhibit or enhance no tissue or cell toxic reactions and/or acute or chronic inflammatory responses on exposure to host tissue, it is however very essential to include such tests in the experimental design and evaluation of such systems (Anderson and Shive, 1997).

The evaluation of mammalian cell growth and viability through the measurements of parameters which quantify toxicity at the cellular level is essential for ascertaining the efficiency of chemical compounds, biomedical agents, and other materials in the medical, pharmaceutical and other scientific fields (Mosmann, 1983; Kato *et al*., 1999). The outcomes of such studies have produced results which have contributed to or served as substitutes to animal experiments (Kato *et al*., 1999). There are several established methods of measuring cell number/proliferation/viability such as counting cells that include/exclude dye (colorimetric assay), measuring released labeled protein after cell lysis and quantifying the incorporation of radioactive nucleotides during cell proliferation (Mosmann, 1983). This study employed the colorimetric assay based on a soluble tetrazolium salt called 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (Mosmann, 1983). The MTT assay is a simple, accurate, versatile and quantitative method which yields reproducible results by quantifying the metabolically viable/live cells through their ability to reduce soluble MTT from yellow to insoluble blue-purple formazan crystals (Mosmann, 1983; Price and McMillan, 1990). The mitochondrial enzyme, succinate dehydrogenase, present within living cells cleave the tetrazolium ring to yield the blue-purple formazan crystals which are measured using spectrophotometry by relating the absorbance values to the number of live cells. (Price and McMillan, 1990). Therefore, an increase or decrease in cell number is directly related to the amount of formazan formed indicating the degree of cytotoxicity caused by the tested material (Mosmann, 1983; Price and McMillan, 1990).

The purpose of this Chapter was to perform detailed histological investigations on the potential toxic effects of the newly fabricated optimized PS or CBZ loaded P-RPM formulation on the pig's buccal mucosa after the delivery system has been applied over the 8 hour period. In addition, the toxicity of the drug loaded P-RPM formulation to mammalian cell cultures (cytotoxicity assay) was evaluated in this Chapter. These objectives were carried out respectively by isolating and evaluating buccal mucosal biopsy specimens under the electron microscope for probable morphological histopathological changes as well as employing three different mammalian cell lines namely the HT-29 (human colonic adrenocarcinoma), CACO-2 (epithelial colorectal adrenocarcinoma representative of normal colon) and MCF-7 (human breast cancer) to visualize the influence of the P-RPM formulations on cell growth/viability.

5.2. EXPERIMENTAL SECTION

5.2.1. Materials

The optimized drug loaded pore-regulated polymer matrices discussed in Chapters two, three and four were employed for this experimental section. Haematoxylin and eosin stains were acquired from Brisben Chemicals, Maharashtra, India and GFS Chemicals, Inc., Powell, OH, USA respectively. The human colonic adenocarcinoma (HT-29), epithelial colorectal adrenocarcinoma (CACO-2) and epithelial colorectal adrenocarcinoma (MCF-7) epithelial colorectal adrenocarcinoma cell cultures were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). Dulbecco's Modified Eagle's Medium (DMEM) and 10% heat-inactivated foetal bovine serum (FBS) were procured from BD Biosciences (California, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), Trypsin - EDTA, trypan blue, dimethylsulfoxide (DMSO) and camptothecan were obtained from Sigma Chemical Company (St. Louis, USA).

5.2.2. Histological studies to assess the potential toxicity and biocompatibility of the newly constructed P-RPM formulations

Biopsy samples of about 3mm deep (1mm^2 area) were taken from the sites of application (buccal mucosa) of the drug loaded (PS and CBZ) and drug free (placebo) optimized P-RPM formulations respectively. In addition, control biopsy samples were collected from equivalent sites where no formulation was applied. In order to rule out the possible interfering effects of the suturing material, nylon 3/0, (employed for application of formulation) with the overall outcome, biopsy samples of the sutured sites with no formulation were also taken (Section 4.2.2.1. in Chapter four). All samples were taken in quadruplicate at the end of the 8 hour study. The buccal mucosal biopsy specimens were stored in 10% formalin for analysis and were processed in an automated tissue processor (Electron Microscopy Sciences, Hatfield, PA, USA). Thereafter, tissue blocks were sectioned at 5µm, the sides that were produced were stained using the haematoxylin and eosin staining method in an automated stainer (Rankin Biomedical Corporation, Holly, MI, USA) before histological evaluation under the electron microscope. The specimens that were collected and underwent histological analysis were classified as:

- A – control tissue specimen (site without formulation or suture)
- B – sutured site without formulation

- C – site with placebo P-RPM formulation
- D – site with PS loaded P-RPM formulation
- E - site with CBZ loaded P-RPM formulation

5.2.3. Cytotoxicity studies

5.2.3.1. Preparation of test formulations and description of controls

For this assay, the PS loaded and CBZ loaded P-RPM formulations were tested. One of each of these formulations was separately dissolved in double distilled water to a final stock concentration of 2mg/mL, filter-sterilized through a 0.22µm Cameo acetate membrane filter (Millipore Co., Bedford, Massachusetts) and stored at 4°C until it was used. Both negative and positive controls were employed for this assay. With the negative control, no formulation or compounds were added while camptothecan, a known chemotherapeutic agent was employed as the positive control. A concentration of 200µg/mL of either the test formulation or compound was employed as the positive control and all tests were conducted in triplicate.

