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RESEARCH PAPER

Biodistribution and Pharmacokinetics of Dapivirine-Loaded Nanoparticles after Vaginal Delivery in Mice

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ABSTRACT

Purpose To assess the potential of polymeric nanoparticles (NPs) to affect the genital distribution and local and systemic pharmacokinetics (PK) of the anti-HIV microbicide drug candidate dapivirine after vaginal delivery.

Methods Dapivirine-loaded, poly(ethylene oxide)-coated poly(epsilon-caprolactone) (PEO-PCL) NPs were prepared by a nanoprecipitation method. Genital distribution of NPs and their ability to modify the PK of dapivirine up to 24 h was assessed after vaginal instillation in a female mouse model. Also, the safety of NPs upon daily administration for 14 days was assessed by histological analysis and chemokine/cytokine content in vaginal lavages.

Results PEO-PCL NPs (180–200 nm) were rapidly eliminated after administration but able to distribute throughout the vagina and lower uterus, and capable of tackling mucus and penetrate the epithelial lining. Nanocarriers modified the PK of dapivirine, with higher drug levels being recovered from vaginal lavages and vaginal/lower uterine tissues as compared to a drug suspension. Systemic drug exposure was reduced when NPs were used. Also, NPs were shown safe upon administration for 14 days.

Conclusions Dapivirine-loaded PEO-PCL NPs were able to provide likely favorable genital drug levels, thus attesting the potential value of using this vaginal drug delivery nanosystem in the context of HIV prophylaxis.

KEY WORDS HIV/AIDS · microbicides · poly(ε-caprolactone) · pre-exposure prophylaxis · vaginal drug delivery

ABBREVIATIONS

λ_z	Apparent terminal elimination rate constant
AE%	Association efficiency
$AUC_{0.25\text{-}24h}$	Area under the concentration-time curve between 15 min and 24 h
C_{max}	Maximum observed concentration
DL%	Drug loading
EC_{50}	Half maximal effective concentration
F_{rel}	Relative bioavailability
IL	Interleukin
KC	Keratinocyte-derived chemokine
MIP-1α	Macrophage inflammatory protein
MPPs	Mucus-penetrating particles
N-9	Nonoxynol-9
NPs	Nanoparticles
PBS	Phosphate buffered saline
PCL	Poly(ε-caprolactone)
PD	Pharmacodynamics
PdI	Polydispersity index
PEG	Poly(ethylene glycol)

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PEO	Poly(ethylene oxide)
PK	Pharmacokinetics
PLGA	Poly(D,L-lactide-*co*-glycolide)
PPO	Poly(propylene oxide)
PrEP	Pre-exposure prophylaxis
rhod-123-PCL	Rhodamine-123-labeled poly(ε-caprolactone)
s.d.	Standard deviation
s.e.m.	Standard error of the mean
$t_{1/2\beta}$	Terminal elimination half-life
t_{max}	C_{max} time

INTRODUCTION

The importance of tackling the HIV/AIDS pandemic is well recognized and topical pre-exposure prophylaxis (PrEP) now stands as a promising prevention strategy (1). The basic idea is fairly simple: the vaginal (and/or rectal) administration around the time of sexual intercourse of antiviral compounds formulated as suitable products, usually termed microbicides, inhibits infection by blocking viral transmission at the mucosal level. Minimum concentration levels of active compounds at genital fluids and tissues in order to prevent HIV transmission are not yet established, or even easily determinable, but it is expected that increasing and sustained levels of antiretroviral drugs will result in enhanced protection (2). For instance, the analysis of animal and human data on vaginally delivered gels containing tenofovir, the only drug so far proven partially effective against HIV acquisition in a clinical trial (3), seems to indicate a direct relationship between genital levels of this drug and protection (4–7). In the case of gels containing dapivirine, a non-nucleoside reverse transcriptase inhibitor, available animal and human data on pharmacokinetics (PK) indicate that this drug is able to achieve considerably higher levels (2–5 logs) in cervicovaginal fluids and tissues upon vaginal administration than those required for protection in cell and tissue explants assays (8–10). Even so, fast decrease of dapivirine concentrations is observed after its administration as traditional dosage forms such as gels. This may lead to short time-frames of protection which limits the development of coitally-independent microbicides. Thus, new delivery approaches that can increase dapivirine levels and residence in genital fluids/tissues may provide increased protection against HIV transmission. One formulation strategy to circumvent this problem has been the development of vaginal rings (11,12). Even so, these prolonged-release delivery platforms may not fit all usage patterns and single-use microbicides may be preferred over products requiring continuous presence in the vagina (13). Another important aspect of the PK of microbicide drugs has to deal with systemic exposure: low systemic drug levels seem to be favored in order to avoid toxicity and circumvent potential viral resistance issues (14,15).

Nanotechnology-based systems are gaining increasing interest as potential carriers for the vaginal delivery of microbicide drug candidates. In particular, nanosystems may potentially help solving solubility and stability issues of active compounds, modulate drug release, enhance mucosal drug distribution and retention, increase tissue penetration, and allow cell targeting (16,17). However, the true impact of nanocarriers in the fate of anti-HIV microbicide drugs is still poorly recognized. Our group has recently reported that drug-loaded poly(ethylene oxide)-coated poly(ε-caprolactone) nanoparticles (PEO-PCL NPs) possess interesting properties for the vaginal delivery of dapivirine. The ability of NPs to tackle (simulated) vaginal fluid barriers (18), enhance intracellular drug delivery to HIV-susceptible cells and provide favorable *in vitro* toxicity profile (19), while allowing lower permeability and higher retention in cell monolayers and vaginal mucosal explants (20), seem to justify further *in vivo* testing of these nanosystems. In the following we detail on *in vivo* studies in a diestrus mouse model of the genital tract distribution of NPs after vaginal instillation, as well as of their ability to influence the PK of dapivirine as compared to the free drug administered as a suspension. Moreover, the safety of daily use of NPs over a 14-day period is reported.

MATERIALS AND METHODS

Materials

Dapivirine was provided by the International Partnership for Microbicides (IPM; Silver Spring, MD, USA). PCL (14.8 kDa) was from Polysciences (Warrington, PA, USA) and poloxamer 338 NF [triblock copolymer of poly(ethylene oxide) (PEO), poly(propylene oxide) (PPO) (PEO-PPO-PEO)] from BASF (Mount Olive, NJ, USA). Other chemicals and reagents were of analytic grade or equivalent.

