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Enhanced vaginal drug delivery through the use of hypotonic formulations that induce fluid uptake

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ABSTRACT

Mucosal epithelia use osmotic gradients for fluid absorption and secretion. We hypothesized that administration of hypotonic solutions would induce fluid uptake that could be advantageous for rapidly delivering drugs through mucus to the vaginal epithelium. We found that hypotonic formulations markedly increased the rate at which small molecule drugs and mucoinert nanoparticles (mucus-penetrating particles, or MPP), but not conventional mucoadhesive nanoparticles (CP), reached the vaginal epithelial surface *in vivo* in mice. Additionally, hypotonic formulations greatly enhanced drug and MPP delivery to the entire epithelial surface, including deep into the vaginal folds (rugae) that drugs or MPP in isotonic formulations failed to reach efficiently. However, hypotonic formulations caused unencapsulated "free" drugs to be drawn through the epithelium, reducing vaginal retention. In contrast, hypotonic formulations caused MPP to accumulate rapidly and uniformly on vaginal surfaces, ideally positioned for localized sustained drug delivery. Using a mouse model of vaginal genital herpes (HSV-2) infection, we found that hypotonic delivery of free drug led to improved immediate protection, but diminished longer-term protection. In contrast, as we previously demonstrated, hypotonic delivery of drug via MPP led to better long-term retention and protection in the vagina. Importantly, we demonstrate that slightly hypotonic formulations provided rapid and uniform delivery of MPP to the entire vaginal surface, thus enabling formulations with minimal risk of epithelial toxicity. Hypotonic formulations for vaginal drug delivery via MPP may significantly improve prevention and treatment of reproductive tract diseases and disorders.

1. Introduction

Vaginal products have traditionally been made with *hyper*tonic formulations, including yeast infection treatments, most sexual lubricants such as KY® warming gel, and most microbicide gels designed to prevent sexually transmitted infections such as HIV. Hypertonic formulations cause rapid, osmotically–driven *secretion* of fluid through the vaginal tissues into the lumen of the vagina. Osmotically–driven secretion has been shown to cause an immediate increase in vaginal product leakage at a rate proportional to the hypertonicity of the formulation [1]. Moreover, recent investigations of candidate vaginal and rectal microbicides, both in animal models and in humans, have revealed that hypertonic formulations cause toxic effects that can *increase* susceptibility to infections [2–4]. The first successful microbicide trial for HIV prevention found that the antiretroviral drug, tenofovir, provided partial protection when delivered in a vaginal gel formulation. Unfortunately, the gel formulation was highly hypertonic, leading investigators in the most recent clinical trial of tenofovir to reduce, but not eliminate, the hypertonic concentration of glycerol. There appears to be no evidence to justify hypertonic formulations for

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vaginal drug delivery, since in addition to the documented toxic effects, hypertonic formulations cause rapid osmotically–driven secretion of vaginal fluid that opposes the delivery of drugs to the epithelium.

In contrast, hypotonic formulations cause fluid absorption, leading to convective transport of drugs and nanoparticles to the vaginal epithelial surface. Hypotonic formulations have been reported to provide enhanced drug and particle delivery to several mucosal surfaces, including improved nanoparticle uptake by M-cells in the gastrointestinal tract [5] and nasal passages [6], improved carrier stability for gene delivery in the lung [7,8], and for paracellular drug absorption across the intestine [9–11]. However, to our knowledge, hypotonic formulations for vaginal drug delivery have not been previously investigated.

The surface of the vagina is highly folded to accommodate expansion during intercourse and childbirth; these folds, or "rugae", are normally collapsed by intra-abdominal pressure, hindering drug delivery to the infolded surfaces. For effective prevention and treatment, sustained drug concentrations must be delivered to, and maintained over the entire susceptible surface. Failure to achieve adequate distribution over the entire vaginal epithelium is a documented failure mode of vaginal microbicides [12].