5.2.3.2. Routine cell culture maintenance

The HT-29 (human colonic adenocarcinoma), CACO-2 (cells representative of the “normal” colon) and MCF-7 (human breast cancer) cells employed for this investigation were routinely maintained in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% heat-inactivated foetal bovine serum (FBS) cell culture media. Cells were maintained in a humidified incubator (Afrox, South Africa) circulated with 95% oxygen, 5% carbon dioxide and maintained at 37°C ± 0.1°C.

5.2.3.3. Trypsinization, cell counting and seeding of cells

The HT-29, CACO-2 and MCF-7 cells were trypsinized using trypsin–EDTA and 0.1M phosphate buffered saline (pH 7.4) and incubated at 37°C ± 0.1°C for 10 minutes. The cells were re-suspended in fresh complete medium and centrifuged at 1000 rpm for 5 minutes to pellet the cells. The cell pellets were then re-suspended in about 5mLof the medium. The cells were then counted using the trypan blue exclusion assay, in which cells were dyed with 0.4% trypan blue. In this case, viable cells appear yellow in colour while the non-viable cells appear blue when viewed under a light microscopy. Only cell suspensions with viability higher than 95% were used in subsequent experiments. Cells were seeded into 96-well plates at a final concentration of 15 000 cells/well. The plates were then incubated at 37 ± 0.1°C overnight to

allow for attachment of the cells to the wells. Following attachment, a 20µL solution of the test formulation were then added to the wells and incubated for a 48 hour period. The MTT assay was then carried out to determine the degree of cell viability.

5.2.3.4. Colorimetric MTT assay

The respective compounds were added to the wells at a final concentration of 200µg/mL The negative control consisted of no compound but only 1% DMSO (as a vehicle control) and the positive control was made up 200µg/mL camptothecan solution. After the 48 hour incubation period (Section 5.2.3.3), the spent medium was aspirated from the cells, and replaced with 200µL of 0.05mg/mL MTT solution. The cells were then incubated in the presence of the MTT solution for 3 hours, after which the MTT was aspirated from the cells, and replaced with 200µL DMSO. The formazan crystals were left to dissolve in the DMSO for 30 minutes and after which the absorbance was read at a maximum wavelength of 540nm against DMSO as a blank using a microtitre plate reader (BioTek Powerwave XS, Winooski, VT, USA).

5.2.3.5. Preparation of calibration curve and computation of viable cell number

The calibration curves obtained for the HT-29, CACO-2 and MCF-7 cell cultures are presented in Figure 6.1 (a), (b) and (c). For each type of cell, series of dilutions were made to produce different cell number per well. The absorbance for each cell culture solution was read at 540nm. Subsequently, linear calibration curves directly relating absorbance to cell number were generated for HT-29, CACO-2 and MCF-7 cell cultures. The absorbance readings generated at the end of the duration of exposure of each substance were then used to compute the number of viable cells present. All results are presented as the mean reading ± standard deviation and the statistical significance of all experimental data was evaluated using the Minitab Statistical Software, Version 14 (Minitab Inc., State College, PA, USA). A probability limit of $p < 0.05$ was considered to be statistically significant. In addition, equation 5.1 was used to quantify the number of viable cells in both test and control sample.

$$\% \text{ cell viability} = \frac{\text{Number of viable test cells}}{\text{Number of viable control cells}} \times 100 \qquad \textit{(Equation 5.1)}$$