Preparation and Characterization of Nanoparticles

Dapivirine-loaded PEO-PCL NPs were prepared by a solvent displacement method as previously described (19). Briefly, PCL (34 mg), PEO-PPO-PEO (6 mg) and dapivirine (6 mg) were dissolved in 2 mL of acetone/ethanol (1:1) and added dropwise to 20 mL of stirring deionized water. NPs were recovered by ultracentrifugation and washed twice with 20 mL of water, before being resuspended as described below. Fluorescent counterparts were also obtained by substituting PCL by rhodamine-123-labeled PCL (rhod-123-PCL) and omitting the incorporation of dapivirine (20). NPs were characterized for hydrodynamic diameter, polydispersity index (PdI) and zeta potential using a ZetaSizer Nano ZS (Malvern Instruments, Worcestershire, UK). In the case of dapivirine-loaded NPs, drug association efficiency (AE%) and drug

loading (DL%) were calculated from the amounts of drug recovered in supernatants resulting from NP ultracentrifugation/washing steps and using HPLC-UV (21). Calculations were performed according to the following equations:

$$AE\% = \frac{\text{used amount of drug} - \text{drug in supernatants}}{\text{total amount of drug}} \times 100 \quad (1)$$

$$DL\% = \frac{\text{used amount of drug} - \text{drug in supernatants}}{\text{total weight of nanoparticles}} \times 100 \quad (2)$$

Preparation and Characterization of Suspensions of Nanoparticles and Dapivirine

Drug-loaded NPs, fluorescent NPs or dapivirine were dispersed in phosphate buffered saline (PBS; pH 7.4±0.1) at a final concentration of 0.02% (*w/v*; expressed as the total content of dapivirine) in order to be used for *in vivo* studies. The amount of sodium chloride in PBS was reduced to half in the case of NPs in order to maintain similar osmolality values to those of dapivirine suspension. In the case of the free drug, dimethyl sulfoxide was used at a final concentration of 0.1% (*v/v*) to disperse the drug in PBS. Dispersions were evaluated for pH and osmolality (20). Particle size and distribution of the dapivirine suspension were assessed using a MasterSizer Hydro 2000S (Malvern Instruments, Worcestershire, UK) at room temperature.

Animals

Animal experiments were approved by the Local Ethics Committee at the Faculty of Pharmacy, University of Porto (Approval Number 3/07/2012, July 23, 2012) and conducted under the guidelines and recommendations of FELASA and the European Directive 2010/63/EU. Female ICR mice (Charles River, Barcelona, Spain) were provided with food and tap water *ad libitum*, and treated at 7 and 3 days prior to the start of experiments with 3 mg of subcutaneous medroxyprogesterone acetate (Depo-Provera®). This treatment induces a diestrus-like state which is characterized by thinning of the vaginal epithelial layer and absence of its cornification, reduced inter-individual variability of vaginal histology, and increased drug permeability and susceptibility to toxic insult (22,23). Conversely, the thickness of mucus increases and provides a more stringent barrier to NP permeation (24). Mice were used at 8–12 weeks old (32.4±2.4 g). For the purpose of the described studies, the genital tract was divided in 4 sections (vagina, lower uterus, upper uterus and ovaries) as detailed in Supplementary Material (Figure S1).

Distribution and Tissue Penetration of Fluorescent Nanoparticles in the Genital Tract

Twenty-five microliters of either fluorescent NP dispersion (30 μg of NPs) or PBS (control) were administered intravaginally to conscious animals. Mice were hold upwards by the base of the tail and tested liquids administered intravaginally using a micropipette with a plastic tip. Care was taken in order to minimize tissue injury or disturbance of vaginal mucus. Animals were maintained in an upwards position for one minute before returning to their cages in order to reduce immediate vaginal leakage. Mice were then euthanized at pre-determined time points by inhalational 8–10% isoflurane overdose followed by intracardiac exsanguination. The vagina was washed 4-times by flushing repeatedly 50 μl of PBS with a micropipette and lavages frozen at −80°C. Previous reports showed that this washing procedure was able to remove nearly completely the vaginal mucus content in mice, including previously administered mucoadhesive NPs (150–170 nm) (25,26). Removal of mucus upon washing was further confirmed by probing the presence of mucin at the epithelial surface of the vaginal mucosa using the alcian blue staining (Supplementary Material, Supplementary Methods and Figure S2). The genital tract was collected during necropsy and representative sections prepared for fluorescent microscopy as detailed in Supplementary Material (Supplementary Methods). The remaining amount of genital tissues was weighted and stored at −80°C. Thawed vaginal lavages were mixed with acetonitrile (1:1) in order to dissolve NPs and precipitate protein content. Samples were then centrifuged (13,000 rpm, 10 min, 4°C) and the supernatant collected for florescence analysis. Tissue samples were minced, mixed with acetonitrile and homogenized using an Ultra-Turrax (IKA®-Werke, Staufen, Germany). Homogenates were centrifuged (4,300 rpm, 15 min, 4°C) and the supernatants recovered. Samples fluorescence was measured in 96-well opaque plates using a plate reader (Synergy 2 Multi-Mode, BioTek, Winooski, VT, USA) at 485/528 nm. The concentration of NPs was calculated against a standard curve obtained by spiking vaginal lavage or tissue samples from PBS-treated mice (control blanks) with known amounts of fluorescent NPs and processing these last as described for test samples. The minimum detectable level of fluorescent NPs was 0.02 μg/mL. All time points were tested using groups of five animals.

Local and Systemic Pharmacokinetics of Dapivirine

The PK profile of dapivirine was determined after vaginal administration of 25 μL of dapivirine-loaded NPs or free dapivirine dispersions (corresponding to a total amount of 5 μg of dapivirine in both cases), as described above. Animals were sacrificed at pre-determined time points by isoflurane overdosing followed by intracardiac exsanguination. Blood

was collected into Vacuette® tubes containing K_3EDTA (Greiner Bio-One GmbH, Kremsmünter, Austria) and plasma recovered by centrifugation (2,500 rpm, 10 min, 4°C). The vagina was washed and necropsy performed in order to collect genital tissues, rectum and selected organs. Vaginal lavages, weighted tissues/organs and blood plasma were frozen and stored at −80°C until further processing. Drug content in all samples was determined by a previously developed and validated HPLC-UV method (27). Specific method validation parameters for considered matrices are presented in Supplementary Material (Table S-I). Calculated PK parameters for each fluid/tissue/organ included: (1) maximum observed concentration (C_{max}) and time at which it occurred (t_{max}); (2) area under the concentration-time curve between 15 min and 24 h ($AUC_{0.25\text{-}24h}$), calculated using the trapezoid rule (GraphPad Prism 5.03, GraphPad Software, La Jolla, CA, USA); and (3) relative bioavailability (F_{rel}) which was defined as the ratio between $AUC_{0.25\text{-}24h}$ values of dapivirine-loaded NPs and the free drug. Also, the apparent terminal elimination rate constant (λ_z) in blood plasma and vaginal fluid was estimated by linear least squares regression of the three terminal points of log-linear concentration-time curves and terminal elimination half-life ($t_{1/2\beta}$) values calculated as $\ln(2)/\lambda_z$. All experimental conditions (treatments and time points) were tested in groups of five animals.