Another significant barrier to effective drug delivery to the vagina is the viscoelastic layer of mucus secreted by the endocervix that coats the vaginal epithelium. Mucus efficiently traps foreign particles and particulates by both steric and adhesive mechanisms, facilitating rapid clearance [13]. Although the use of mucoadhesive dosage forms has been proposed for increasing residence time in the vagina [14], mucus clearance occurs rapidly (on the order of minutes to hours), limiting the residence time of mucoadhesive systems that do not penetrate the outermost mucus layer [15]. We recently demonstrated that MPP that do not adhere to mucus are capable of rapidly diffusing through human and mouse cervicovaginal mucus (CVM), enabling them to penetrate deep into the more slowly cleared mucus layers, including those in the rugae, thereby effectively coating the entire vaginal surface and residing in the vagina longer than conventional mucoadhesive nanoparticles (CP) [16].

One key to the improved vaginal distribution and retention by MPP was administering the nanoparticles in a hypotonic solution. When delivered hypotonically, MPP rapidly accumulated on the entire vaginal surface, arriving there much more rapidly than expected based on diffusion alone. We demonstrated that MPP flow through the mucus along with pressure-induced fluid flow [16]. We anticipated that a similar effect would occur if drugs were delivered to the vagina in hypotonic formulations. Here, we investigate hypotonic vaginal delivery of free drug and optimize hypotonic delivery of MPP to provide improved distribution, retention, and safety.

2. Materials and methods

2.1. Animal model

Female 6–8 week-old CF-1 mice were purchased from Harlan (Indianapolis, IN). Mice were housed in a reverse light cycle facility (12 h light/12 h dark), to enable selection of mice in a naturally cycling estrus state. The mouse vagina during the estrus phase of the estrous cycle is most similar to the human vagina [17,18]. Also, we previously demonstrated that barrier properties to nanoparticles in estrus phase mouse mucus closely mimic the barrier properties to nanoparticles in human CVM [16]. Thus, mice in estrus phase, as determined visually by the appearance of the vaginal introitus, were used for all distribution and retention studies [19]. Mice used for vaginal HSV-2 protection and susceptibility studies were given a subcutaneous flank injection of 2.5 mg Depo-Provera (Pharmacia & Upjohn Company, New York, NY) in 100 uL phosphate-buffered saline (PBS) 7 days prior to experiments. This treatment is commonly used to increase susceptibility to vaginal HSV-2 infection [20,21]. All experimental protocols were approved by the Johns Hopkins Animal Care and Use Committee.

2.2. Nanoparticle preparation and characterization

Fluorescent, carboxyl(COOH)-modified polystyrene (PS) nanoparticles 100 nm in diameter were purchased from Molecular Probes (Eugene, OR). To produce MPP, PS particles were covalently modified with 5 kDa amine-modified PEG (Creative PEGworks, Winston Salem, NC) as previously described [22]. Particle size and ζ-potential were determined by dynamic light scattering and laser Doppler anemometry, respectively, using a Zetasizer Nano ZS90 (Malvern Instruments, Southborough, MA). Size measurements were performed at 25 °C at a scattering angle of 90°. Samples were diluted in 10 mM NaCl solution (pH 7) and measurements performed according to instrument instructions. A near neutral ζ-potential was used to confirm PEG conjugation, and particles were tested for mucus-penetrating ability in human CVM as previously described [23,24]. These particles were previously shown to rapidly penetrate estrus phase mouse vaginal mucus [16]. The osmolality of solutions was measured using a Wescor Vapro vapor pressure osmometer.