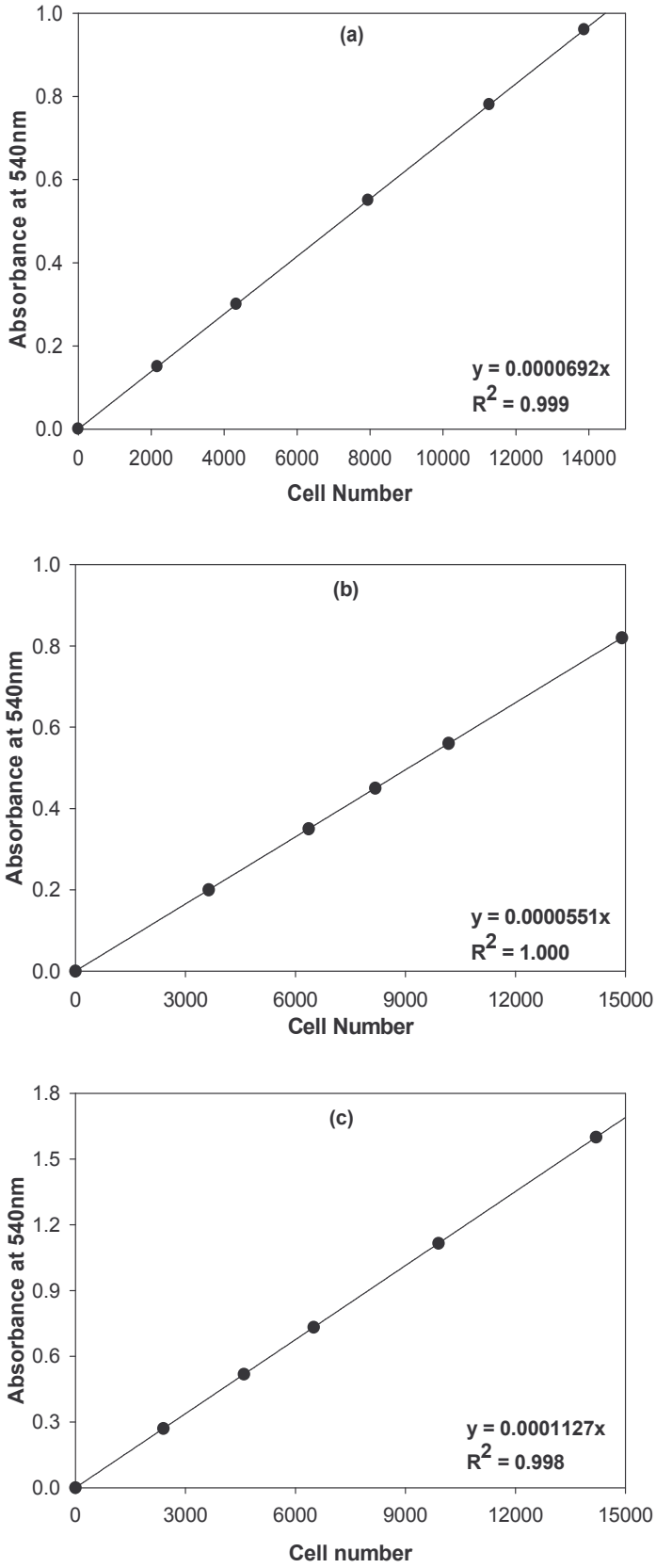

Figure 5.1: Calibration curves for (a) CACO-2, (b) HT-29 and (c) MCF-7 cell cultures for the MTT colorimetric assay.

5.3. RESULTS AND DISCUSSION

5.3.1. Histomorphological evaluation of the buccal mucosal tissue specimens post administration of the optimized P-RPM formulations

Typical electron micrographs showing the porcine buccal mucosal epithelium before application of the drug loaded P-RPM formulation (normal), the effects of the suture, the influence of the PS or CBZ loaded optimized P-RPM formulations are displayed in Figures 5.2 - 5.6.

The epithelial hyperplasia (increased cellularity and rete ridge formation resulting in acanthosis or thickening of the epithelial layer) was non-specific, possibly of no significance and negative for all samples (A, B, C, D and E) (Table 5.1) which indicates that it had no significance in assessing the toxicity of the formulations. Epithelial spongiosis (intercellular oedema characterized by widening of the intercellular spaces with prominent intercellular bridges and irregular spongiform appearance) and exocytosis (trans-epithelial leukocyte migration by inflammatory cells which are mainly neutrophils) correspond relatively well and indicate absence of epithelial damage associated with inflammation for samples A, C, D and E except for sample B which displayed a moderate level of epithelial spongiosis and exocytosis which was majorly due to the effects of suturing of the formulation to the buccal mucosa.

Generally, in the lamina propria the purulent inflammation (characterized by exudation of acute inflammatory cells into the stroma of the lamina propria) and leukostasis (early leukocyte chemotaxis with adhesion of the leukocytes to the capillary endothelium-margination) of inflammatory type appear to occur mildly in the specimens B, C, D and E with the exclusion of the control spceimen A. Also, the extent of inflammatory leukostasis was slightly higher (moderate) for specimen B representing the site with suture only. The oedema (widening spaces in the loose connective tissue especially in the epithelial stroma due to vascular permeability and transudation of the fluid from the blood capillaries into the lamina propria), leukostasis that is followed by transmigration into the lamina propria at a later stage and purulent inflammation in the lamina propria indicates acute, insignificant stomatitis. The perivascular inflammation observed for all of the specimens can be desribed as mild, non-specific and of incidental occurence. Table 5.1 summarizes the extent of damage displayed by the tissue specimens collected for histological and cytological examinations.