Fourteen-Day Toxicity of Dapivirine-Loaded Nanoparticles

Toxicity of the vaginal administration of drug-loaded NPs was assessed by histological analysis (H&E) and by measuring interleukin (IL)-1β, IL-6, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 1α (MIP-1α) in vaginal lavages. Briefly, dapivirine-loaded NPs were administered intravaginally once daily during 14 days as described above. After the first administration, the vaginas of mice were washed with PBS before a new administration and collected samples used for drug and chemokine/cytokine assay. At day 14, mice were sacrificed and biological fluids/tissues/organs collected for drug assay as described for PK studies. Results were compared to those of mice daily administered with dapivirine in suspension and 2% (*w/v*) nonoxynol-9 (N-9) in PBS. Two groups of mice treated daily with PBS or without any treatment (including daily vaginal washing) were also included as controls. Detailed methods are described in Supplementary Material (Supplementary Methods).

Statistical Analysis

Differences between dapivirine levels obtained with drug-loaded NPs and the free drug were assessed using two-tailed, Student's *t*-test and assuming equal variance of both groups (95% confidence interval). One-way ANOVA was used to investigate differences between the amounts of recovered fluorescent NPs at various time points, and between cytokine/chemokine content in vaginal lavages of mice exposed to NPs, free drug, N-9 and controls. Post-hoc comparisons were conducted according to Tukey's HSD test. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and $P<0.05$ was accepted as denoting significance. Results are presented as mean ± standard deviation (s.d.) unless otherwise stated.

RESULTS AND DISCUSSION

Nanoparticles and Suspensions of Nanoparticles and Dapivirine

Drug-loaded PEO-PCL NPs and fluorescent counterparts presented similar mean hydrodynamic diameter values of 199±13 nm and 186±3 nm, respectively. Drug-loaded nanoparticles were previously optimized for diameter in order to fit in the range of around 200 nm by adjusting the amount of PEO-PPO-PEO and the composition of the organic phase used during their preparation (Supplementary Material, Table S-II). This size range was selected as potentially optimal in terms of vaginal drug delivery, particularly when trying to target the epithelial layer, based on previous reports by different groups (28–30). PdI values were low in both cases (≤0.114), indicating that NPs were monodisperse. Zeta potential was negative for dapivirine-loaded NPs and fluorescent NPs, presenting values of −29.2±4.5 mV and −32.3±3.0 mV, respectively. Partial coating of this type of NPs with non-ionic PEO chains, as formerly determined by electron spectroscopy for chemical analysis (ESCA) (31), justifies these negative values due to the contribution of the negatively-charged PCL matrix. In the case of dapivirine-loaded NPs, AE% was 97.6%±0.1% and DL% was 12.7%±0.1%. Also, we have previously shown that dapivirine-loaded PEO-PCL NPs were stable for at least one year when stored as aqueous suspension at a concentration of 0.02% (expressed as dapivirine concentration) between 5°C and 40°C (32). Moreover when incubated with a simulated vaginal fluid, no apparent changes were observed in the properties of NPs, including the formation of particle aggregates. The addition of both types of NPs and dapivirine to PBS at considered concentrations did not change its pH value (7.4). Osmolality for drug-loaded NPs, fluorescent NPs and dapivirine suspensions was 354±12 mOsm/kg, 338±14 mOsm/kg and 345±6 mOsm/kg, respectively, thus presenting near iso-osmolal values with blood plasma. Mean particle size for free dapivirine in suspension in PBS was 27.1±0.6 μm, with 90% of particles presenting diameter values equal or lower than 54.0±1.8 μm. Release studies from dapivirine-loaded PEO-PCL NPs in two types of medium, namely a simulated vaginal fluid pH (SVF; 4.2) and PBS (pH 7.4) added with 2% (*w/v*) polysorbate 80 (in order to

maintain sink conditions), were formerly described (19,32). Release was slightly slower in PBS than in SVF and characterized by a fast initial burst effect: around 45% (PBS) and 65% (SVF) of dapivirine was recovered from media in the first 15 min, while an additional 10% (SVF) and 25% (PBS) of the drug was released up to 2 h. These release profiles were able to justify the maintenance of the *in vitro* antiviral activity of the drug at the low nanomolar range, with variable half-maximal effective concentration (EC_{50}) values depending on used cell setup (1.5–49.8 nM) (19).

Distribution and Penetration of Fluorescent Nanoparticles in the Genital Tract

PEO-rhod-123-PCL NPs were used as surrogates of dapivirine-loaded NPs for fluorescence microscopy. Confocal imaging of genital tissues (Fig. 1a–d) showed that NPs were present at the vaginal level and to some extent in the uterine tissue, but not in the ovaries. In the case of the control group (*i.e.* mice treated with PBS alone), no interfering auto-fluorescence signal was detected (data not shown). NPs were able to penetrate the vaginal epithelium up to a few tens of micrometers deep and distribute evenly throughout the tissue, including at vaginal folds. This may be interesting since HIV-susceptible cells in humans are mostly located at the epithelial and sub-epithelial layers (33).

Penetration of NPs was not only observed at discrete sites, where mucus disruption might have occurred (24), but also throughout the entire vagina, thus supporting that NPs were able to, at least partially, traffic through the mucus barrier. This is in line with our previous data showing that PEO-PCL NPs can interact mildly and transiently with mucin but still present the ability to traffic through a simulated vaginal fluid in a sub-diffusive fashion as assessed by *in vitro* multiple particle tracking (18). Although not presenting the same ability to avoid interactions with mucin and be able to distribute almost unhindered through mucus as densely low molecular weight poly(ethylene glycol) (PEG)-coated NPs (usually referred to as mucus-penetrating particles – MPPs) (34–36), PEO-PCL NPs can still migrate at considerable pace through mucus-like fluids. For example, transport rates of PEO-PCL NPs were only around 3–7 times lower than those reported by Lai *et al.* for same sized MPPs (28). Still, trafficking ability of NPs through the mucus should be interpreted with caution, namely due to the differences in vaginal pH between female mice (6.5–7.5) and women (4.0–5.0). Our previous investigations also showed that the ability of PEO-PCL NPs to diffuse through simulated vaginal fluid was mildly influenced by pH: diffusivity was increased around 2-times when the pH was changed from 4.2 to 7.0 (18). Parallel behavior was also found by Lai *et al.* for negatively-charged HIV-like particles (120 nm) *in vitro* when varying the pH of human cervicovaginal mucus from 4 to 6 (37). Although other variables can influence the ability of nanosystems to traffic and cross mucus barriers (*e.g.* mucus composition and its thickness/continuity alongside the epithelial lining, coitus, presence of semen, health state) it may be expected that tested NPs affect differently the PK of dapivirine at different pH values. Despite the previous, mixed ensemble transport behavior can be considered as potentially valuable since transient adhesion to mucus may contribute to retention while not completely impairing movement across mucus (38). Also, dense PEGylation of MPPs is known to impair phagocytosis, which can be a disadvantage in the case of drugs with intracellular mechanisms of action such as