2.3. Drug and nanoparticle distribution in the vagina

Doxorubicin (NetQem, Durham, NC) was dissolved at 1 mg/ml concentration in either PBS (isotonic) or ultrapure water (hypotonic). Doxorubicin was vaginally administered both isotonically and hypotonically to mice in two different conditions. The "non-ambulatory" group was anesthetized with an intraperitoneal injection of Avertin working solution (prepared according to Johns Hopkins ACUC guidelines) and remained supine for 1 h prior to tissue collection. The "ambulatory" group was anesthetized with fast-acting, inhalable isoflurane, such that the mice immediately awoke and were ambulatory for 10 min prior to tissue collection. The vaginal tissues were then collected, sliced open longitudinally, flattened, and clamped between two glass slides sealed shut with super glue. This procedure completely flattens the tissue, exposing the surfaces that were infolded from the lumen. Tissues were imaged on an epifluorescence microscope (Nikon E6100) at 2× magnification. Doxorubicin is fluorescent (ex/em 470/590). Untreated control tissues were imaged to ensure that the fluorescent signal from Doxorubicin was well above the tissue autofluorescence. The low magnification captured large portions of the tissue, so only 2–3 images were needed to observe the entire tissue surface. The images were 'thresholded' to draw region of interest boundaries around the fluorescent signal, and then the area covered quantified using ImageJ software. An average percentage coverage was determined for each mouse, and these values were averaged over groups of $n = 5$ mice.

To capture the distribution of MPP due to immediate fluid absorption dynamics, 20 μl of either isotonic (PBS) or hypotonic (ultrapure water) MPP solution was administered vaginally. This volume of solution helped ensure that the lumen would be filled with fluid, and the particles were diluted (0.01% w/v) such that concentration gradients would be visually evident. The mice were anesthetized with isoflurane, and sacrificed immediately after particle administration. The vaginal tissue was quickly excised and flash-frozen in Tissue-Tek O.C.T. Compound. Transverse sections were obtained at various points along the length of the tissue using a Leica CM-3050-S cryostat. The thickness of the sections was set to 6 μm to achieve single cell layer thickness. The sections were then stained with ProLong Gold antifade reagent with DAPI to visualize cell nuclei and retain particle fluorescence. Fluorescent images of the sections were obtained with an inverted epifluorescence microscope (Zeiss Axio Observer). For MPP distribution with varying solution osmolality, particle solutions (0.08% w/v) were prepared from varying ratios of PBS and ultrapure water. Mice were anesthetized with isoflurane, and 5 μl of particle solution was administered vaginally. After 10 min, tissues were collected, flash-frozen, sectioned, and stained following the procedures outlined for observing fluid absorption dynamics. In order to quantify MPP vaginal tissue coverage, mice were anesthetized with isoflurane, and 5 μl of nanoparticle solution was administered vaginally. Within 10 min, tissues were excised, sliced open longitudinally, and then flattened as described for drug distribution experiments. Control tissues were imaged to ensure that the fluorescent signal was well above the tissue autofluorescence. Tissues were imaged at 10× magnification using an inverted epifluorescence microscope (Zeiss Axio Observer), and 8 images per tissue were acquired. The coverage was quantified as previously outlined for drug distribution experiments.

2.4. Drug and nanoparticle retention in the vagina

Doxorubicin was dissolved at a concentration of 1 mg/ml in PBS (isotonic) or ultrapure water (hypotonic). Mice were anesthetized with an intraperitoneal injection of Avertin prior to intravaginal administration of 5 μl of Doxorubicin solution. The mice remained supine for 10 min to ensure that solution "drip out" would not affect the retention measurement. Then, the whole cervicovaginal tracts were excised and placed in a standard tissue culture dish. Fluorescence images of the tissues were obtained using the Xenogen IVIS Spectrum imaging device (Caliper Life Sciences). To account for potential differences in Doxorubicin solution fluorescence, vials of both isotonic and hypotonic Doxorubicin solutions were included in the image. The ratio of the intensity of the two solutions was used to normalize the tissue fluorescence for each group. Quantification of fluorescent counts per unit area was calculated using the Xenogen Living Image 2.5 software. The average for the isotonic and hypotonic groups was normalized to the isotonic group.