Table 5.1: Summary of histopathological state of buccal mucosal specimens collected

Histological Measures of Toxicity	A	B	C	D	E
Epithelium					
Hyperplasia	-	-	-	-	-
Spongiosis of the epithelium	-	2+	-	-	-
Exocytosis in the epithelium	-	2+	-	-	-
Lamina Propria					
Oedema of lamina propria	-	3+	1+	-	-
Perivascular inflammation	1+	1+	1+	1+	1+
Purulent inflammation	-	1+	1+	1+	1+
Inflammatory Leukostasis	-	2+	1+	1+	1+

Note: *A – control tissue specimen with normal epithelium (site without formulation nor suture); B – sutured site without formulation to assess the effects of the suture on the buccal mucosal tissue; C - site to which the placebo (drug-free) formulation was applied; D - phenytoin sodium-loaded formulation site of application and E – site to which the carbamazepine-loaded formulation was administered.*

Legend: *Negative (**-**); Mild (**1+**); Moderate (**2+**) and Severe (**3+**)*

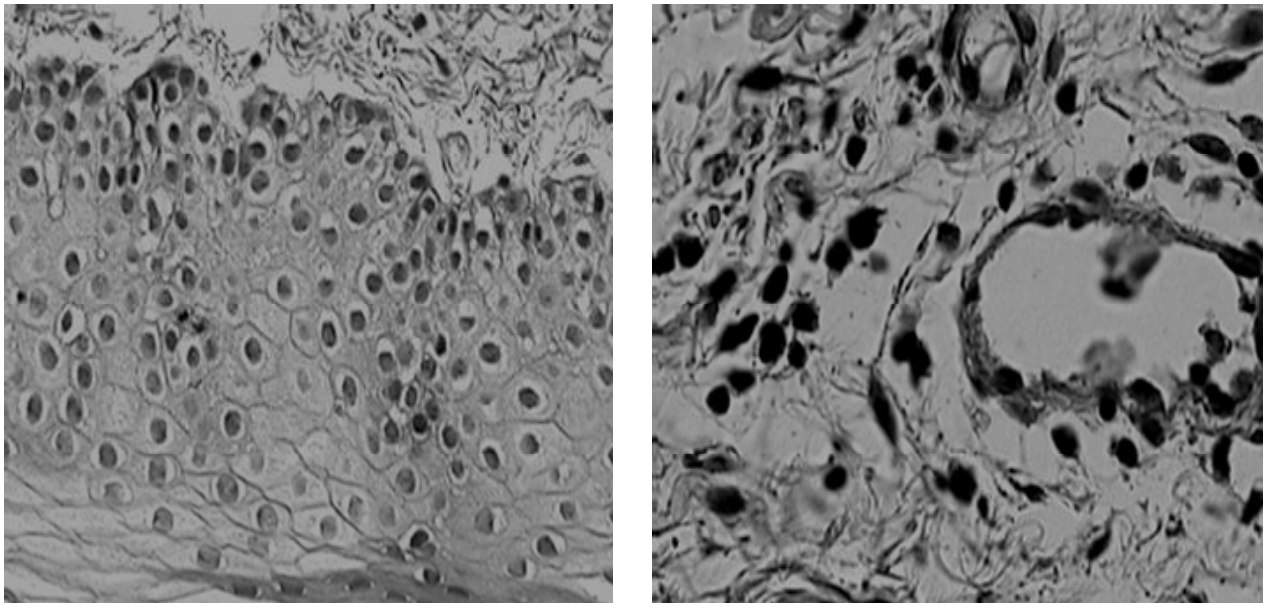

Figure 5.2: Micrographs of the normal porcine buccal epithelium with no formulation or suture applied taking at different angles (control) (magnification x40).

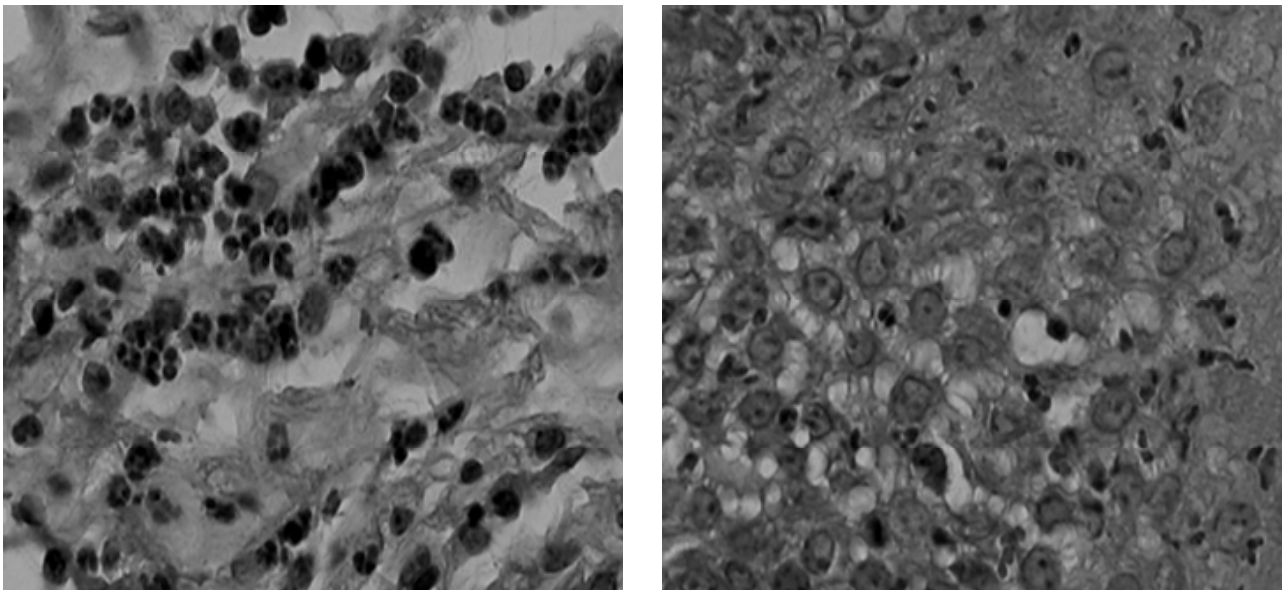

Figure 5.3: Micrographs of the sutured porcine buccal epithelium with no formulation applied taking at different angles (magnification x40).