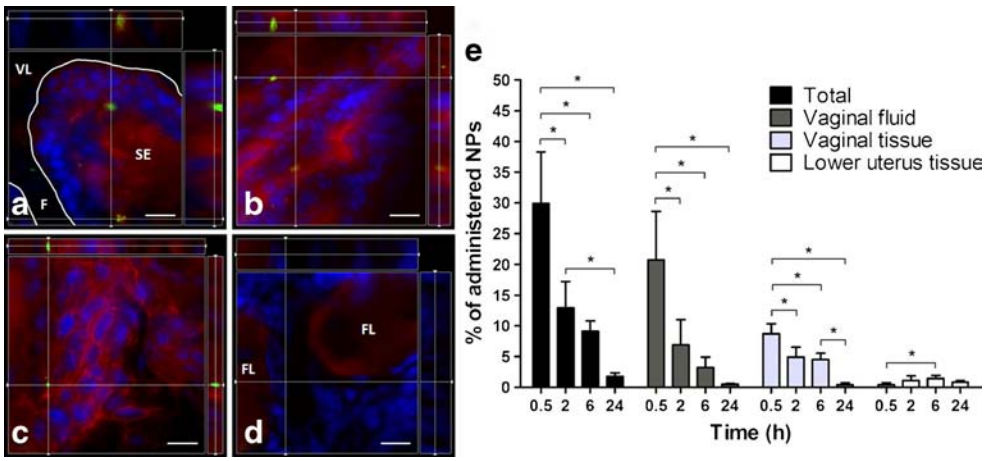

Fig. 1 Tissue penetration and distribution of fluorescent PEO-PCL NPs in the genital tract. Fluorescent confocal microscopy images of the (**a**) vagina, (**b**) lower uterus, (**c**) upper uterus and (**d**) ovaries were taken 2 h after vaginal administration of PEO-rhod-123-PCL NPs (in *green*). Images are representative of (**a**) and (**d**) transverse and (**b**) and (**c**) longitudinal sections. Blue and red signals are from Hoechst 33342 (DNA) and WGA, Alexa Fluor® 594 conjugate (sialic acid/N-acetylglucosaminyl residues at cell membranes/mucin), respectively. Scale bars = 10 μm. The mucosal tissue surface in (**a**) (*white lining*) has been included for clarity. VL: vaginal lumen; SE: sub-epithelium; F: vaginal folding; FL: follicle. Recovery of fluorescent NPs from vaginal lavages, and vaginal/lower uterine tissues is presented in (**e**). Results are expressed as the percentage of the initial amount of NPs administered intravaginally. Columns represent mean values and bars the s.d. ($n = 5$). (*) denotes $P < 0.05$ when comparing amounts of fluorescent NPs at different time points.

dapivirine; this contrasts with the ability of PEO-PCL NPs to be taken up by different cell types, in particular HIV-target cells, as demonstrated previously *in vitro* (19).

Confocal microscopy results (Fig. 1) are consistent with two previous studies showing that polymeric NPs (75–300 nm) could effectively penetrate the vaginal epithelium when administered intravaginally to mice (30,39). The relatively low amount of fluorescent NPs associated with the vaginal tissue may be related with NP loss due to natural leakage and removal during vaginal washing. Even so, the epithelial distribution pattern of PEO-PCL NPs is comparable to the one obtained by Woodrow *et al.* (30) for polyvinyl alcohol-coated, poly(D,L-lactide-*co*-glycolide) (PLGA) NPs (100–300 nm). Thus, it seems that at least a fraction of NPs can migrate through mucus, reach the underlying epithelial lining and penetrate the tissue. Moreover, fluorescent imaging supports that NPs were able to distribute to the uterus and penetrate the epithelial cell lining (Fig. 1b and c).

In order to quantify the distribution of NPs through the genital tract, the amount of rhod-123-PCL recovered from vaginal lavages and genital tissues was determined by measuring fluorescence signals (Fig. 1e). After as little as 30 min, around 70% of the total amount of administered NPs was already cleared. Leakage from the vagina is the most probable cause for this fact. Self-grooming may also have contributed to NP loss since no measures were adopted in order to restrict mice movements. The total amount of NPs recovered further decreased to less than 10–15% by 2–6 h, being minimal at 24 h (<2%). These results seem to be in agreement with the only two studies reporting on the quantification of NPs recovered from the genital tract of mice after intravaginal administration (26,36). Cu *et al.* (26) showed that only around 30–44% and 8–22% of differently-surface coated PLGA-based NPs (150–170 nm), administered as PBS dispersions, were able to be recovered after 30 min and 2 h, respectively. Contrasting with the present investigation, recovery included NPs present in vaginal lavages, reproductive tissues and also leaked fluids. The contribution of this last was estimated around 3–13% of total administered NPs by using absorbent paper floor-linings.

By 30 min, 21% of administered NPs were present in vaginal lavages, corresponding to around 70% of the total amount recovered, but rapidly decreased to 7% and 3% after 2 h and 6 h respectively (Fig. 1e). These results reinforce that NPs were only able to reside transiently in the vaginal lumen, being rapidly removed either by leakage or due to migration through mucus and subsequent penetration of the epithelial barrier. Again, these results are in line with those reported by Cu *et al.* for 150–170 nm MPPs (26). The rapid elimination of NPs from the vaginal lumen is most probably related with the use of PBS as vehicle rather than a dosage form that can promote, at least to some extent, longer residence. Despite the fact that the amount of administered fluid was relatively small (25 μL), leakage is still inevitable. Even so, the delivery of NPs and free dapivirine as PBS dispersions was preferred in order to avoid a cumulative effect with specific dosage forms (*e.g.* gels or films), thus allowing to better understand the sole behavior of NPs and its influence in the PK of dapivirine.