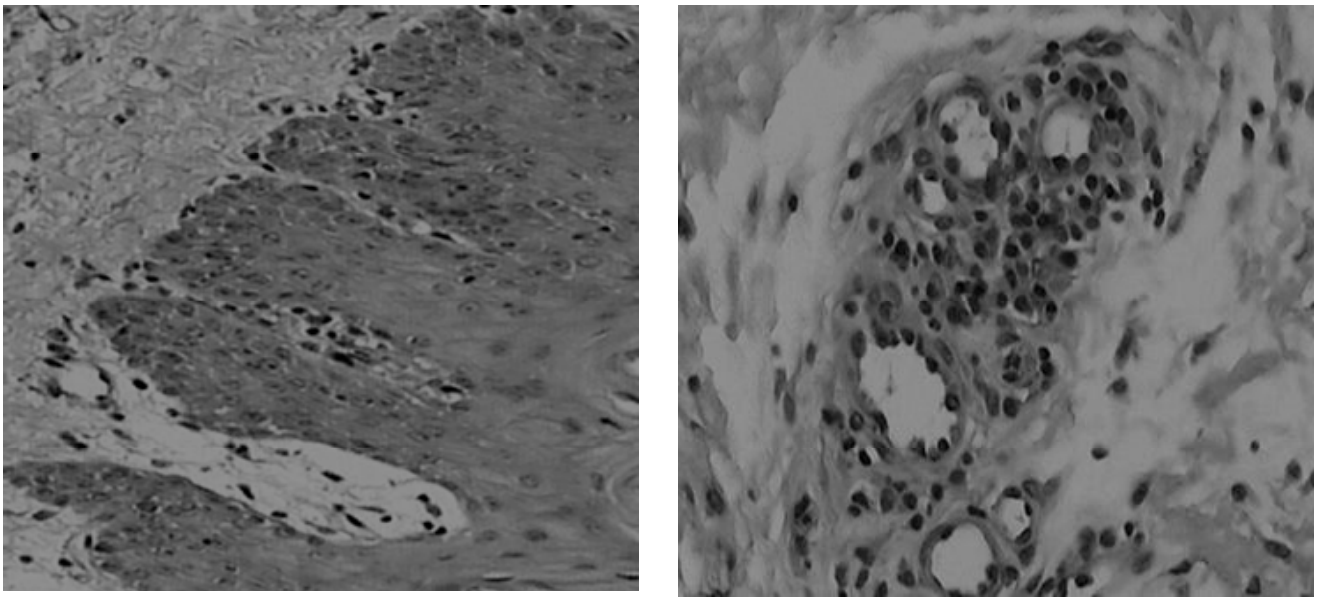

Figure 5.4: Micrographs of the porcine buccal epithelium after removal of minute remnants of the placebo P-RPM formulation taking at different angles (magnification x40).