Recovery of the fluorescent polymer from the ovaries and the upper uterus was below the minimum detectable at all time points (0.2% of the total amount of administered NPs). This suggests that NPs were nearly incapable of reaching these anatomical sites, being their distribution mostly confined to the vagina and lower areas of the uterus. In the case of vaginal tissue, nearly 9% and 5% of administered NPs were recovered at 30 min and 2 h, respectively. Although relatively low, these values seem to provide evidence that a fraction of NPs can rapidly migrate through the mucus layer and associate with/penetrate the vaginal epithelium as previously shown *in vitro* (18). These results seem to be again in line with those for PEG-coated PLGA NPs as reported by Cu *et al.* (26). Also, no differences were observed for the amount of NPs present in the vaginal tissue between 2 and 6 h thus indicating the onset of equilibrium between incoming and eliminated NPs. As for the lower uterus, results point out that NPs are able to reach this site. More important, increasing amounts of recovered NPs up to 6 h suggest that nanosystems require a time lag for translocation from the vagina and later tissue penetration. The amount of NPs at different sections of the lower uterus was not assessed but it is expected that the majority is located near the cervix, presenting a gradient pattern from higher NP-concentrated areas around the vagina to nearly absent towards the middle segment of the uterine horns. Overall decreasing levels in both vaginal and lower uterine tissues (from 6 h to 24 h) may probably be attributed to self-cleansing mechanisms of the genital tract associated with mucus turnover and epithelial cell shedding.

Local and Systemic Pharmacokinetics of Dapivirine

The assessment of local and systemic PK of antiviral candidate drugs is currently regarded as an important step in the development of microbicides (40). Increased cervicovaginal drug levels are thought to be beneficial, while systemic exposure may be potentially detrimental. Therefore, strategies that can modulate the local distribution and absorption of microbicide compounds upon vaginal delivery are required.

The PK of dapivirine as modulated by its incorporation into NPs was determined after vaginal administration and compared with the PK of dapivirine in suspension. The amount of drug recovered from vaginal lavages (Fig. 2) showed that the residence of the drug was clearly increased when associated to NPs. Drug levels were around 2- to 17-fold higher between 30 min and 16 h than those achieved with the free drug. This fact is probably related with the ability of NPs to transiently associate with mucus and increase their

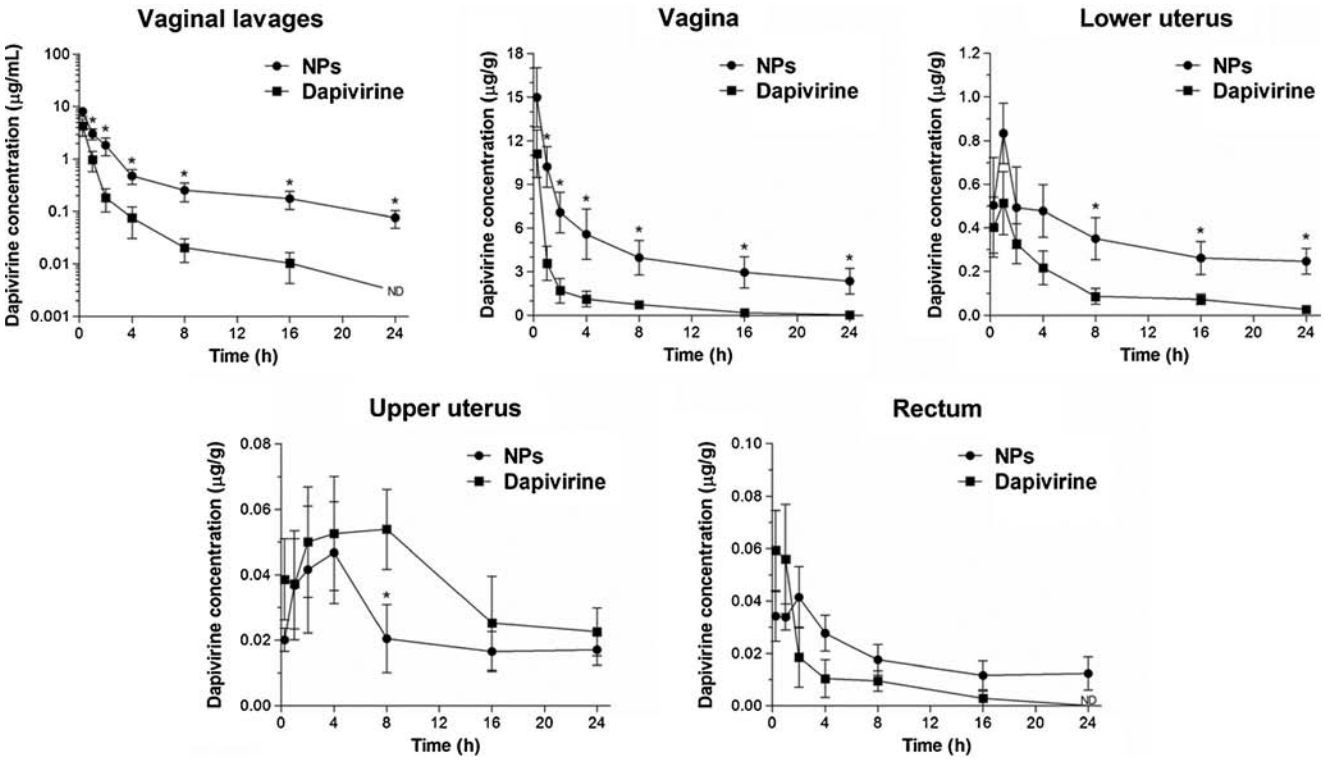

Fig. 2 Dapivirine levels in vaginal lavages, genital tissues and rectum following vaginal administration of dapivirine-loaded PEO-PCL NPs or the free drug in PBS. Note the different scales and units in *y*-axes, including the log-scale for vaginal lavages. Individual points represent mean values and vertical bars the standard error of the mean (s.e.m.; $n=5$). (*) denotes $P<0.05$ when comparing NPs with free dapivirine at the same time point. ND: not detected.

residence at the vaginal canal (18). However, a fast decrease in drug levels was observed: for example, drug levels diminished nearly 30-fold and 200-fold in the case of NPs and free dapivirine, respectively, between 15 min and 4 h post-administration. This reduction was also noticeable during the first 15 min when an estimated 60–80% of dapivirine was cleared from the vaginal canal, as determined by the difference between the initial amount of drug administered and the one recovered from lavages. Even if drug/NP permeation of the cervicovaginal mucosa, systemic drug absorption and drug/NP diffusion to the uterus may also have contributed, leakage is likely the main reason for this decrease (41). This also seems to be in agreement with data obtained for the amount of NPs present in vaginal lavage after administration, as reported above. Overall, NPs may be able to increase the retention of the drug at the vaginal canal as further revealed by detailed PK parameters, namely the F_{rel}, as presented in Table I. Values for $t_{1/2\beta}$ were higher for NPs than for free dapivirine (9.2 h *vs.* 5.8 h) but lower than the values previously reported for vaginal gels in humans (16–17 h) (9). Besides obvious interspecies differences, the ability of polymeric gels to enhance drug residence may also explain these dissimilarities.

The levels of dapivirine in different genital tissues between 15 min and 24 h post-administration (Fig. 2), as well as detailed PK parameters (Table I), were also determined. The amounts of dapivirine associated with the vaginal mucosa were at least one log higher than those observed in the lower uterus for the same time points. In the case of the upper uterus, drug levels were around 2–3 log lower than in vaginal tissue. Dapivirine was undetectable in the ovaries. These observations suggest a decreasing concentration gradient from the vagina towards the ovaries, in line with the distribution of NPs. This relative inability of the drug to reach higher sites of the genital tract may be regarded as advantageous since the transmission of the virus in humans occurs at the vagina and cervix (33). Thus, lower drug exposure at non-virus target sites seems favorable. When comparing drug levels obtained with NPs and free dapivirine, it seems clear that nanocarriers were able to increase the amount of drug associated with the vaginal tissue. The increase in drug levels was less obvious at 1 h (around 3-fold) but peaked at 24 h (nearly 2-log increase). Value for F_{rel} (Table I) revealed that NPs provided around 5-times higher levels of dapivirine as compared to the drug in suspension between 15 min and 24 h after vaginal administration. Taken together, data support that NPs are able to maintain higher levels of dapivirine at this tissue, a fact that may be associated, at least partially, with the distribution of nanosystems throughout the mucosa and, possibly, the sustained release of the drug from these last (19). A similar trend for drug levels was observed at the lower uterine tissue. In this last case maximum levels were only observed at 1 h post-administration rather than at 15 min, which appears to

Table I PK Parameters of Dapivirine in Different Fluids/Tissues/Organs After Intravaginal Delivery of Drug-Loaded NPs or Free Dapivirine. Results are Presented as Mean (± s.e.m., if Applicable; $n=5$)

Biological sample	Dapivirine Formulation	$AUC_{0.25\text{-}24h}$ (μg.h/g) [a]			C_{max} (μg/g) [b]			F_{rel}	t_{max} (h)	$t_{1/2\beta}$ (h)
		mean (± s.e.m.)	Range	P [c]	mean (± s.e.m.)	Range	P [(c)]			
Vaginal lavage	NPs	13.07 (± 0.71)	11.39–15.49	**0.000003**	8.07 (± 1.62)	3.06–12.13	0.121	4.15	0.25	9.2
	Suspension	3.15 (± 0.52)	1.68–4.72	–	4.23 (± 1.51)	0.30–8.12	–	–	0.25	5.8
Vagina	NPs	98.34 (± 12.46)	56.60–123.60	**0.0003**	14.97 (± 2.04)	10.68–19.82	0.176	5.10	0.25	–
	Suspension	19.29 (± 2.38)	12.41–27.00	–	11.10 (± 1.62)	5.34–14.63	–	–	0.25	–
Lower uterus	NPs	8.27 (± 1.52)	3.86–12.05	**0.010**	0.834 (± 0.139)	0.468–1.232	0.709	2.80	1	–
	Suspension	2.96 (± 0.42)	2.10–3.98	–	0.513 (± 0.145)	0.059–0.864	–	–	1	–
Upper uterus	NPs	0.566 (± 0.079)	0.363–0.818	**0.031**	0.047 (± 0.016)	0.015–0.090	0.728	0.60	4	–
	Suspension	0.946 (± 0.098)	0.620–1.129	–	0.054 (± 0.012)	0.028–0.098	–	–	8	–
Ovaries	NPs	0	–	–	0	–	–	–	–	–
	Suspension	0	–	–	0	–	–	–	–	–
Rectum	NPs	0.435 (± 0.099)	0.173–0.696	0.059	0.041 (± 0.012)	0.014–0.078	0.382	2.47	2	–
	Suspension	0.176 (± 0.028)	0.116–0.277	–	0.059 (± 0.015)	0–0.082	–	–	0.25	–
Liver	NPs	0.372 (± 0.085)	0.157–0.651	0.155	0.033 (± 0.016)	0–0.090	0.943	0.59	1	–
	Suspension	0.628 (± 0.098)	0.315–0.856	–	0.032 (± 0.005)	0.021–0.042	–	–	1	–
Kidney	NPs	0.020 (± 0.020)	0–0.098	0.956	0.003 (± 0.003)	0–0.016	0.734	0.74	8	–
	Suspension	0.027 (± 0.016)	0–0.081	–	0.005 (± 0.005)	0–0.027	–	–	1	–
Heart	NPs	0	–	–	0	–	–	–	–	–
	Suspension	0	–	–	0	–	–	–	–	–
Lungs	NPs	0	–	–	0	–	–	–	–	–
	Suspension	0	–	–	0	–	–	–	–	–
Blood plasma	NPs	0.166 (± 0.034)	0.108–0.300	**0.002**	0.026 (± 0.011)	0.006–0.070	**0.005**	0.44	0.25	8.2
	Suspension	0.377 (± 0.043)	0.315–0.534	–	0.099 (± 0.015)	0.066–0.124	–	–	0.25	12.7

[a] μg.h/mL in the case of vaginal lavage and blood plasma

[b] μg/mL in the case of vaginal lavage and blood plasma

[c] when comparing with the free drug for the same biological sample

support that a time lag was required for the diffusion of the drug and NPs proceeding from the vagina. Also, drug levels obtained were higher when considering NPs, varying from 4-fold at 8 h to nearly 9-fold increases at 24 h and with a mean F_{rel} value of 2.8. Taken as whole, higher dapivirine concentrations at the cervicovaginal level obtained using drug-loaded NPs may be encouraging in terms of protection against viral transmission. As for the upper uterus, there was a trend for reaching higher drug concentrations in the case of the drug in suspension, even if significant differences were only observed at the 8 h time point. The total bioavailability of the drug at this tissue was reduced by around 40% for NPs. Despite these small differences it seems that NPs may have slightly reduced drug exposure to the upper genital tract.