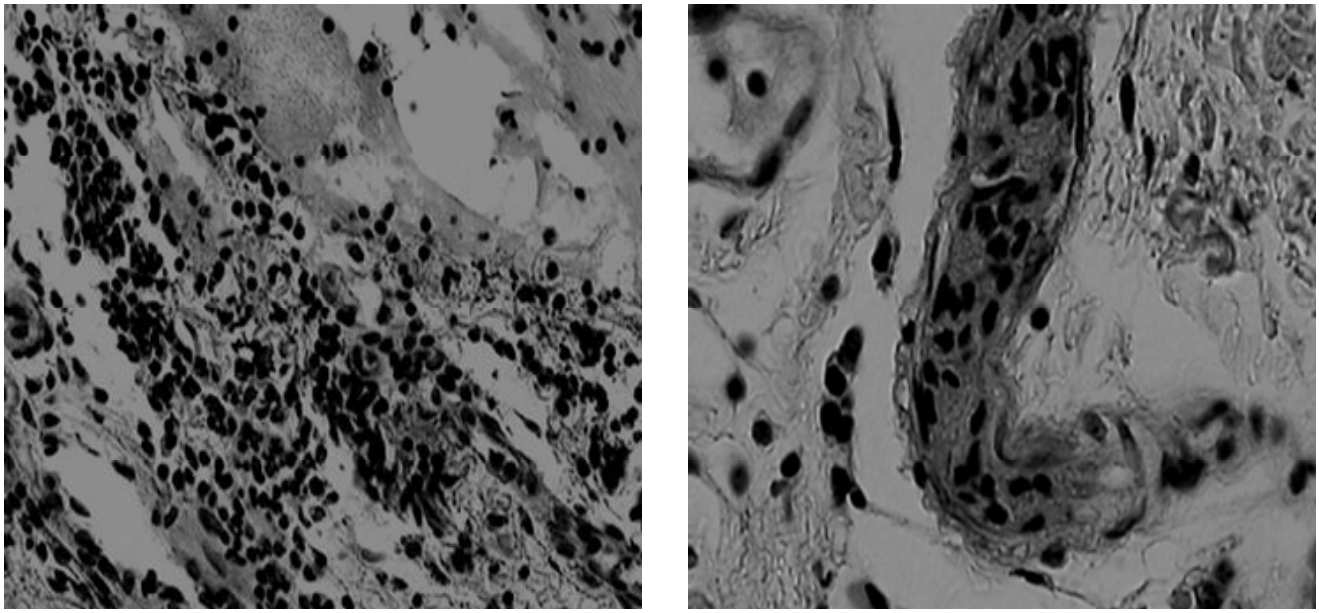

Figure 5.5: Micrographs of the porcine buccal epithelium after removal of minute remnants of the PS loaded P-RPM formulation taking at different angles (magnification x40).

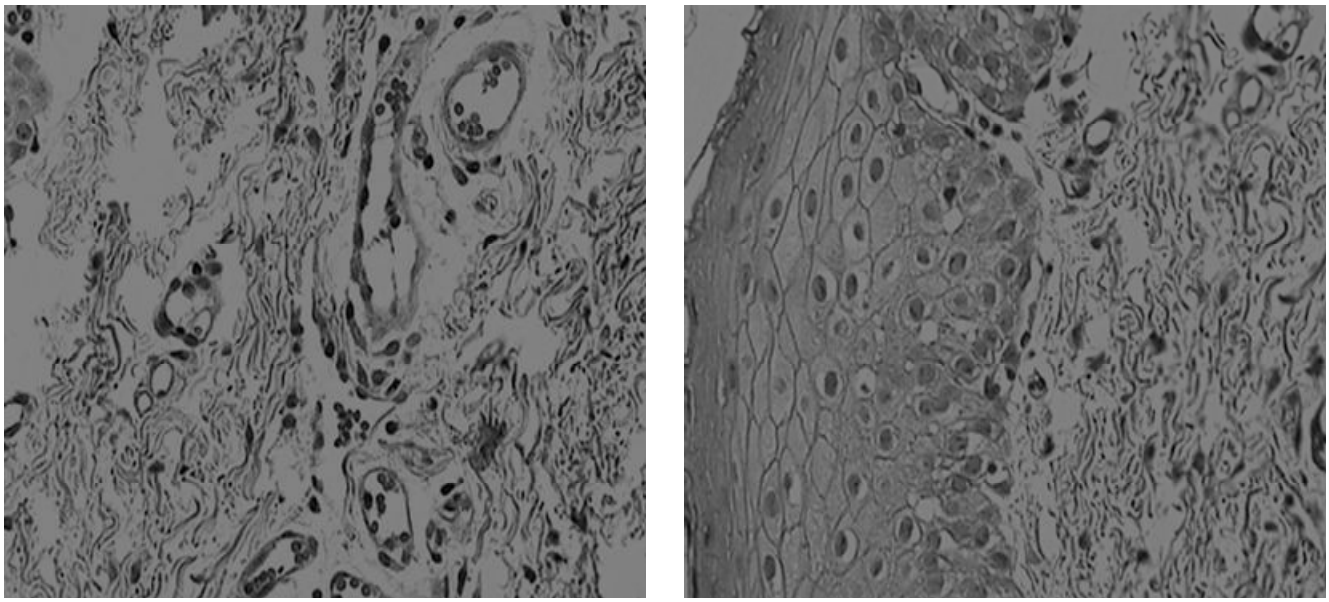

Figure 5.6: Micrographs of the porcine buccal epithelium after removal of minute remnants of the CBZ loaded P-RPM formulation taking at different angles (magnification x40).

Overall, compared with the normal epithelium, the suturing procedure employed to administer the P-RPM formulation (to the buccal mucosa of the pig) to prevent it from being chewed or swallowed can be said to moderately damage the epithelium and lamina propria. However, the application of the P-RPM placebo (drug free) formulation, C, resulted in a reduction in the extent of damage. In addition, the damage of the buccal mucosal epithelium was further reduced with the administration of the respective PS and CBZ loaded P-RPM formulations (specimens D and E). It can be inferred from the outcome of this analysis (Table 5.1 and Figures 5.2 - 5.6) that this set of formulations (both placebo and drug loaded had minimal, non significant pathological effects on the buccal epithelium and lamina propria of the buccal mucosa which implies that both the polymeric and non-polymeric components of the P-RPM placebo formulation as well as the drugs employed (PS and CBZ) in the testing of the newly developed, novel P-RPM formulation had no irritable nor toxic effects on the porcine buccal mucosa and makes it potentially biocompatible and suitable for its intended transbuccal drug delivery application.

5.3.2. Cytotoxicity assay

Generally, the PS and CBZ loaded P-RPM formulations displayed no potential cytotoxicity on the cell cultures employed after the 48 hour exposure duration. Graphical representations of the reaction of each cell culture to the tested P-RPM formulations are presented in Figures 5.7 -5.9. All comparisons were made relative to the positive and negative control experiments. For the PS loaded P-RPM formulation, a significant ($p < 0.005$) enhancement of growth of the CACO-2 and HT-29 cell lines (± 12 and ± 8% respectively) were observed (Figures 5.7 and 5.8) while a non-significant ($p > 0.005$) minimal inhibition of the growth of the MCF-7 cell line (± 14%) was observed (Figure 5.9). The CBZ loaded P-RPM formulation on the other hand produced a similar significant enhancement of growth of the HT-29 cells (± 14%; $p < 0.005$) (Figure 5.8), but inhibited the growth of both the Caco-2 and MCF-7 cells insignificantly (± 15% and ± 4% respectively; $p > 0.005$) (Figures 5.7 and 5.9 respectively). This reduction in cell viability is statistically insignificant ($p > 0.005$) and would not be considered as an indication of cytotoxicity, as seen in the positive control which significantly ($p < 0.005$) inhibited the growth of the three cell lines (CACO-2, MCF-7 and HT-29; Figure 5.7 – 5.9). Based on the outcome of this assay, it can be said that the drug loaded P-RPM formulation are potentially biocompatible and non-toxic.

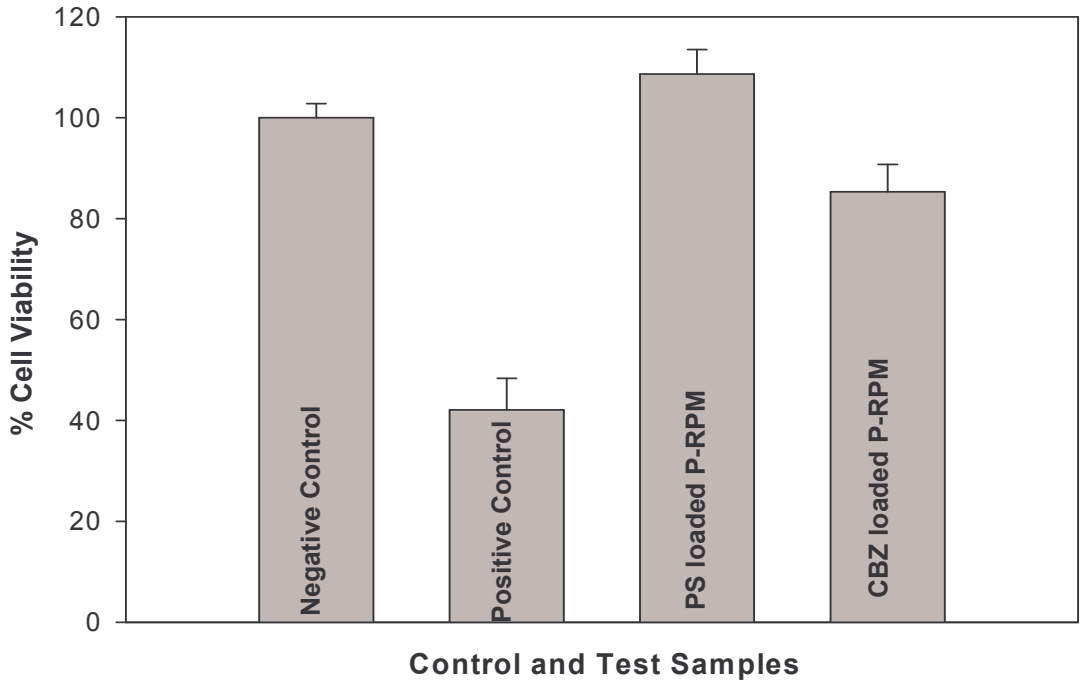

Figure 5.7: A graphical representation of the percentage cell viability after a 48 hour separate exposure to the PS and CBZ loaded P-RPM formulation relative to the positive and negatives controls using the CACO-2 cell culture.