The concentrations of dapivirine at the rectum were further determined (Fig. 2). Levels of dapivirine were comparable to those obtained in the upper uterus but no significant differences were detected between NPs and the free drug ($P=0.059$). Even so, NPs appear to have provided higher bioavailability as revealed by the analysis of $AUC_{0.25\text{-}24h}$ and F_{rel} values (Table I). The distribution of the drug to the rectum occurred fairly rapid, with the drug being detected after as little as 15 min post-vaginal administration. Two explanations may justify these observations: first, leakage from the vagina and diffusion across the anus seems to be plausible due to the short genital-anal distance; second, the close proximity of the vaginal and rectal tissues suggests that passive diffusion or phenomena similar to the so called "uterine first pass effect" (42) may have occurred. Other hypotheses such as drug transfer *via* the blood stream seem improbable since drug levels detected in blood plasma were very low (Fig. 3). In a recent study, Nuttall *et al.* (43) observed a similar trend for tenofovir formulated as a gel after vaginal administration to rhesus macaques. Similar explanations to the ones hereby provided were also suggested. Despite uncertainties on the actual mechanism of the vaginal-rectal drug transfer, presented data suggest that rectal protection may be achievable after vaginal administration of dapivirine.

Alongside plasmatic drug levels, systemic exposure was further assessed by measuring dapivirine levels in selected

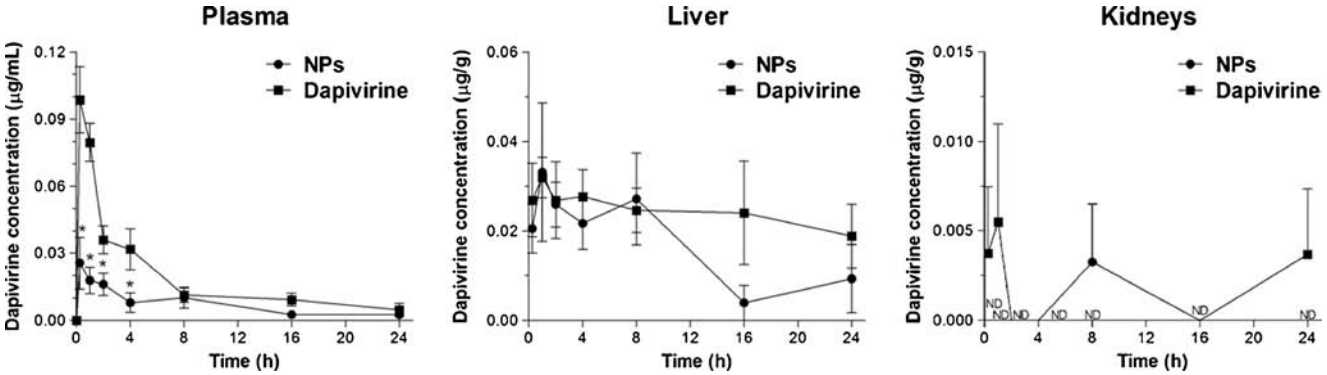

Fig. 3 Systemic exposure to dapivirine following vaginal administration of dapivirine-loaded NPs or the free drug. Note the different scales and units in *y*-axes. Individual points represent mean values and vertical bars the s.e.m. ($n = 5$). (*) denotes $P < 0.05$ when comparing NPs with free dapivirine at the same time point. ND: not detected.

organs (Fig. 3). Plasmatic levels were higher at 15 min indicating that the drug was rapidly absorbed. Even so, the fraction of drug present in the blood was relatively low: at 15 min only around 3% and 0.8% of the total administered drug was found in the blood plasma in the case of free dapivirine and NPs, respectively, considering a mean total plasma volume of 1.5 mL in mice (44). These levels rapidly decreased up to 4–8 h and were residual around 24 h (0.08–0.14% of the total amount of drug administered). Plasmatic levels of dapivirine were also significantly lower for NPs from 15 min to 4 h as compared to the free drug, thus indicating a trend for reduced total systemic exposure. The analysis of values for $AUC_{0.25\text{-}24h}$, C_{max} and F_{rel} (Table I) further supports this claim. Values for $t_{1/2\beta}$ were substantially lower than those reported for vaginal gels of dapivirine in humans (73–90 h) (9). Again, interspecies differences and the use of PBS suspensions in the present study may justify this disparity. As for selected organs, exposure to dapivirine was low in the liver and kidneys (Fig. 3 and Table I). Dapivirine was only detected and quantifiable in the kidneys of three and one animal administered intravaginally with the free drug and NPs, respectively. No differences were observed between NPs and free dapivirine in both cases. Overall, data seems to indicate that the association of dapivirine to NPs was able to mildly reduce its systemic exposure.

Further, the PK of dapivirine over 14 days was assessed using samples collected from animals included in the 14-day toxicity study. Figure 4a presents drug concentrations in vaginal lavages collected 24 h after the last administration. There was a slight increase in dapivirine levels over the first week for the free drug but this trend was not apparent for NPs. It seems that no considerable accumulation of the drug occurred between 7 and 14 days. At all time points, NPs allowed obtaining significantly higher drug concentrations in vaginal lavages as compared to the free dapivirine (around 3–17-fold). These results suggest that increased levels of drug may still be observable for NPs upon continuous use, as compared to free dapivirine. In the case of genital tissues and tested organs, drug levels at 24 h after the last vaginal administration were relatively similar when comparing single treatment or daily treatment for 14 days (Fig. 4b). Differences were only observed for ovaries in the case of dapivirine-loaded NPs, where the drug was quantifiable after 14 days in 1 out of 5 animals. A significant increment in the drug levels was also noticed at the rectal tissue, which may be considered beneficial in terms of dual protection. As for dapivirine in suspension, significant increases in drug levels were observed in the case of vaginal, lower uterine and rectal tissues; the drug was also quantifiable in the ovaries and the heart (1 out of 5 mice in both cases), contrasting with day one when the drug was bellow the limit of detection of the dosing method (0.01 μg/g and 0.006 μg/g, respectively). Also noteworthy, significantly higher concentrations of dapivirine were observed in the vaginal and lower uterine tissues for NPs as compared to the free drug after 14 days. Drug levels were 25- and 7-times higher in the vagina and lower uterus, respectively. Overall, PK results obtained after daily administration for 14 days reinforce that NPs may be advantageous even in the case of continuous use. It is also worth mentioning that differences between day 1 and 14 may be related with facilitated contact of NPs and the free drug with the vaginal mucosa due to continuous depletion of mucus by daily washing with PBS. Even though vaginal washing was mainly concerned with chemokine/cytokine assay, this procedure may also simulate to some extent vaginal intercourse thus providing an additional interesting feature to this model in the context of microbicide research.

Although promising, the true impact of presented PK differences between NPs and free dapivirine, namely at the genital tract, is not easily assessable. The lack of established PK/pharmacodynamics (PD) relationships for dapivirine and other microbicide drugs (45), as well as interspecies differences (*e.g.* differences in vaginal histology and mucus composition between diestrus mice and women), limits data analysis. Other animal models, namely non-human primates, could probably reduce the interspecies barrier even though their highly restricted use and still limited correlation with human data

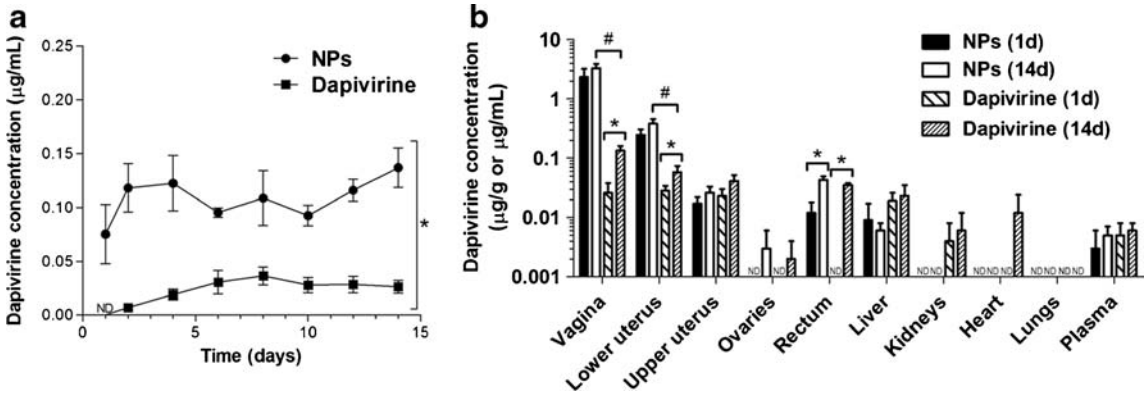

Fig. 4 Dapivirine levels in (**a**) vaginal lavages, and (**b**) different tissues/organs and blood plasma following daily vaginal administration of dapivirine-loaded PEO-PCL NPs or the free drug for 14 days. Results are from 24 h after the last administration. Note the different scales and units in *y*-axes, including the log-scale in (**b**). Individual points represent mean values and vertical bars the s.e.m. ($n=5$). In panel (**a**), (*) denotes $P<0.05$ when compared with free dapivirine; in panel (**b**), (*) denotes $P<0.05$ when comparing the same treatment (NPs or free drug) after 1 and 14 days, while (#) indicates $P<0.05$ when comparing NPs and free dapivirine after 14 days. ND: not detected.

would impair its usefulness at this stage (46). However, the ability to achieve concentrations of dapivirine in genital fluids/tissues above the levels required for preventing HIV infection *in vitro* seems to be a reasonable threshold (10,47). For example, Fletcher *et al.* (48) determined that concentrations around 10–100 nM (0.033–0.33 μg/mL) were able to completely inhibit the infection in human mucosal explants. The only reported study on the efficacy of vaginal dapivirine in a humanized mouse model showed that a gel containing 2.25 μM (0.74 μg/mL) of the drug was able to provide nearly full protection against HIV-1 (49). In a rather simplistic analysis, and considering at least one-log dilution of vaginal secretions in the present investigation due to vaginal washing, it may be anticipated that a concentration of around 0.074 μg/mL in vaginal lavages could provide substantial protection. For example, NPs were able to provide mean levels of dapivirine which were equal or above this last value in vaginal lavages up to 24 h; as for the suspension of free drug, mean concentrations equal or above 0.074 μg/mL were only observed up to 4 h. This analysis, although speculative, seems to indicate that the incorporation of dapivirine in NPs may indeed provide enhanced protection when compared to the unformulated drug. Prolonged protection, in particular, may be expectable and seems to support the usefulness of NPs in obtaining coitally-independent microbicides. However, *in vivo* efficacy data are required in order to verify these assumptions.

Fourteen-Day Toxicity of Dapivirine-Loaded Nanoparticles

The evaluation of the toxicity of drug-loaded NPs and free dapivirine revealed that both were potentially safe. Histological analysis of the genital tract and other selected organs, as well as inflammatory chemokine/cytokine release profiles in vaginal lavages upon once-daily administration for 14 days failed in identifying any signs of toxicity. Tested cytokines/chemokines included IL-1β, IL-6, KC and MIP-1α. Detailed results are presented and further analyzed in Supplementary Material.

CONCLUSIONS

In the present investigation, we showed that PEO-PCL NPs were able to distribute mostly in the vaginal canal upon administration to mice but also reached the uterus. The elimination of these nanosystems was fast and most likely due to leakage; however, NPs could still be traceable in vaginal lavages up to at least 24 h post-administration. NPs rapidly penetrated and distributed in the vaginal and lower uterine tissues, thus providing *in vivo* evidence that a considerable amount of nanocarriers can tackle the mucus barrier and reach the epithelium. Dapivirine-loaded PEO-PCL NPs allowed modifying local and systemic PK of dapivirine when compared to the free drug in suspension. Significantly higher levels of dapivirine were recovered from vaginal lavages and vaginal and lower uterine tissues; at the same time, lower systemic exposure was observed. These data appear to substantiate that NPs are able to provide dapivirine with a distinguished PK profile and potentially contribute to higher protection against HIV transmission. Increased levels of dapivirine detected in vaginal lavages and tissues appear to be associated with enhanced luminal retention of NPs (which may release their content *in loco*) and, at least partially, the ability of NPs to tackle mucus and penetrate the epithelium. However, presented drug level results should further be carefully analyzed since they do not offer information on the fraction of dapivirine that still remains associated with NPs; also, drug levels at tissues do not account for intracellular (namely in HIV-target cells) and extracellular fractions thus limiting their full interpretation. Thus, further studies to address these issues are required. Results from the 14 day

toxicity study indicate that the once-daily vaginal use of dapivirine-loaded NPs may be regarded as safe. Overall, presented *in vivo* results appear to evidence that drug-loaded PEO-PCL NPs can provide an interesting platform for the vaginal delivery of dapivirine in the context of anti-HIV microbicides. Further testing of the ability of these NPs to prevent vaginal HIV transmission in an animal model seems to be justified.

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