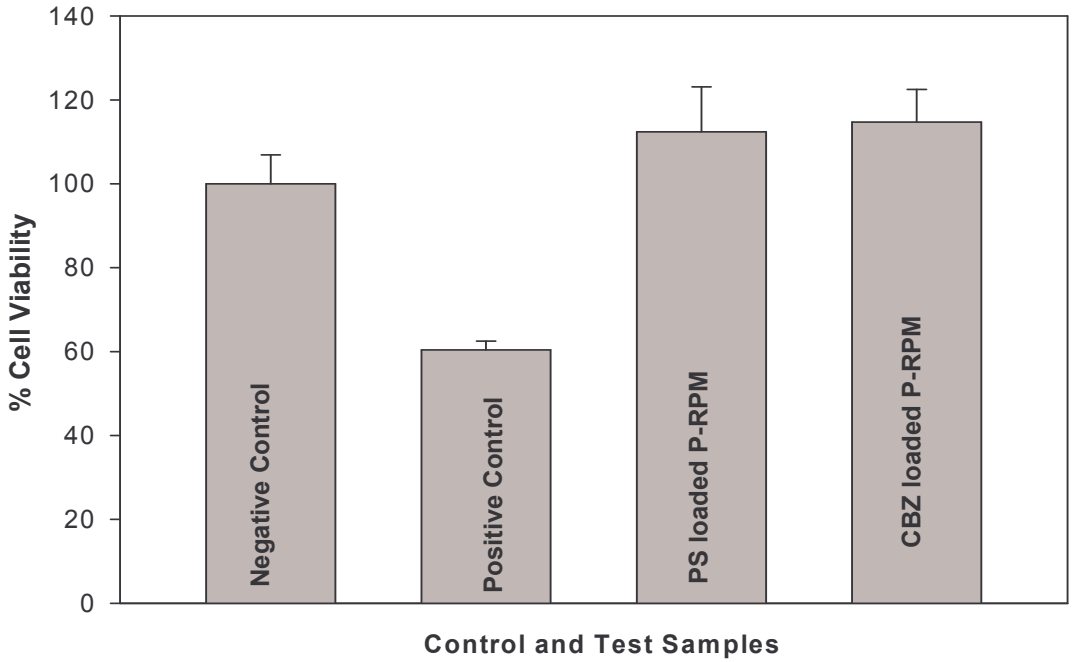

Figure 5.8: A graphical representation of the percentage cell viability after a 48 hour separate exposure to the PS and CBZ loaded P-RPM formulation relative to the positive and negative controls using the HT-29 cell culture.

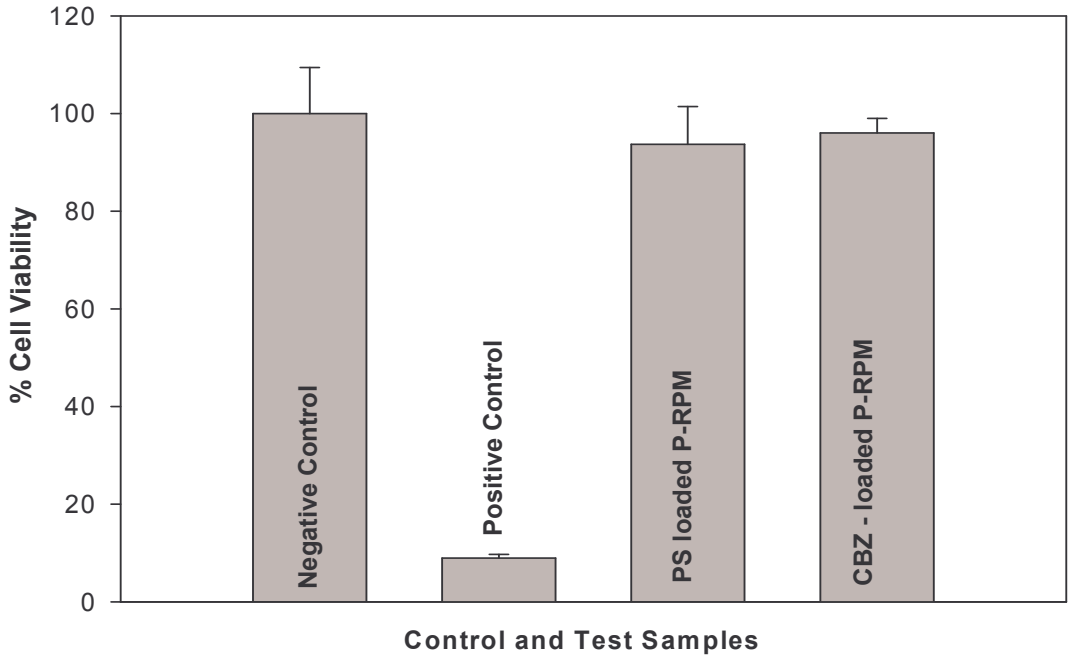

Figure 5.9: A graphical representation of the percentage cell viability after a 48 hour separate exposure to the PS and CBZ loaded P-RPM formulation relative to the positive and negatives controls using the MCF-7 cell culture.

5.4. CONCLUDING REMARKS

This Chapter evaluated the potential toxicity and biocompatibility of the newly developed optimized P-RPM formulation loaded with either PS or CBZ. Morphological histopathological studies of the optimized, drug loaded P-RPM formulations showed that the placebo and drug loaded formulations had insignificant toxic and irritable effects on the buccal mucosal specimens evaluated over the duration of application and action. Furthermore, the assessment at the cellular level showed that both formulations either significantly ($p < 0.005$) enhanced or slightly insignificantly inhibited ($p > 0.005$) the growth of the employed cell cultures over the prolonged 48 hour exposure duration. This outcome further substantiates the histological evaluations and overall, the P-RPM formulation can be described as potentially biocompatible and non-toxic. The next chapter will be attempting to elucidate the possible mechanisms of formation and action of the P-RPM drug delivery system employing the experimental verification and computational modeling approaches.